Cloning of the dextranase gene(*lsd11*) from *Lipomyces starkeyi* and its expression in *Pichia pastoris.*

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ABSTRACT

Dextranase (α-1,6-D-glucan-6-glucanogydrolase:E.C. 3.2.1.11) catalyzes the hydrolysis of α-(1.6) linkages of dextran. A *lsd1* gene encoding an extracellular dextranase was isolated from the genomic DNA of *L. starkeyi*. The *lsd11* gene is a synthetic dextranase (*lsd1*) after codon optimization for gene expression with *Pichia pastoris* system. A open reading frame of *lsd11* gene was 1827 bp and it was inserted into the pPIC3.5K expression vector. The plasmid linearized by *Sac* I was integrated into the 5'*AOX* region of the chromosomal DNA of *P. pastoris*. The *lsd11* gene fragment encoding a mature protein of 608 amino acids with a predicted molecular weight of 70 kDa, was expressed in the methylotrophic yeast *P. pastoris* by controling the alcohol oxidase-1 (*AOX1*) promoter. The recombinant *lsd11* was optimized by using the shake-flask expression and upscaled using fermentation technology. More than 9.8 mg/L of active dextranase was obtained after induction by methanol. The optimum pH of LSD11 was found to be 5.5 and the optimum temperature 28℃.

INTRODUCTION

Dextranase (EC 3.2.1.11, α-1,6 glucan-6-glucanohydrolase) catalyzes the hydrolysis of the α-1,6 glucosidic linkages of the dextran. Dextranase are important since these enzymes can depolymerise various troublesome microbial dextran deposits [1]. *Lipomyces starkeyi*, an ascosporogenous yeast, produces an endo-dextranase and/or an α-amylase [2]. This dextranase has been demonstrated to be an effective agent for removing dextran during sugar processing [3]. Except for a few bacterial dextranase, microbial dextranase generally are inducible [4]. Kim and Day reported on the development of a derepressed and partially constitutive mutant (ATCC 74054) for dextranase and amylase and described the characterization of its enzyme [5]. Ryu *et al.* also reported purification and characterization of a novel glucanhydrolase, which exhibited either dextranase or amylase activity, from *L. starkeyi* KSM22 [6]. There have been a number of recent reports on cloning and expression of bacterial genes encoding dextranase [7, 8]. However, the cloning of a yeast dextranase gene was few reported. We believe that the cloning and characterization of the dextranase gene as well as the expression of this gene in the methylotrophic yeast *P. pastoris* would make it possible to generate large amounts of enzyme with relative ease and would be of potential utility in industrial filed. In this paper, we first report the cloning of *L. starkeyi* dextranase gene after codon optimization and expression of its encoded dextranase by recombinant industrial strains of *P. pastoris*.

MATERIALS AND METHODS

Construction of the recombinant plasmid for expression

The DNA fragment corresponding to the dextranase gene (*lsd11*) was amplified by PCR. PCR fragment was cloned into vector pPIC3.5K (Invitrogen, USA) resulted in the plasmid pPIC3.5K-*lsd11*.

Transformation into P. pastoris.

The plasmid pPIC3.5K-*lsd11* was linearized by digestion with *Sac* I or *Bgl* II for 2 hour at 37℃ prior to transformation, and was transformed using method described by Gietz *et al* [9]. Also the linearized DNA was used to transform

by electroporation. The electroporation was carried out essentially by the method of Becker and Guarente (1991), with cells pulsed in 0.2 cm electroporation cuvettes at 1500V, 25 μF, 400 Ohm, using a BioRad Gene Pulser with Pulse Controller.

The transformed cells were selected on YPD plates containing 4 mg/mL of geneticin(G418). Then, each single colony was transferred to minimal methanol (MM) agar plates with 0.5% blue dextran (Sigma Co., USA), and the dextranase expression was induced with methanol in vapor phase by the addition of 0.5 ml portions of 100% methanol under the lids of inverted plates. The plates were incubated overnight at 30℃ and the formation of clear halos of dextran hydrolysis around the colonies were observed. For isolation of intracellular protein MM broth was inoculated to 2 OD_{600} from overnight culture and grown at 30℃ for 72 h. Cell were harvested and resuspeneded in 1/10 volume breaking buffer (20mM citrate phosphate, pH 5.5). 0.45 mm glass beads were added and the cells were vigorously vortexed. After centrifugation, the supernatant was carefully removed and used for the enzyme assay.

Fermentation

The jar fermentor containing chemically defined growth medium (glycerol 30g/L, $CaSO_4$ 0.93g/L, K_2SO_4 18.2g/L, $MgSO_4.7H_2O$ 14.9g/L, KOH 4.13g/L, K_2HPO_4 7g/L, H_3PO_4 22.7mL/L) adjusting the pH to 5.0 using ammonium hydroxide and with 0.435% PTM1 ($CuSO_4.5H_2O$ 6g/L, NaI 0.08g/L, $MnSO_4$. H_2O 3.0g/L, $Na_2MoO_4.2H_2O$ 0.2g/L, H_3BO_3 0.02g/L, $CoCl_2$ 0.5g/L, $ZnCl_2$ 20g/L, $FeSO_4.7H_2O$ 65g/L, $FeSO_4.7H_2O$ 65g/L, Biotin 0.2g/L). Fermentations were started by the inoculation of 1 L of MGY medium in a 2 L flask. An overnight preculture in MGY was inoculated with shaking at 30℃to OD600 = 4. Fermentation was to grow the cells in excess glycerol to repress the expression followed inducement of protein production by adding methanol after glycerol was exhausted. The temperature was set at 30℃. RPM was kept 900 and with aeration.

RESULTS AND DISCUSSIONS

Construction of the recombinant expression plasmid

The DNA fragment encoding *lsd11* was amplified by PCR. PCR fragment was cloned into vector pPIC3.5K. Transformation by LiCl method of the GS115 (*His4*) strain with this DNA yielded His^{+} transformants. A screening step to find the clones producing active dextranase was performed on minimal agar plates containing blue dextran, and the expression was induced with method. All the clones tested presented a halo of dextran hydrolysis.

Properties of dextranase expressed by P. pastoris transformants.

We tested the ability of non-dextranlytic yeast P. pastoris GS115 to secrete active dextranase when transformed with pPIC3.5K-*lsd11*. GS115 transformants were able to form blue halos on BDMD plates within 72 h. Intracellular extracts of pPIC3.5K-*lsd11* were used in the determination of the lsd11 activity at different condition. The optimum temperature for lsd11 was 37℃. The optimum pH of dextranase is 5.5.

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