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MULTIPLE XYLITOL DEHYDROGENASE SYSTEM OF *ASPERGILLUS ORYZAE*

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ABSTRACT

Aspergillus oryzae P5 has some xylanolytic enzymes such as xylanase of 8.8 U/mg, α-L arabinofuranosidase of 1.2 U/mg, β- xylosidase of 1.9 U/mg and xylose reductase activities were 11.3 U/mg and 13.4 U/mg by using NADH and NADPH. However, xylitol dehydrogenase activities were 5.5 U/mg and 1.8 U/mg using NAD^+ and $NADP^+$ as cofactor, respectively. In genome of *A. oryzae*, there are 4 fragments, which are homologous with DNA xylitol dehydrogenase genes of other strains. Based on DNA sequences of these fragments, 2 genes were cloned. One of them (*xdhA)* gene encodes for NAD^+-dependent xylitol dehydrogenase activity. The gene was knock out in *A. oryzae* P5, however the mutant still showed NAD^+-dependent xylitol dehydrogenase activity. Therefore, *A. oryzae* P5 has multiple xylitol dehydrogenases.

1. INTRODUCTION

Aspergillus oryzae is one of the most important microorganisms, which have been applied in food processing and fermentation industries. *A. oryzae* has xylanolytic enzymes for xylan degradation such as two xylanase genes of family 11 and at least four xylanase genes of family 10 [1 - 6] and a β-xylosidase gene [6]. Recently, NAD+-dependent xylitol dehydrogenase

gene (*xdhA*) and NAD+ -dependent l- arabinitol 4 dehydrogenase gene (*ladA*) of *A. oryzae* were cloned [17, 18]. Lignocellulose, comprising cellulose, hemicellulose and lignin, is one of the most abundant renewable resources and has great potential as feedstock for the production of value products including a number of useful chemicals and liquid fuels. Many researches were done using yeasts. However, researches using mold was limited. Finding out enzymes, responding for using lignocellulose, is one of important step for new application of *A. oryzae*. In this report, study of multiple xylitol dehydrogenase system in *A. oryzae* was carried out.

2. MATERIALS AND METHODS

2.1. Microorganisms, plasmids and media

Aspergillus oryzae P5, a mutant of *A. oryzae* KBN 616 without pyrG, was used in this study. The fungus was incubated at 30^{o}C at 130 rpm in 500 ml flask with 100 ml carbon-polypepton medium (containing 20.0 g carbon source, 10.0 g polypeptone, 5.0 g KH_2PO_4, 1.0 g $NaNO_3$ and 0.5 g $MgSO_4 \cdot 7H_20$ per liter) and addition of 0.15 g uridine and 0.07 g uracil per liter.

Plasmids pT7 Blue T-vector was purchased from Takara, Japan, pyrG100 vector consisted of pUC119 vector and pyrG gene [6]. The Gateway technology cloning kit was purchased from Invitrogen USA. *Escherichia coli* DH5α was used for propagation of plasmids according to Sambrook *et al.* [14].

2.2. Preparation of the free cells extracts for enzyme assays

Aspergillus oryzae P5 was grown in 100 ml carbon-polypepton medium with different carbon sources and the addition of uracil and uridine. The culture was incubated at 30^{o}C and 130 rpm for 3 days. Then the cells were harvested by vacuum filtration, disrupted with a mortar and pestle in liquid nitrogen. The powder was transferred to an eppendorf tube. An amount of 1 ml of 0.5 M phosphate buffer (pH 7.5) was added and centrifuged at 12,000 rpm for 20 min. After that the supernatant was collected and used as a crude enzyme. Protein was measured by Bradford method using Bio-Rad protein assay reagent (Bio-Rad, Hercules, Calif., USA) with bovine serum albumin (BSA) as standard. All enzymes assays were measured by DU 64

spectrophotometer (Beckman USA). Specific activity of enzyme was expressed in units per milligram of protein.

2.3. Enzyme assays

Xylose reductase, xylitol dehydrogenase, xylanase, xylose - isomerase β-xylosidase and α-L- arabinofuranosidase activities were measured according to the method as mention previously [1, 2, 4, 10, 15, 19].

