2011 International Conference on Food Engineering and Biotechnology
IPCBEE vol.9 (2011) © (2011)IACSIT Press, Singapore

Surface display of a bacterial amylase enzyme on yeast

Nadzarah A.Wahab [1], Zainon Mohd Noor [1] and Mohamad Faiz Foong Abdullah [1] +

[1] Dept of Biomolecular Science, Faculty of Applied Sciences
Universiti Teknologi MARA Malaysia

Abstract. An E.coli-yeast shuttle vector for the anchoring of heterologous protein to the yeast host's cell wall was constructed using the backbone from pGAD424. A construct comprising the signal sequence from the yeast sucrose isomerase gene (SucSg) , a multiple cloning site sequence and a DNA fragment encoding 67 amino acids from the carboxyl -terminal of the yeast cell wall protein 2 (CWP2) was constructed in vitro. The construct was designed such that a gene sequence cloned into the MCS will be translated in-frame with the SucSg and CWP2. The construct was then inserted into the HindIII site on pGAD424, replacing the GAL4 fusion tag and the original MCS sequence. DNA sequencing confirmed the correct insertion of both signal and anchor proteins in the vector. A bacterial amylase gene was cloned into the vector and tested for functionality.

Keywords: surface display,bacterial amylase, yeast, pGAD424.

1. Introduction

Display of heterologous protein on microorganisms has been reported since 1980's as a replacement to its current display system on bacteriophage due to size limitation of attached protein. The concept of protein display on the yeast *Saccharomyces cerevisiae* has been introduced few decades ago [1]. Methods for anchoring proteins onto the yeast's cell wall by using either agglutinin or flocculin has been used in displaying enzymes such as amylase and glucoamylase [1,2], cellulolytic enzymes [3, 4, 5] and lipase [6], and also displaying histidine oligopeptide (Hexa-His) for chelating heavy metal ions [7,8] and displaying the endocrine disruptor group of proteins [9]. Other application include the displaying of fluorescent protein as a reporter gene [10], protein A (SpA) for immunoglobulin binding [11], functional hetero-oligomeric protein such as antibody Fab fragment [12] and also combinatorial protein library by means of random DNA priming [14]. Surface display permits more than single modification to be carried-out on the subject protein, and some has reported a combination of protein engineering and display system e.g. when lipase of *R.oryzae* origin was displayed on cell surface of *S.scerevisiae* in organic solvents, the catalytic activity was several folds higher than the original construct [15]. *S.cerevisiae* is an ideal host system for surface display as the organism has GRAS status which allows its safe usage in food and drugs application, its complete genome sequence is known, it is genetically tractable and it possesses protein-folding and secretory machineries similar to mammalian cells. In this paper we report the purification, cloning and expression of a bacterial amylase enzyme in a newly constructed vector pYDSM01.

2. Experimental

2.1. Construction of pYDSM01

The design of vector is based on the plasmid pGAD424, a yeast expression vector (Clontech, USA). The plasmid was harvested using a commercial extraction kit (QIAGEN, USA). The original size of the plasmid is 6.6 kb. The plasmid was digested with the restriction enzyme *HindIII* (NEB, UK) to remove the original

+ Corresponding author. Tel.: + (603-55444547); fax: +(603-55435576).
E-mail address: (mohdf184@salam.uitm.edu.my).

GAL4 AD domain fusion tag and the multiple cloning site (MCS). This resulted in a linearised plasmid of 6.0 kb with *HindIII* overhangs. This fragment was resolved on a 0.8% agarose gel by electrophoresis and recovered using a gel extraction kit (QIAGEN, USA). The linearised plasmid was then dephosphorylated using a Antartic phosphatase (NEB).

We have chosen to use the *S.cerevisiae* sucrose isomerase signal sequence for the signal sequence, which is necessary to ensure that the expressed protein is efficiently transported out of the cell, and the C-terminal of the *S.cerevisaie* Cell Wall Protein 2 *(CWP2)* as the anchor sequence [16]. The DNA sequence for the corresponding gene, *CWP2*, was downloaded from the public domain www.yeastgenome.org, and primers were designed to amplify a fragment coding for 67 amino acids on the C-terminal of *CWP2*. The sequence for the forward primer (YS1F) also include an in-frame multiple cloning site sequence and a section of the signal sequence. The reverse primer (YS2R) targets the partial *CWP2* sequences and includes a flanking *HindIII* restriction site.

