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Direct conversion of inulin into single cell protein by the engineered *Yarrowia lipolytica* carrying inulinase gene

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ABSTRACT

The *INU1* gene encoding exo-inulinase cloned from *Kluyveromyces marxianus* CBS 6556 was ligated into the expression plasmid pINA1317 and expressed in the cells of the yeast *Yarrowia lipolytica* with high content of protein. The activity of the secreted inulinase was found to be 43.1 ± 0.9 U/ml after cell growth for 80 h. When the engineered yeast cells were grown in the medium containing 4.0% inulin in 2-l fermentor, crude protein in the cells and cell mass could reach 47.5% and 20.1 g/l, respectively within 72 h. Furthermore, when the engineered yeast cells were grown in the medium containing 8.0% the meal of Jerusalem artichoke tuber in 2-l fermentor, crude protein and cell mass could reach 53.7% and 20.8 g/l, respectively within 80 h. Therefore, the engineered *Y. lipolytica* could be further used to produce single cell protein from inulin and inulin-containing materials.

1. Introduction

In nature, inulin is a mixture of oligo- and polysaccharides composed of fructose unit chains (linked by β-(2-1)-D-fructosyl-fructose bonds) of various lengths with a glucose molecule at the end of each fructose chain. Inulin is present as a reserve carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, dahlia, and yacon. The dried materials of the tubers contain over 50% inulin [1]. The inulinases are classified among the hydrolases and target on the β-2,1 linkage of inulin and hydrolyze it into fructose and glucose [2]. Inulin and its hydrolysate as important materials in microbial biotechnology have been used to produce single cell protein, single cell oil, citric acid, bioethanol, ultra-high fructose syrup production, 2,3-butanediol, lactic acid, mannitol and sorbitol [3–10].

Single-cell protein has many applications in food and feed industries as it has high content of protein, high percentage of essential amino acids and other nutrients [3,11,12]. In addition, after the single cell proteins were hydrolyzed by alkaline protease, the hydrolysates produced have angiotensin converting enzyme inhibitory activity and antioxidant activity [13]. The microorganisms which can be used as single-cell protein include a variety of marine microalgae, bacteria, yeasts, and molds [3]. In general, the protein contents in the single cells for protein production should be between 39 and 73% [14]. In our previous study [11], *Yarrowia lipolytica* SWJ-1b isolated from the marine fish gut was found to contain 47.6 g of crude protein per 100 g of cell dry weight and had potential use as single cell protein. However, the yeast strain cannot synthesize inulinase so that it cannot be used to produce single cell protein from inulin and inulin-containing materials directly. Therefore, in the current study, the *INU1* gene encoding exo-inulinase cloned from *Kluyveromyces marxianus* CBS 6556 was ligated into the expression plasmid and expressed in the cells of the marine-derived yeast *Y. lipolytica* with high content of protein. Then, the engineered yeast which can actively secrete the recombinant inulinase was used to produce single cell protein from inulin and meal of Jerusalem artichoke tubers directly.

2. Materials and methods

2.1. Strains and media

The single cell protein producer used in this study was *Y. lipolytica* SWJ-1b which was isolated from gut of the marine fish at Bohai Sea. It was found that the yeast strain contained over 52% (w/w) of crude protein in its cells [11]. Uracil mutant of *Y. lipolytica* SWJ-1b was isolated from *Y. lipolytica* SWJ-1b using 5′-FOA (5′-fluororotic acid) [11]. Yeast strains were grown in yeast peptone dextrose (YPD) (1.0% yeast extract, 2.0% bacto peptone, 2.0% glucose). The yeast transformants were selected on YNB-N_{5000} (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 1.0% glucose, 0.5% ammonium sulfate). In order to determine secreted inulinase by yeast transformants, the PPB medium containing sucrose (2.0%), yeast extract (0.132%), NH_4Cl (0.132%), KH_2PO_4 (0.032%), $MgSO_4{\cdot}7H_2O$ (0.024%), thiamine (0.33 mg/l), and pH 7.4 was used [15]. The *Escherichia coli* strain used in this study for plasmid recovery and cloning experiments was DH5α [F− endA1 hsdR17 (rK−/mK+) supE44 thi-1λ− recA1 gyr96ΔlacU169 (φ80lacZΔM15)] and was grown in Luria–Bertani broth (LB). The *E. coli* transformants were grown in LB medium with 100 μg/ml of ampicillin or 30 μg/ml of kanamycin. Single cell protein (SCP) production medium was 4.0% inulin, 4.0% hydrolysate of soybean meal, 0.1% ammonium

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Table 1
Inulinase activities of different transformants and *Y. lipolytica* SWJ-1b.