2.4. Design of primer for XDH gene

Based on Genbank data base and the expressed sequence tag (EST) data base of *A. oryzae*, 4 pair primers were constructed (table 1). Polymerase chain reaction was conducted with genomic DNA of *A. oryzae* P5 as template and the following parameters: initial denaturation (94^oC, 2 min), 30 cycles of denaturation (94^oC, 30 s) annealing (52^oC, 30 s) and extension (72^oC, 1 min) and final extension (72^oC, 4 min). The interesting bands of PCR products were purified by using Seakem GTG agarose (Biowhittaker Molecular Application, USA) and were cloned in *E. coli* DH 5α. Sequencing was performed in both strands using an Applied Biosystem ABI prims 3100 Genetic Analyzer, USA.

3. RESULTS AND DISCCUSIONS

3.1. Xylanolytic enzymes of *A. oryzae*

The specific activities of these enzymes of *A. oryzae* P5 are shown in table 1. *A. oryzae* P5 have XDH activity 5.5 U/mg and 1.8 U/mg using NAD^+ and $NADP^+$ as cofactor, respectively. XR activities of mutant 5 were 11.3 U/mg and 13.4 U/mg by using NADH and NADPH, respectively. Xylanase, α-L arabinofuranosidase, β-xylosidase, xylose isomerase activities were 8.8 U/mg, 1.2 U/mg, 1.9 U/mg and 0.01 U/mg, respectively. The results showed that *A. oryzae* has xylanases to break down the backbone of xylan into smaller molecules and

Table 1. Some enzymatic activities of *A. oryzae*

Enzyme	Co-factor	Specific activities (U/mg Protein) P5 (wild-type)
Xylitol dehydrogenase	(NAD^+)	5.5
	($NADP^+$)	1.8
Xylose reductase	(NADH)	11.3
	(NADPH)	13.4
Xylose isomerase		0.01
Xylanase		8.8
β-xylosidase		1.9
α-L-arabinofuranosidase		1.2

α-L arabinofuranosidase, β-xylosidase to cut further the smaller molecules into D-xylose unit. In addition, *A. oryzae* has xylose reductase, which converts D-xylose into xylitol. Probably this strain can convert xylan to xylitol. Interestingly, xylose isomerase activity of *A. oryzae* P5 is quite low of only 0.01 U/mg. Therefore, conversion of D-xylose to xylulose is negligible. However, *A. oryzae* also has xylitol dehydrogenase, which converts xylitol in to xylulose. In *A. oryzae* NAD^+-dependent XDH was 3 times of $NADP^+$- dependent XDH.

3.2. PCR products

Aspergillus oryzae P5 has XDH activity 5.5 U/mg and 1.8 U/mg using NAD^+ and $NADP^+$ as cofactor, respectively, as mentioned above. Wittenveen et al. [20] had found that *A. niger* has both NAD^+-dependent and $NADP^+$- dependent XDH. Therefore, probably the genome of *A. oryzae* also has multiple XDH genes. Base on Genbank data base and the expressed sequence tag (EST) data base [8] four pairs of primers were designed AG, 34, 48, 54. From PCR products (Fig. 1), seven bands of PCR were cloned but only DNA sequences of 4 clear bands (each pair primer has one clear band) showed homologous with XDH genes of another strains (Table 2). The DNA sequences of fragments 48, 54 have higher homology with XDHs of *Galactocandida mastotermitis*, *Hypocea jecorina, Saccharomyces cerevisiae*. The fragment 48 and 54 were cloned.

Table 2. Primer sequences for four fragments: AG, 34, 48 and 54

Primer name	Sequences
AG-upper	5' - AGTGATAGCCGCCTTGATGG- 3'
AG-lower	5' - TGGATGCCGTGTTGTTGTGG- 3'
34- upper	5' - CCACATCAAACCGCATAATC - 3'
34- lower	5' - TTCCTCATCCACCACGACAT - 3'
48- upper	5' - CGCCACCCAAAACCGCTCAG - 3'
48-lower	5' - GGGCAGGACATAATACTTGG - 3'
54-upper	5' - ACCAACACCAACCACGACTT - 3'
54- lower	5' - GCGAGAGGCACACTGAGAGG - 3'

The cells were harvested after 3 days cultivation at 30°C in carbon-polypepton medium with shaking. Xylitol dehydrogenase [15], xylose reductase [19], xylanase [4], α-L-arabinofuranosidase [4], activities were measured as described previously.

