Construction of pY-Af Vector for Expression of Thermostable α-L-Arabinofuranosidase in *Saccharomyces cerevisiae*

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Abstract

In this research, construction of expression vector for thermostable α-L-arabinofuranosidase in *Saccharomyces cerevisiae* BJ1824 was conducted. The *Escherichia coli*/*S. cerevisiae* shuttle vector, pYES2 was used as parental vector in construction. The *abfA* gene encoding α-L-arabinofuranosidase from *Geobacillus thermoleovorans* IT-08 was amplified by PCR, in which the plasmid pTP510 was used as a template. The amplification product was treated with *Sac*I and *Xho*I and then subcloned to the pYES2 vector, which was previously digested with *Sac*I and *Xho*I. The recombinant plasmid was designated as pY-Af and propagated first in *E. coli* Top10, and then transformed into *S. cerevisiae* BJ1824. For α-L-arabinofuranosidase (AbfA) production, the yeast transformants were grown in YNBG selective medium and YPG rich medium, using galactose as an inducer. The AbfA activity was assayed by measuring the amount of *p*-nitrophenol (pNP) released from *p*-nitrophenyl-α-L-arabinofuranoside (pNPA) substrate at pH 6.0 and 70°C for 30 min. The recombinant AbfA activity was detected in either of culture medium (0.98%), cell-associated (14.17%) and intracellular (84.85%) when recombinant yeast was grown in YPG rich medium.

Key words: α-L-arabinofuranosidase; *Saccharomyces cerevisiae*; expression vector

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Introduction

α-L-Arabinofuranosidases (α-L-arabinofuranosidase arabinofuranohydrolase, EC 3.2.1.55, AF) are accessory enzymes that cleave α-L-arabinofuranosidic linkages and act synergistically with other hemicellulases and pectic enzymes for the complete hydrolysis of hemicelluloses and pectins. The action of this enzyme alone or in combination with other lignocellulose degrading enzymes represents a promising biotechnological tool as alternatives to some of the existing chemical technologies such as chlorination in pulp and paper industry, synthesis of oligosaccharides and pretreatment of lignocelluloses for bioethanol production (Saha, 2000; Saha, 2003). A thermostable α-L-arabinofuranosidase gene (*abfA*) from *Geobacillus thermoleovorans* IT-08 (Gene-bank Accession No. DQ387046) was successfully cloned and expressed in *Escherichia coli* DH5α harboring plasmid pTP510 (Puspaningsih, 2004).

L-Arabinose is useful in preventing postprandial hyperglycemia in diabetic patients**.** Therefore, effective L-arabinose production is a vital prerequisite for its use in this respect as well as for its importance in food industry (Numan & Bhosle, 2006). To achieve this goal, it is necessary to use arabinose-releasing enzymes α-L-arabinofuranosidases that can be used safely in food industry. *E. coli* is not as safe commercial host for big scale enzyme production. Yeasts, mainly *Saccharomyces cerevisiae* has been used for centuries in food production and considered as Generally Recognized As Safe (GRAS). *S. cerevisiae* has been extensively used in industrial processes and it possesses a number of attributes that render it an attractive host for

the production of foreign proteins. *S. cerevisiae*, however, it can not degrade polysaccharides, including xylan (Kim *et al*, 2000). The advantage of *S. cerevisiae* is that it produces practically no hydrolases attacking polymeric (hemi)cellulosic substrates and that possible background activities against oligosaccharides can be easily checked (Clark *et al*, 1996).

The expression of α-L-arabino-furanosidase from *Aspergillus niger* (*abfB*) in *S. cerevisiae* was reported by Torres *et al* (1996). Cloning and expression of α-L-arabinofuranosidase from *A. niger* (*ABF2*) in *S. cerevisiae* was also published by Crous *et al* (1996). The α-L-arabinofuranosidase from *Trichoderma reesei* was cloned and expressed in di *S. cerevisiae* and secreted using signal peptide itself (Clark *et al*, 1996). The α-L-arabinofuranosidase from *G. thermoleovorans* IT-08 (*abfA*) has never been expressed in *S. cerevisiae.* We are interested to choose this enzyme because it has a thermostable characteristic that can be applied in industry. The aim of the present study was construction of the expression vector of *abfA* for *S. cerevisiae* system from a recombinant plasmid pTP510 containing the *abfA* gene.

