

RESEARCH PAPER



Construction of recombinant *Yarrowia lipolytica* and its application in bio-transformation of lignocellulose

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ABSTRACT

Lignocellulose is a polysaccharide and an abundant biomass resource that widely exists in grains, beans, rice, and their by-products. Over 10 million tons of lignocellulose resources and processing products are produced every year in China. Three recombinant *Y. lipolytica* strains with cellulase (β -glucosidase, endoglucanase and cellobiohydrolase) were constructed. The enzymatic activities of these enzymes were 14.181 U/mL, 16.307 U/mL, and 17.391 U/mL, respectively. The whole cell cellulases were used for a stover bio-transformation. The celluloses in the stover were partly degraded by the cellulases, and the degradation products were transformed into single cell protein (SCP) by the *Y. lipolytica* cells. After 15 d of fermentation with the whole cell cellulases, the protein content of the maize stover and the rice straw reached 16.23% and 14.75%, which increased by 168.26% and 161.52% compared with the control, respectively. This study provides a new stage for the efficient utilization of stover in the feed industry.

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Introduction

Lignocellulose is a cheap, renewable, abundant biore-source.¹ There are about 180 billion tons of biomass both above ground and in the soil, and an additional 40 million tons in the oceans.² These resources are widely used in stockbreeding, energy, biomaterials, chemicals, and other fields.^{3–5} Due to the large amounts of production and waste of lignocellulose resources, they have not been used efficiently.⁶ Based on the demonstrated potential, determining how to increase the utilization rate of cellulose in new and traditional fields is the subject of a great deal of research attention.

Among a variety of lignocellulose resource applications, using lignocellulose to produce bio-ethanol has attracted the most attention. It is a good way to solve the problem of wasted cellulose resources and a lack of energy.^{7–9} Lignocellulose widely exists in grains, beans, maize, rice, and some subsidiary agricultural products. Using lignocellulose in the feed industry is another important application. In lignocellulose,

lignin and hemicellulose form a solid layer closely surrounded by cellulose,^{10–12} which results in its inefficient use in feed.^{13,14} Ruminants can use part of the cellulose with the aid of rumen microbes, but monogastric animals like pigs and chickens lack cellulase in their gastrointestinal tract. This greatly limits the use of cellulose in stock breeding. For ruminants, adding some cellulase to their feed can partly decompose cellulose into its component monosaccharides and oligosaccharides, which can be absorbed more easily by ruminants.^{15,16}

Protein is the main nutrient in ruminant feed, and the nutritional value of this feed is directly affected by its protein content. Currently the shortage of protein feed resources is the main problem in the development of animal husbandry and the feed industry in China. Feed high in single cell protein that are rich in amino acids, vitamins, and growth factors has a high nutritional value, and it is an important protein feed resource for animals. Producing single cell protein *via* microbial fermentation could be increasingly used in

the feed industry as a solution to the shortage of feed protein. *Y. lipolytica*, which is generally regarded as safe, can be used in food and drug production.¹⁷⁻²⁰ It is an ideal microorganism for single cell protein production. With its strong secretory expression, *Y. lipolytica* is an efficient exogenous gene expression system.²¹

In China, there are a large number of lignocellulose resources like maize stover and rice straw, but the utilization of these bioresources is extremely limited. This limited utilization of bioresources leads to environmental pollution, wasted resources, and other issues. This research presents a novel strategy for stover bioresource transformation using simultaneous saccharification and fermentation (SSF) with modified *Y. lipolytica* that expresses cellulases. The degraded part of lignocellulose in stovers was transformed into single cell protein (SCP) to increase the crude protein content and nutrients in animal feedstuffs.

Results

Construction of expression vectors

The restructured plasmids were used as templates for polymerase chain reaction (PCR), and the fragments were sequenced to confirm that the cellulase genes had been inserted into the pINA1297 vector successfully. The 3 recombinant plasmids containing the inserted cellulase genes β -glucosidase, endoglucanase, and cellobiohydrolase were named pINA1297-bg, pINA1297-eg, and pINA1297-cbh, respectively, as shown in Fig. 1.

Screening of transformants and enzymatic activity assay

The transformants were screened via colony PCR using total DNA from the transformants. The same size fragments as target genes were obtained from the

recombinant transformants. The recombinant *Y. lipolytica* strains were named polh-1297-bg, polh-1297-cbh, and polh-1297-eg, respectively.