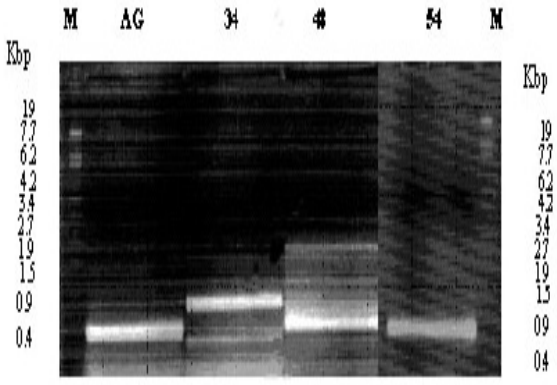

Figure 1. PCR products

M: marker, AG: AG fragment, 34: 34 fragment, 48: 48 fragment, 54: 54 fragment.

The results in table 3 showed that fragment 48 and 54 have high identity with XDH gene of other microorganisms. Base on DNA sequence of these fragments two gene had be cloned and expressed. Genes of fragment 48 and fragment 54 were designated as *xdhA* and *ladA* genes. XdhA encodes a xylitol dehydrogenase [17, 18] and *ladA* encodes a L- arabinitol 4-dehydrogenase. We had disrupted *xdhA* gene of this fungus by using restriction

enzyme-mediated integration (REMI) method. The mutant showed very lower activity of XDH comparing to the wild strain with NAD^+ as cofactors. However, $NADP^+$-dependent XDH activity was also found and maintained in the mutant (data not shown). From the results we concluded that *A. oryzae* has at least three XDH genes.

Table 3. Comparison DNA sequences of the four fragments with XDH genes from Genbank

Fragment name	Microrganisms	Gen- bank number	Identity (%)
Fragment AG	*Galactocandida mastotermitis*	AF072541	35
Fragment 34	*Galactocandida mastotermitis*	AF072541	34
	Pichia stipitis	X55392, AF127801	34
Fragment 48	*Galactocandida mastotermitis*	AF072541	**71**
(*xdhA*)	*Hypocea jecorina*	AF428150	**67**
	Saccharomyces cerevisiae	NC-001144	61
	Morganella morganii	L34345	55
	Pichia stipitis	X55392, AF127801	53
Fragment 54	*Galactocandida mastotermitis*	AF072541	**53**
(ladA)	*Agrobacterium tumefaciens*	NP53479, G98119	47
	Morganella morganii	AB308, AAL45112	44
	Pichia stipitis	L34345	44

Xylose reductase (XR) and xylitol dehydrogenase catalyze the initial catabolic pathway for xylose in yeast and fungi. The overall efficiency of xylose metabolism is connected through a complex regulatory network with the ability of XR and XDH to provide a high flux of carbon through the initial pathway. Therefore, XRs and XDHs are important target for metabolic engineering of yeast and fungi towards utilization of xylose in a manner that meets the requirement of biotechnological industry. Recently, some XR and XDH of yeasts were intensively studied [3,5, 9,11,12,13, 18, 15, 16,19]. But for fungi very few researches of XDHs were reported. *A. oryzae* is well known microorganism in traditional fermented food industry, therefore further studies on XDHs of *A. oryzae* would be open a new application of *A. oryzae* for another field of industry such as xylitol production or ethanol production.

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TÓM TẮT

HỆ THỐNG ĐA GIEN XYLITOL DEHYDROGENASE CỦA *ASPERGILLUS ORYZAE*

Aspergillus oryzae P5 có hoạt tính của một số enzim phân huỷ xylan như enzim xylanase 8,8 U/mg, α-L arabinofuranosidase 1,2 U/mg, β- xylosidase 1,9 U/mg và xylose reductase lần lượt là 11,3 U/mg và 13,4 U/mg. Tuy nhiên hoạt lực của xylitol dehydrogenase là 5,5 U/mg và 1,8 U/mg khi sử dụng NAD+ và NADP+ là cofactor. Trong hệ gien của *A. oryzae* có 4 đoạn có độ đồng nhất cao với các gen xylitol dehydrogenase của các chủng khác. Dựa trên trình tự của các đoạn này 2 gien đã được tách dòng. Một gen xdhA mã hoá cho enzym xylitol dehydrogenase với cofactor là NAD+. Khi làm bất hoạt gen này thì ở các mutant vẫn còn hoạt tính của xylitol dehydrogenase. Do vậy *A. oryzae* P5 c ó nhiều gien xylitol dehydrogenase.