Biochemical Engineering Journal 43 (2009) 266–271

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Inulinase overproduction by a mutant of the marine yeast *Pichia guilliermondii* using surface response methodology and inulin hydrolysis

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ARTICLE INFO

Article history:
Received 20 March 2008
Received in revised form 7 October 2008
Accepted 16 October 2008

Keywords:
Pichia
Inulinase
Mutagenesis
RSM
Overproduction

ABSTRACT

In this study, in order to isolate inulinase overproducers from the marine yeast *Pichia guilliermondii*, its cells were treated by using UV light and LiCl. The mutant M-30 with enhanced inulinase production was obtained and was found to be stable after cultivation for 20 generations. Response surface methodology (RSM) was used to optimize the medium compositions and cultivation conditions for inulinase production by the mutant M-30 in liquid fermentation. Inulin, yeast extract, NaCl, temperature, pH for maximum inulinase production by the mutant M-30 were found to be 20.0 g/l, 5.0 g/l, 20.0 g/l, 28 °C and 6.5, respectively. Under the optimized conditions, 127.7 U/ml of inulinase activity was reached in the liquid culture of the mutant M-30 whereas the predicted maximum inulinase activity of 129.8 U/ml was derived from RSM regression. Under the same conditions, its parent strain only produced 48.1 U/ml of inulinase activity. This is the highest inulinase activity produced by the yeast strains reported so far. We also found that inulin could be actively converted into monosaccharides by the crude inulinase.

1. Introduction

Inulin is a widespread polyfructan, which is accumulated, in underground organs of several plants, such as Jerusalem artichoke, dahlia tubers, chicory root and yacon [1]. It consists of linear β-2, 1-linked polyfructose chains displaying a terminal glucose unit. Inulinase (β-2, 1-D-fructan fructanohydrolase (EC 3.2. 1.7) targets on the β-2, 1 linkage of inulin and hydrolyzes it into fructose [2]. Fructose is widely used in pharmaceutical industry, many foods and beverages instead of sucrose and also can be converted into fuel ethanol by fermentation. Therefore, inulin and inulinase can be used for production of either ultra-high fructose syrups, with D-fructose content over 95% by exo-enzymatic hydrolysis, or for production of oligofructose syrups by endo-enzymatic hydrolysis or for production of ethanol by fermentation [3–5]. In our previous study [1], we found that that under the optimal conditions, over 85.0 U/ml of inulinase activity was produced by the marine yeast *Cryptococcus aureus* G7a within 42 h of fermentation at shake flask level when cell growth was at the early stationary phase. In inulin, the highest production of inulinase was 2.8 U/ml using *Candida pseudotropicalis*. 14.6 U/ml for *Candida kefyr*, 18.7 U/ml for *C. pseudotropicalis*, 18.4 U/ml for *Kluyveromyces marxianus* var. *bulgaricus*, and 14.3 U/ml for *K. fragilis* [6]. Inulinase yield of 40.5 U/ml of *K. marxianus* var. *bulgaricus* in an optimized medium at 30 °C under agitation (150 rpm) has been obtained at shake flask level [7]. Inulinase gene *inuA*1 from *Aspergillus niger* AF10 was overexpressed in *Pichia pastoris* and inulinase activity reached 50.6 U/ml in the fermentation liquid [8]. The *INU1* gene encoding an exoinlinase from *K. marxianus* KW02 was expressed in *P. pastoris*. The enzyme activity of the recombinant *P. pastoris* in the fermentation liquid was 52.0 U/ml [9]. Maximum enzyme production (55.4 U/ml) was obtained by *K. marxianus* YS-1 at an agitation rate of 200 rpm and aeration of 0.75 vvm in a stirred tank reactor with a fermentation time of 60 h [10]. In another study [3], we found that marine yeast strain 1, isolated from the surface of a marine alga and identified as a strain of *Pichia guilliermondii* could produce over 60.0 U/ml of inulinase under the optimal conditions. In this study, the mutant M-30 with enhanced inulinase production was obtained from the marine yeast *P. guilliermondii* using UV radiation and LiCl treatment. After optimization of the production medium and cultivation conditions by using response surface methodology (RSM), 127.7 U/ml of inulinase activity was reached in the culture of the mutant M-30 while the predicted maximum inulinase activity of 129.8 U/ml was derived from RSM regression. This is the highest inulinase activity produced by the yeast strains reported so far.

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doi:10.1016/j.bej.2008.10.018

2. Materials and methods

2.1. Yeast strain and media

The yeast strain *P guilliermondii* 1 was isolated from the surface of a marine alga collected at 100 m depth of seawater at Changdao island in Penglai, China [3]. This yeast strain was maintained in YPD medium (prepared with seawater) containing 20.0 g/l glucose, 10.0 g/l yeast extract, and 20.0 g/l polypeptone at 4 °C. The medium (prepared with seawater) for mutagenesis contained 20.0 g/l glucose, 10.0 g/l yeast extract, 5.0 g/l LiCl, and 20.0 g/l polypeptone. The liquid medium (prepared with seawater) for inulinase production contained 20.0 g/l inulin, 5.0 g/l yeast extract, 20.0 g/l NaCl and initial pH 6.5.