The crude enzymes were prepared via fermentation with the recombinants. The enzymatic activities of the 3 transformants were calculated according to the standard curve as shown in Fig. 2. The enzymatic activities of 3 recombinants polh-1297-bg, polh-1297-cbh, and polh-1297-eg were 14.181 U/mL, 16.307 U/mL, and 17.391 U/mL, respectively. The recombinants were used as the whole-cell enzymes for the bio-transformation of stovers.

Bio-transformation of stover with whole-cell cellulase

Fermentation of the maize stover and the rice straw were both performed by mixed culture of the 3 recombinant strains, equal volume culture was used and marked as the 'MIX' group. At the same time, fermentation of maize stover and rice straw with *Y. lipolytica* polh were marked as the 'polh' group. After 10 to 15 d of fermentation, the crude protein content of the bio-transformed stover samples was determined. As shown in Fig. 3, the crude protein content of the maize stover 'MIX' group reached 14.54% after 10 d and 16.23% after 15 d. The crude protein content in the 'polh' group reached 13.82% after 10 d and 14.84% after 15 d. Similar results were found with the fermentation of the rice straw (Fig. 4). The crude protein content of rice straw after 10 d and 15 d was 12.72% and 13.47%, respectively, using fermentation with *Y. lipolytica* polh. The crude protein content of rice straw via fermentation with the 3 mixed recombinant strains reached 13.28% after 10 d and 14.75% after 15 d. The crude protein contents increased both in the maize stover fermentation system and in the rice straw fermentation system. These results indicated that bio-transformation was efficient for increasing the crude protein content in both systems. Compared with the

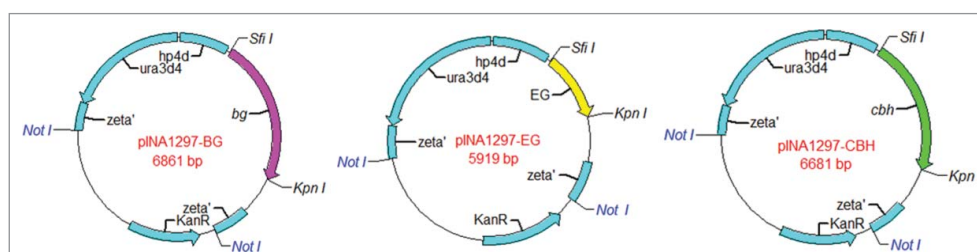


Figure 1. The recombinant plasmids of pINA1297-bg, pINA1297-eg, and pINA1297-cbh.

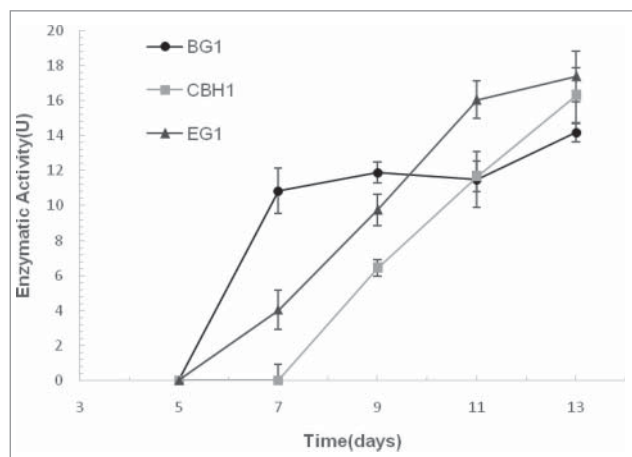


Figure 2. Enzymatic activity of the transformants expressing the BG1, CBH1, and EG1 genes.

untreated stovers, the crude protein content was obviously improved with the bio-transformation.

Discussion

In theory, the *Y. lipolytica* polh cannot use the lignocellulose as a carbon source, and it cannot grow using lignocellulose as the sole carbon source. The crude protein in the maize stover and the rice straw fermented with the *Y. lipolytica* polh for 15 d increased from 6.05% to 14.84% (maize stover) and from 5.64% to 13.47% (rice straw). Before being used as a fermentation carbon source, the maize stover and rice straw were subjected to high-temperature sterilization, which could have partly degraded the lignocellulose such that the hydrolyzed carbohydrate was the carbon source for *Y. lipolytica* polh growth.