Materials and Methods

Microbial strains, plasmid, and culture conditions. A yeast host strain used in this study was *S. cerevisiae* strain BJ1824 (*MATa ura3 trp1 leu2 pep4*). *E. coli* strain Top10 (Invitrogen, USA) was used for subcloning. The plasmid pTP510 that isolated from *E. coli* DH5α/pTP510 was used for the source of the *abfA* gene, which was originated from *G. thermoleovorans* IT-08 (Puspaningsih, 2004). For the construction of yeast expression vector, the plasmid pYES2 (Invitrogen, USA) was used as a parental plasmid (Figure 1). *E. coli* containing recombinant plasmid was cultured at 37°C in Luria–Bertani medium supplemented with 100 μg/ml ampicillin. Yeast host cells were grown at 30°C in YPD medium (2% Bactopeptone, 1% yeast extract, and 2% glucose). For the selection and maintenance of yeast transformants, YNBD selective medium (0.67% yeast nitrogen base without amino acids supplemented with appropriate nutrients and 2% glucose) was used. YNBG selective medium (0.67% yeast nitrogen base without amino acids supplemented with appropriate nutrients and 2% galactose) or YPG (2% Bactopeptone, 1% yeast extract, and 2% galactose) was used for the expression of α-L-arabinofuranosidase from yeast transformants using a pYES2 derivative.

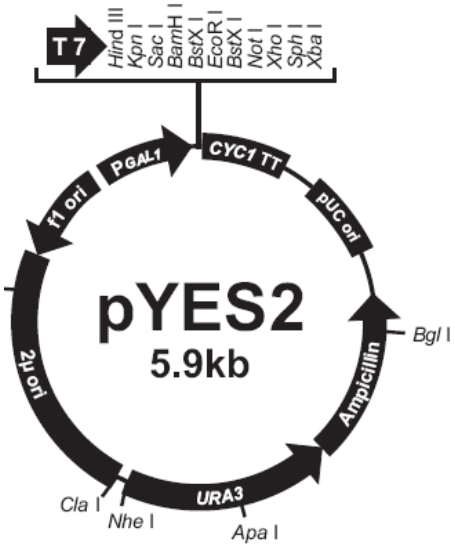

Figure 1. A physical map of plasmid pYES2 (Invitrogen, USA).

Subcloning and construction of expression vector. The *abfA* gene of 1.5 kb was amplified by PCR, in which the plasmid pTP510 was used as a template. For construction of *S. cerevisiae* expression vector, PCR was done with a foward primer pF*Sac*IAf (5'-GCGAGCTCATGGCT ACAAAAAAAGCAACC-3'), with a *Sac*I restriction cleavage site (underlined) and a reverse primer pR*Xho*IAf (5'-GCCTCGAGTTATCGTTTTCCTAAACGA ATCAC-3'), with a *Xho*I restriction cleavage site (underlined). After amplification, the *abfA* gene was treated with *Sac*I and *Xho*I and then subcloned to the pYES2 plasmid, which was previously digested with *Sac*I and *Xho*I. The recombinant plasmid was designated as pY-Af. This recombinant plasmid was introduced in *E. coli* Top10 using $CaCl_2$ method (Sambrook *et al*, 1989). After restriction analysis, the pY-Af was introduced into *S. cerevisiae* BJ1824 for the α-L-arabinofuranosidase (AbfA) production. Yeast transformation was carried out with a modified lithium acetate method as described

by Gietz (Gietz *et al*, 2002), followed by plating on YNB selective medium and incubation at 30°C for 48-72 h to recover transformants. Expression of AbfA was under the control of the inductive *GAL1* promoter. The vector also includes the *E. coli* ampicillin resistance gene and the yeast selectable marker URA3.