Yeast strains	Inulinase activity (U/ml)
C5	28.3 ± 0.52
C43	30.1 ± 0.76
C47	31.2 ± 0.58
C55	35.7 ± 0.45
C58	33.5 ± 0.60
Y. lipolytica SWJ-1b	0

Data are given as means ± SD, $n = 3$.

sulfate, pH 6.0 or 8.0% meal of Jerusalem artichoke tubers and 4.0% hydrolysate of soybean meal, pH 6.0.

2.2. Plasmids

The expression vector pINA1317 was constructed at CBAI, AgroParisTech, 78850 Thiverval-Grignon, France. pMD19-T and pMD-19T simple vectors were purchased from TaKaRa (Japan). pMD18-T-*INU1* carrying the inulinase gene cloned from *K. marxianus* CBS 6556 was kindly supplied by Dr. Zongbao K. Zhao at Division of Biotechnology, Dalian Institute of Chemical Physics, CAS, Dalian, China.

2.3. Preparation of the hydrolysate of soybean meal

Thirty-two grams of soybean meal was mixed with 250.0 ml of tap water containing 0.25 M HCl. The mixture was autoclaved at 121 °C for 25 min. After cooling, pH of the mixture was adjusted to 5.5 with 1.0 M NaOH solution and the suspension was filtered. The filtrate was diluted to 400.0 ml [16]. The soybean meal was purchased from the local market.

2.4. Preparation of the meal of Jerusalem artichoke tuber

Jerusalem artichoke tubercles were collected from the farms in Taian, Shandong Province, China in December 2009. About 10.0 kg of the Jerusalem artichoke tubercles was washed, peeled and cut. The cut tubercles were dried at 80 °C until their weight was constant. The dried tubercles were grounded by the mechanical ways. The grounded tubercle (100 mesh) was named the meal of Jerusalem artichoke tubers.

2.5. Isolation of DNA and RNA, restriction digestions, and transformation

DNA manipulations were carried out using standard methods [17]. Bacterial plasmid DNA was purified using Perfectprep plasmid minikit (Eppendorf). Restriction endonuclease digestions and DNA ligations were performed according to the manufacture's recommendations. *E. coli* was transformed with plasmid DNA according to Sambrook et al. [17]. Transformants were plated out onto LB medium containing 100 μg/ml of ampicillin or 30 μg/ml of kanamycin. *Y. lipolytica* was transformed according to the methods described by Xuan et al. [18].

2.6. Inulinase gene expression in the cells of Y. lipolytica SWJ-1b

In order to amplify the *INU1* gene encoding the inulinase from pMD18-T-*INU1* by PCR, the forward primer was ppu: 5′-GGCCGTTCTGGCC TCAGTTATCAATTACAAGAGAGATGGTGACAGC-3′ (boxed bases encode *Sfi*I restriction site) and the reverse primer was ppd: 5′-GGATCC TCAATGGTGATGGTGGTGATGAAGGTTAAATTGGGTAACG-3′ (boxed bases encode *BamH*I restriction site and underlined bases encode 6× His tag). The gene amplification by PCR and the gene expression in cells of *Y. lipolytica* were performed as described by Yue et al. [19]. After ligation to pINA1317, the resulting plasmid carrying the *INU1* gene was designated as pINA1317-*INU1* (Fig. 1). The positive transformants carrying the *INU1* gene were grown in PPB liquid medium for 96 h. The culture was centrifuged at 5000 × *g* and 4 °C and the supernatant obtained was used as the crude inulinase preparation. The inulinase activity in the supernatant obtained was determined as described below. *Y. lipolytica* SWJ-1b cells only carrying yeast cassette without the *INU1* gene were used as controls. Finally, we found that the transformant C55, one of the transformants, could produce 35.7 U/ml of inulinase activity (Table 1). Therefore, the transformant C55 was used in the subsequent investigation.

2.7. Confirmation of integration of the target gene into Y. lipolytica genome

To get the evidence that DNA fragments carrying the inulinase gene have been integrated into the genome of the yeast *Y. lipolytica* SWJ-1b with high content of protein, genomic DNAs from the corresponding transformant C55 and *Y. lipolytica* SWJ-1b were extracted and used as templates for PCR, respectively. The forward primer used for this checking was ppu and the reverse primer was ppd (sequences described earlier). PCR amplification was performed as described earlier. The sizes of the PCR products were estimated using the Automated Gel Documentation & Analysis System (Gene-Genius, USA).