The hydrolysis carbohydrate contents in the maize stover and the rice straw increased after

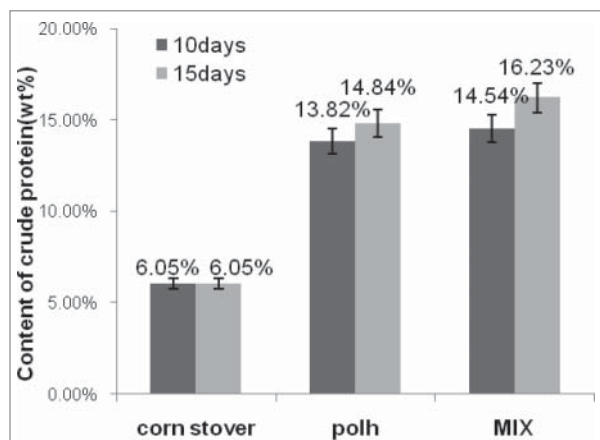


Figure 3. Crude protein content after maize stover fermentation.

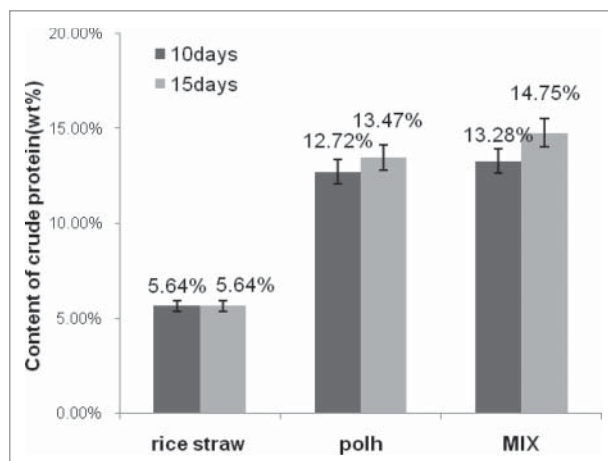


Figure 4. Crude protein content after rice straw fermentation.

high-temperature sterilization, which was later confirmed by thin layer chromatography (TLC), as shown in Fig. 5. The TLC was performed on a sheet of glass, which was coated with a thin layer of silica gel. In the 'polh' group, the hydrolysis carbohydrate contents decreased continuously, accompanying the increase in crude protein contents. The hydrolysis carbohydrate was used as carbon source for the growth of *Y. lipolytica* polh and transformed into single cell protein (SCP). In the 'MIX' group, the crude protein content increased, but the hydrolysis carbohydrate content did not decrease. The lignocellulose in the maize stover and rice straw was degraded and partly transformed into SCP by the whole-cell cellulose. The protein content of the maize stover and the rice straw was 16.23% and 14.75%, respectively. The maize stover and rice

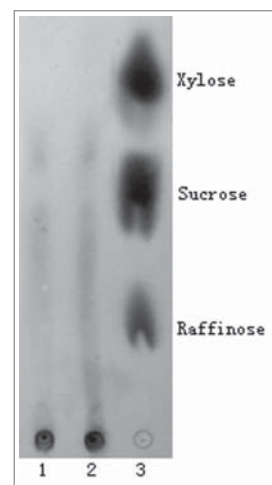


Figure 5. The TLC result of the hydrolysis carbohydrate. 1, The maize stover sample after high-temperature sterilization; 2, The rice straw sample after high-temperature sterilization; 3, Control.

straw increased by 168.26% and 161.52%, respectively, compared with the control. Continuous degradation was performed by the whole-cell cellulase, so the SCP contents increased and the hydrolysis carbohydrate contents did not decrease.

The recombinant *Y. lipolytica* with cellulase was more effective for the stover bio-transformation. The high SCP contents and the high hydrolysis carbohydrate contents, including oligosaccharide, in the bio-transformed stover translates to high quality feeds.

Materials and methods

Materials

Corn stovers and rice straws were sampled from the farmland around Wuhan, China. All chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Wuhan, China). T4 DNA ligase (Takara, 2011A), *Sfi* I (Takara, 1244A), *Kpn* I (Takara, 1068A) and *Not* I (Takara, 1166A) were purchased from Takara Biotechnology Company. *Escherichia coli* were preserved in the laboratory. *Y. lipolytica* and vector pINA1297 were a gift from Prof. Catherine Madzak (INRA/CNRS/AgroParisTech CBAI, Thiverval Grignon, France). *E. coli* was grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0). *Y. lipolytica* was used as the host for expression of exogenous cellulolytic enzyme genes. The yeast cells were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 28°C.