Expression of the *abfA* gene in *S. cerevisiae*. A single colony of *S. cerevisiae* BJ1824 harboring pY-Af was grown 1 day at 30°C in YNBD medium. The saturated cultured was then diluted 1 : 100 in 100 ml of YNBG or YPG medium, grown 6 days with shaking at 30°C, and measured the enzyme activity every 24h. The yeast culture broth was centrifuged, and then the supernatant (culture medium) and cells (cell-associated) were used for the measurement of extracellular α-L-arabinofuranosidase activity. Intrace-lullar crude extracts was prepared by glass beads treatment of cells. The enzyme activity was determined by the measuring change in absorbance at 410 nm due to *p*-nitrophenol (pNP, Sigma USA) release using a UV-Vis spectrophotometer. The α-L-arabinofuranosidase activity was assayed by measuring the amount of (pNP) released from *p*-nitrophenyl-α-L-arabinofuranoside (pNPA, Sigma USA) at pH 6.0 (in 50 mM phosphate buffer) and 70°C for 30 min. One unit of the enzyme activity was defined as the amount of enzyme liberating 1 μmol pNP from pNPA per min under assay condition (Puspaningsih, 2004).

Results

The 1.5-kb PCR product containing the α-L-arabinofuranosidase gene from *G. thermoleovorans* IT-08 was amplified from pTP510. An electrophoregram of PCR product is shown in Figure 2. This fragment was fused in-frame with the *E. coli*/*S. cerevisiae* shuttle vector pYES2 and introduced into *E. coli* Top10. The recombinant plasmid was designated as pY-Af. The pY-Af was treated with *Sac*I or *Xho*I and resulted one band 7.4 kb in size (Figure 2). This result was combination between insert 1.5 kb (*abfA* gene) and vector 5.9 kb (pYES2). A physical map of plasmid construction is showed on Figure 3. The pYES2 vector carries a 2μ as origin of replication and are maintained episomally in high copy. This vector also contains the *URA3* selection marker for selection in yeast.

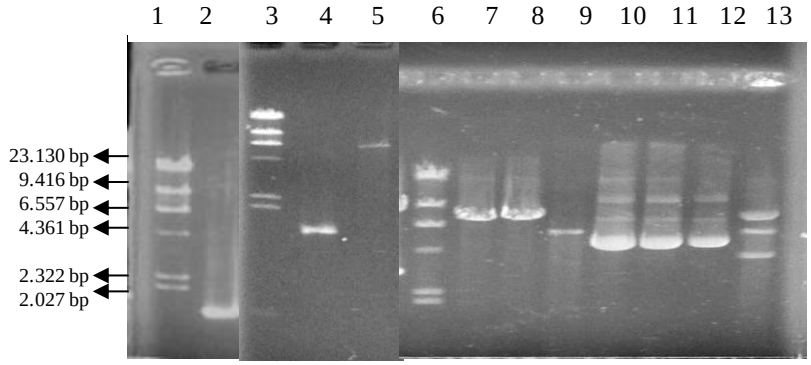

Figure 2. Marker (DNA λ was cut by *Hind*III) (1, 3, and 6), PCR amplification of a 1.5 kb fragment of the *abfA* gene (2) and after *Sac*I and *Xho*I digestion (4), pYES2 after *Sac*I and *Xho*I digestion (5), pY-Af was cut by *Sac*I (7) and *Xho*I (8), pYES2 was cut by SacI (9), uncut pY-Af (10, 11, and 12), and uncut pYES2 (13).