2.8. Determination of the recombinant inulinase activity

The recombinant inulinase activity in the supernatant obtained above was determined according to Gong et al. [20]. The reaction mixture containing 0.1 ml of the supernatant obtained above and 0.9 ml of the sodium acetate buffer (0.1 M, pH 4.5) containing 2.0% (w/v) inulin (Sigma) was incubated at 50 °C for 15 min. The reaction was inactivated immediately by keeping the reaction mixture at 100 °C for 10 min. The reaction mixture containing 0.1 ml of the supernatant heated at 100 °C for 10 min and 0.9 ml of the sodium acetate buffer (0.1 M, pH 4.5) containing 2.0% (w/v) inulin was used as control. The amount of reducing sugar in the reaction mixture was assayed by the method of Nelson–Somogyi [21]. One inulinase unit (U) was defined as the amount of enzyme that produces one micromole of reducing sugar per minute under the assay conditions used in this study.

2.9. SDS-PAGE analysis and Western blotting

The recombinant inulinase was confirmed in noncontinuous denaturing SDS-PAGE [22] with a two-dimensional electrophoresis system (Amersham Biosciences, Sweden) and stained by Coomassie Brilliant Blue R-250 [23]. The molecular mass standards for SDS-PAGE comprised phosphorylase B (97.2 kDa), bovine serum albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa). To confirm the recombinant inulinase expression as a His-tagged fusion protein, Western blot analysis was carried out according to methods described by Li et al. [24]. The mouse anti-His antibody (GE Healthcare, American) was used at a dilution of 1:1500 as the primary antibody and the goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP; TianGen, Beijing, China) was used at a dilution of 1:2000 as the secondary antibody.

2.10. Single cell protein production at flask level

All the experiments were performed in 250-ml conical flasks, containing 50.0 ml of the single cell protein production medium with 4.0% (w/v) inulin, sterilized at 115 °C for 30 min and inoculated with 5.0 ml of 24-h-old pre-culture ($2.0–2.5 \times 10^6$ cells/ml). All the cultures were incubated in an orbital shaker at a shaking speed of 160 rpm and incubation temperature of 28 °C for 78 h.

2.11. Single cell protein production during 2-l fermentation

Seed cultures were prepared by inoculating the yeast cells grown on a YPD agar slant into 500-ml Erlenmeyer flasks that contained 100 ml of PPB medium, and cultivating them at 28 °C for 72 h with vigorous shaking. The fermentation was carried out in a Biostat B2 2-l fermentor (B. Braun, Germany) equipped with baffles, a stirrer, alkali pump, heating element, oxygen sensor, and temperature sensor. 165 ml of the seed culture was transferred into 1500 ml of the single cell protein production medium containing 4.0% (w/v) inulin or 8.0% meal of Jerusalem artichoke tubercles. The fermentation was performed under the conditions of agitation speed of 250 rpm, aeration rate of 10 l/min, temperature of 28 °C and fermentation period of 80 h. Samples were taken approximately every 12 h to determine inulinase activity, biomass production, total sugar, reducing sugar and protein content.

2.12. Estimation of protein content in the yeast cells

The cells in 10 ml of the cultures obtained above were collected and washed three times with sterile saline water by centrifugation at 5000 × *g* and 4 °C for 10 min. The pellets obtained were dried at 80 °C until cell dry weight was constant [16]. 50 mg of the dried cells was digested at 180 °C with High Temperature Digester KDN-08 (Xinjin Electron Co., Ltd., Shanghai, China) for 1.5 h. The crude protein content in the pellets was determined by using the method of Kjehldahl with ninhydrin detection [25]. The solution with 20 mg of bovine serum albumin was served as standard [26].

2.13. Measurement of cell dry weight

The cells in 30.0 ml of the culture obtained above were collected and washed by centrifugation three times with sterile water at 5000 × *g* and 4 °C. The washed cells were dried at 80 °C until the weight was constant.

2.14. Determination of reducing sugar and total sugar in the fermented media

Reducing sugar in the fermented media was determined by the Nelson–Somogyi method [21]. Total sugar was measured as reduction of sugar after hydrolysis of the fermented media [16].