Plasmid construction

The cellulase genes, including β -glucosidase (BG, GenBank: AF163097), endoglucanase (EG, GenBank: EU169241) and cellobiohydrolase (CBH, GenBank: AY861348), were cloned into pINA1297 with the 2 restriction enzyme recognition sites *Sfi* I (Takara, 1244A) and *Kpn* I (Takara, 1068A). The restructured plasmids were sequenced. Primers used for amplification of the 3 cellulase genes are listed in Table 1.

Table 1. Primers used for cellulase gene amplification.

Primer	Nucleotide Sequences (5'–3')
BG-prime-F	ATAGGCCGTTCTGGCCTCTATTGACTTTGCA
BG-prime-R	TTAGGTACCCTAAAAGCCTTCAAT
EG-prime-F	ATAGGCCGTTCTGGCCTACCAGAACGGTT
EG-prime-R	TTAGGTACCTTAGGAGCTAGATTGTGGA
CBH-prime-F	ATAGGCCGTTCTGGCCGCGGCCATTTGCGT
CBH-prime-R	TTAGGTACCTCAGAACGGAGGGTT

Transformation and screening of transformants

The pINA1297 vector contains a hybrid promoter expression cassette (*hp4d* promoter) that drives recombinant protein expression in *Y. lipolytica*.^{21–23} The control (pINA1297 vector) and the 3 recombinant plasmids were linearized by restriction endonuclease *Not* I (Takara, 1166A) and then transformed into the *Y. lipolytica* strain Polh.²¹ The transformation of *Y. lipolytica* was performed with the lithium acetate chemical transformation method.⁶

After transformation, the recombinant clones were cultured on YNBD plates (minimal medium without uracil) at 28°C for 2 weeks. Total DNA of the transformants was extracted and used as the template for PCR to verify that the target genes were successfully transformed into *Y. lipolytica*. The PCR program was as follows: 94°C denaturation for 15 min, followed by 30 cycles of amplification (94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min), and a final extension for 5 min at 72°C.

Enzymatic activity analysis

The recombinants were inoculated to 50 mL parts per billion in liquid medium and cultured at 28°C at 200 rpm. The supernatant was used as crude enzyme to analyze the activity of cellulase expressed by the recombinants.

The dinitrosalicylic acid (DNS) method was used for the cellulase activity analysis. A 1-mL sample was mixed with 0.1 g of microcrystalline cellulose and placed in a constant-temperature water bath reaction for 90 min at 50°C. After centrifuging at 12,000 rpm for 1 min, 0.5 mL of supernatant was mixed with 1.5 mL of DNS reagent and placed in boiling water for 5 min. Next, 1 mL of reaction liquid was diluted to 5 mL and tested for absorbance at 540 nm. Enzyme activity was calculated according to the standard curve. One unit (U) was defined as the amount of enzyme required to produce 1 microgram of glucose after 1 min at 50°C. According to the standard curve, the concentration of glucose (y) was calculated according to Eq. 1,

$$Y(\text{mg} / \text{mL}) = 1.1713x + 0.0271 \quad 1$$

where x is the value of OD₅₄₀ and Y is the concentration of glucose (mg/mL).

Shallow tray fermentation of straws with recombinants

A total of 50 g of substrates (maize stover or rice straw) were sterilized at 121°C for 30 min and mixed with 5 g ammonium chloride. The recombinant and control strains were incubated over night at 28°C in YPD liquid medium. After the OD₆₀₀ reached 2.0, the 3 recombinant strains were mixed. The 500 mL cultures were centrifuged at 4,000 rpm, and the collected cells were suspended in 300 mL PBS. The recombinant cultures were then mixed with the straw substrates, and shallow tray fermentation was performed at 28°C (for straw bio-transformation). This process was repeated 3 times for every group, and the data were measured 3 times in-parallel.

Protein content analysis

The protein content was assayed via Kjeldahl's determination method.²⁴ To eliminate the influence of ammonium chloride, 2 mL of 0.1 M sodium hydroxide was added to 1.0 g of sample and heated to 250°C for 20 min until no more ammonia was released. Digestion was accomplished with concentrated sulfuric acid, followed by distillation and titration once total digestion was achieved. The specific experimental method was based on the Kjeldahl determination²⁴ and the determination of crude protein in feedstuffs.²⁵

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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