The recombinant plasmid, pY-Af was introduced into *S. cerevisiae* BJ1824 and obtained four yeast transformants. The expression analysis of AbfA was carried out in the YNBG (selective medium) and YPG (rich medium). Each single colony of yeast transformants was cultivated 6 days at 30°C with shaking 150rpm. Every 24 hours in 6 days cells cultures were taken for activity assay in the grown medium. Last day the yeast culture broth was centrifuged and the activity of AbfA was measured in the three fractions (culture medium, cell-associated, and intracellular). The expression analysis of AbfA was also conducted in *S. cerevisiae* BJ1824 harboring plasmid pYES2 for control. The

results of the expression analysis of AbfA were presented at Table 1 and Table 2.

Table 1. The expression analysis of the AbfA enzyme in *S. cerevisiae* BJ1824/pY-Af and *S. cerevisiae* BJ1824/pYES2 in the YNBG selective medium.

Transformant	The activity of AbfA enzyme in the culture medium (U/ml) on to (day):					The activity of AbfA enzyme (U/mL) on the final day (6^{th} day)		
						Extracellular		Intracellular
	1^{st}	2^{nd}	3^{rd}	4^{th}	5^{th}	Culture medium	Cell-associated	
A	nd	nd	nd	nd	nd	nd	0.008 (1.17%)	0.673 (98.83%)
B	nd	nd	nd	nd	nd	nd	0.011 (1.59%)	0.681 (98.41 %)
C	nd	nd	nd	nd	nd	nd	0.014 (1.88%)	0.729 (98.12%)
D	nd	nd	nd	nd	nd	nd	0.010 (0.66%)	0.648 (99.34%)
Control	nd	nd	nd	nd	nd	nd	nd	nd

nd = not detected.

Table 2. The expression analysis of the AbfA enzyme in *S. cerevisiae* BJ1824/pY-Af dan *S. cerevisiae* BJ1824/pYES2 in the YPG rich medium.

Transformant	The activity of AbfA enzyme in the culture medium (U/ml) on to (day):					The activity of AbfA enzyme (U/mL) on the final day (6^{th} day)		
						Extracellular		Intracellular
	1^{st}	2^{nd}	3^{rd}	4^{th}	5^{th}	Culture medium	Cell-associated	
A	nd	Nd	0,022	0,085	0,016	0,016 (1,00%)	0,272 (16,98%)	1,314 (82,02%)
B	nd	Nd	0.031	0,072	0,010	0,014 (0,90%)	0,249 (16,11%)	1,283 (82,99%)
C	nd	Nd	0.035	0,089	0,009	0,011 (0,75%)	0,186 (12,72%)	1,265 (86,52%)
D	nd	Nd	0,015	0.064	0,093	0,019 (1,27%)	0,162 (10,87%)	1,310 (87,86%)
Control	nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd

nd = not detected.

The AbfA activity was only detected about 0.66-1.59% from total AbfA activity in cells-associated when *S. cerevisiae* BJ1824 harboring pY-Af was cultivated in the selective medium (YNBG) after 6 days incubation. The intracellular AbfA activity was higher than extracellular fraction (culture medium and cells-associated), when this yeast was grown in the selective medium and also in the rich medium after 6 days incubation. When recombinant *S. cerevisiae* BJ1824/pY-Af was cultivated in the rich medium (YPG), the AbfA activity was detected about 0.75-1.27% and 10.87-16.98% from total AbfA activity in culture medium and cells-associated after 6 days incubation, respectively.

Discussion

The expression of α-L-arabinofurano-sidase in the recombinant *S. cerevisiae* BJ1824 harboring pY-Af could be detected particularly in the intracellular fraction after 6 days incubation., when this yeast was cultivated in the medium containing galactose both selective and rich (non-selective) medium used in this study.

The expression of the *abfA* gene in the pY-Af was under the control of the inductive *GAL1* promoter (Figure 3). The AbfA expression was occured if *GAL1* promoter was induced by galactose in growth medium This promoter was repressed by glucose molecule. If there was glucose in the growth medium, gene expression with the promoter *GAL1* will be pursued. The advantage of this regulation system is organizable expression periodically (Mylin *et al*, 1990).