2.2. Formation of ascospores and mutagenesis

Ascospore formation of *P. guilliremondii* was carried out on 50 g/l malt extract agar at 25 °C for 5–15 days as described by Kurtzman and Fell [11]. Ascospores released from asci of *P. guilliremondii* were suspended in sterile seawater and ascospore concentration of the suspension was adjusted to 10^7 cells/ml. The ascospore suspension was diluted with sterile seawater and the dilute with 100 ascospores per ml was spread on the plate containing 20 ml of the medium (plus 20.0 g/l agar) for mutagenesis. A 15 W UV-lamp was used as the source of radiation. Ultraviolet mutagenesis was applied with exposure time of 5, 10, 15, 20, 25 and 30 s at a fixed distance of 25 cm. The mutated cells were fixed by further cultivating the treated cells in the medium for mutagenesis overnight in the dark. Approximately 0.1 ml of each of the UV and LiCl-treated cell suspensions was transferred to the YPD plates. Colonies that appeared within 72 h of incubation at 28 °C were transferred to YPD slants. Potential mutant strains were cultured in 50 ml of the inulinase production medium (prepared with seawater) by shaking at 170 rpm and 28.0 °C for 2 days. The culture was centrifuged at 4500 × *g* and 4 °C for 5 min and the supernatant obtained was used the crude inulinase preparation. Inulinase activity in the crude inulinase preparation was determined as described below. The mutants with enhanced inulinase production were selected for further studies. Finally, the mutant M-30 that produced 115.0 U/ml of inulinase activity in the liquid culture was obtained.

2.3. Determination of inulinase activity

The reaction mixture containing 0.1 ml of the crude inulinase preparation obtained above and 0.9 ml of phosphate buffer (0.1 M, pH 6.0) containing 2.0% (w/v) inulin was incubated at 60 °C for 10 min. The reaction was inactivated immediately by keeping the reaction mixture at 100 °C for 10 min. The amount of reducing sugar in the reaction mixture was assayed by the method of Nelson-Somogyi [12]. One inulinase unit (U) was defined as the amount of enzyme that produces 1 μmol of reducing sugar per minute under the assay conditions used in this study.

2.4. DNA extraction, PCR and sequencing

The total genomic DNA of the wild type yeast strain and the mutant M-30 was isolated and purified by using the methods as described by Sambrook et al. [13]. The specific primers for amplification of the inulinase gene (accession number: EU195799) in the yeasts were used, the forward primer: PP, 5′-ATGAGAGCTTTTCTTGCCTTAATT-3′ and the reverse primer PR, 5′-CTATGAAGTTGCCCTCAATTTTAA-3′. The reaction system (25 μl) was composed of 10× buffer 2.5 μl, dNTP 0.8 μM, MgCl2 1.5 mM, PP or PR 0.5 μM, Taq DNA polymerase 1.25 U, template DNA 1.0 μl and H_2O 16.6 μl. The conditions for the PCR amplification were as follows: initial denaturation at 94 °C for 10 min, denaturation at 94 °C for 1 min, annealing temperature at 53 °C for 1.0 min, extension at 72 °C for 2.0 min, final extension at 72 °C for 10 min. PCR was run for 32 cycles and PCR cycler was GeneAmp PCR System 2400 made by Perkin–Elmer. PCR products were separated by agarose gel electrophoresis and recovered by using UNIQ-column DNA gel recovery kits (BIOASIA, Shanghai). The recovered PCR products were ligated into pGEM-T easy vector and transformed into the competent cells of *Escherichia coli* JM109. The transformants were selected on LB plates (tryptone 10.0 g, yeast extract 5.0 g, NaCl 10.0 g, agar 15.0 g, distilled water 1000 ml, pH 7.2) with ampicillin (100 μg/ml). The plasmids in the transformant cells were extracted by using the methods as described by Sambrook et al. [13]. In order to confirm that the PCR products had been ligated into the vector, the purified plasmids were used as templates for amplification of the inulinase gene in the yeast strains, respectively. The reaction system and the conditions for PCR amplification were the same as described above. The PCR products inserted on the vector were sequenced by Shanghai Sangon Company.

2.5. Effects of added glucose on inulinase production

The mutant M-30 and its wild type were aerobically grown in the production medium containing different glucose concentrations (0, 1.0, 2.0, 3.0 and 4.0 g per 100 ml) for 48 h. Inulinase activity in the supernatant of the cultures was determined as described above. At the same time, OD value of the cultures was measured at 600 nm using spectrophotometer. The specific inulinase activity was defined as units of inulinase per OD_{600nm}. The inulinase activity of the cultures in the absence of added glucose was defined as 100%.

2.6. Screening of physical and chemical parameters using Central Composite Designs

The Central Composite Design for 5 variables which includes physical and chemical parameters at five levels (+2.38, +1, 0, −1, and −2.38) (Tables 1 and 2) [14] was used for screening. Among the physical and chemical parameters, temperature, pH, the amount of inulin, inoculation size and the amount of yeast extract were tested for their significance in the inulinase production by the mutant M-30.

2.7. Statistical analysis of the data

The statistically planned experimentation was to identify the significant variables and their corresponding coefficients, so that the levels of variables could be managed to obtain a desired output. Hence, the coefficients, sum of squares in percentage (SS%) and coefficient of variation (CV) were analyzed using the experimental results of the inulinase activity produced. Using the design expert (Static Made Easy, Minneapolis, MN, USA; Version 7.0.0, 2005), the experimental plan, the analysis and the results were obtained.

Table 1
Range of the factors investigated in the experimental design.

Variables	Code	Levels				
		−2.38	−1	0	+1	+2.38
Temperature	A	23.24	26	28	30	32.76
pH	B	3.62	5	6	7	8.38
The amount of inulin	C	0.81%	1.5%	2%	2.5%	3.19%
Inoculation size	D	0.31%	1%	1.5%	2%	2.69%
The amount of yeast extract	E	0.26%	0.4%	0.5%	0.6%	0.74%