The *GAL1* is one of the regulated promoters that can be controlled by controlling the availability of certain nutrients. This allows to augment yeast cell mass prior to heterologous gene expression, so that the cell population can be optimized before the regulated promoters are turned on (Feldmann, 2005).

Compared with selective YNBG medium (0.648-0.729 U/mL) in intracellular fraction, about two-fold higher intracellular α-L-arabinofuranosi-dase activity was achieved in rich YPG medium (1.263-1.314 U/mL). YPG medium is cheaper than YNBG medium. Base on this result it can be considered in the application study for the industry.

The activity of α-L-arabinofuranosida-se was also detected in culture medium in low level and in cell-associated when the recombinant *S. cerevisiae* BJ1824/pY-Af was grown in the rich YPG medium. These results

were interesting, because base on prediction of signal peptide using the program Signalp 3.0 server (online at http://www.cbs.dtu.dk/services/SignalP/) showed that the AbfA popypeptide was not found a signal peptide at the end of N-terminal.

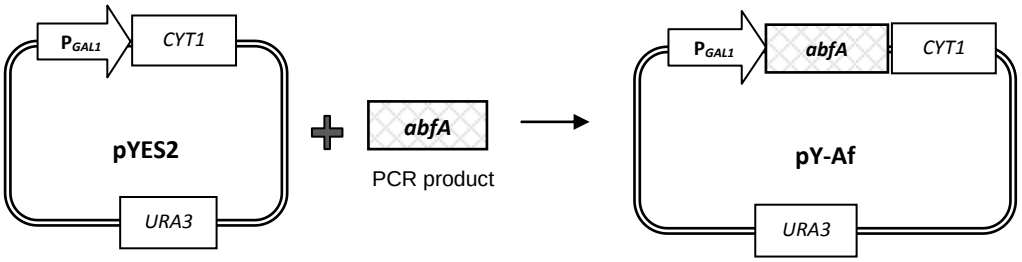

Figure 3. A physical map of plasmid construction in this research. The plasmid pYES2 is as parental vector.

Protein secretion in yeast is a complex process and there is no generally accepted signal peptide which directs secretion. Although several foreign proteins could be secreted under the direction of their own signals, homologous signal peptides were much more successful and could result in highly expressed heterologous proteins recoverable from the extracellular medium (Feldmann, 2005). Signal peptide mediates co-translational translocation into the endoplasmic reticulum. The activity of AbfA could be detected in culture medium in low level, perhaps because lysis cells had been occurred.

The AbfA in *G. thermoleovorans* IT-08 was secreted to growth medium, but the AbfA in *E. coli* DH5α/pTP510 was produ-ced as an intracellular enzyme (Puspa-ningsih, 2004). In this research, the AbfA in the recombinant *S. cerevisiae* BJ1824/pY-Af was expressed intracellu-larly. The Abfa was only secreted in low level in YPG rich medium. Levansucrase, a *Bacillus subtilis* extracellular enzyme, was not secreted in the culture medium when produced in yeast (Scott *et al*, 1996). In this study, the secretion of AbfA in *S. cerevisiae* BJ1824/pY-Af was still low. The secretion pathway is complex. Numerous efforts have been made to elevate secretion level, including overexpression of molecular chaperones and foldases, overexpression of genes associated with the secretory pathway, optimization of gene copy num-ber, manipulation of promoter strength, engineering of the leader sequence, engineering of the expressed protein, and optimization of the expression conditions. Although most of these approaches can be successful in producing higher levels of the particular proteins of interest, results tend to be protein-specific (Huang *et al*, 2008).

Conclusions

In conclusion, pY-Af expression vector that containing *abfA* was constructed. The AbfA was expressed particularly in the intracellular fraction in the recombinant *S. cerevisiae* BJ1824 harboring pY-Af. In the next research, we are going to construct the *S. cerevisiae* secretion vector for AbfA with signal peptide addition.

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