Yeast genomic DNA was extracted using QIAGEN's Dneasy kit and used as the target DNA. The required fragment was generated by polymerase chain reaction (PCR) with a denaturation temperature of 94°C for 15 seconds, annealing at 56°C for 30 seconds, extension at 68°C for 1 minute, for 30 cycles on an Eppendorf Mastercycler. Oligonucleotides synthesis and sequencing reactions were performed using a third party service provider (Research Biolabs). The resulting amplification product from the first amplification contains part of the 3' end of the sucrose signal sequence, an in-frame MCS and sequences coding for the N-terminal 67 amino acids of Cwp2p.

A second set of primers was used to make the complete insert. The forward primer (YS3F) consists of most of the 5' end of the sucrose signal sequence. The internal *HindIII* site in the signal sequence was abolished by site-directed mutagenesis by substituting A for T in the fifth codon.The resulting mutation is silent. A flanking *HindII* site was added immediately before the start codon. The reverse primer (YS4R) is similar to YS2R. The PCR product was purified and verified by sequencing. The fragment was then digested with *HindIII* and ligated into the linearised pGAD424 backbone. Post ligation, the reaction mix was transformed into *E.coli* DH5α cells.

Screening was carried-out by plating on Luria-Bertani (LB) agar plates supplemented with ampicillin (100mg/l). The resulting vector PYDSM01 was recovered from positive transformants and verified by restriction enzyme mapping and DNA sequencing.

2.2. Amplification of bacterial amylase

Bacillus subtilis ATCC 6633 was use as the source of bacterial amylase. Genomic DNA was extracted using QIAGEN's DNeasy kit. PCR was carried out using primers BA1F and BA2R targeting the entire ORF minus the start and stop codons. PCR was performed at 94°C for 15 seconds, annealing at 55°C for 30 seconds, extension at 68°C for 3 minutes, for 30 cycles on an Eppendorf Mastercycler;

Table 1. Primers used in this study (restriction sites are italicized)

YS1F	ATC GAG AAT TCC CGG GGA TCC GTC GA C CTG CAG AGA TCT ATA TTT CTC AAA TCA CTG ACG GTC
YS2R	ATCGA*AAGCTT*TTATAACAACATAGCAGCAGCAG
YS3F	ACATCG*AAGCTT*ATGCTTTTGCAAGCATTCCTTTTCCTTTTGGCTGGTTTT
YS4R	ATCGA*AAGCTT*TTATAACAACATAGCAGCAGCAG
BA1F	ATCGTT*GGATCC*GCCTTACAGCACCGTCGATCAAAA
BA2R	ATCGAA*GGATCC*GTCTTTTAACAAAACTGTATTTCTCGG

2.3. Cloning of bacterial amylase into pYDSM01

The amplified fragment was gel purified using a commercial kit (Qiagen) and verified by sequencing. Following that it was cloned into pYDSM01 at the *BamHI* site in the MCS. Post ligation, the reaction mix was transformed into DH5 alpha cells as previously described. Plasmids were recovered from positive transformants and the insertion was verified by sequencing. The recombinant plasmid was transformed into a *Δleu2* yeast strain according to the method of Gietz (1992). Yeast transformants were selected on synthetic complete media lacking leucine.

2.4 Screening for yeast expressing bacterial amylase

Yeast colonies from the synthetic media were subcultured and individually grown in 96-well plates in 100 ul of YEPD. For screening, 100 ul of 1% starch solution were added to each well and incubated for 1 hour at 30°C. A drop of Gram's iodine was added to detect for residual starch. Transformant displaying amylase activities were selected. These were then grown in YEPD supplemented with 100mg/l ampicillin overnight at 30°C to rule out bacterial contamination. The cells were pelleted by centrifugation and washed twice, and resuspended in phosphate buffer (pH 7). Both the washed cells and the culture supernatant were then assayed for amylase activity by assaying for the production of free glucose from starch. Glucose assay was carried-out by using the AMPLEX Glucose kit (INVITROGEN, USA). All samples were treated as described before and carried-out following the manufacturer's manual. Extracted plasmid from previous positive transformants was used to transformed into a *Δleu2* yeast strain according to the method of Gietz (1992). Yeast transformants were selected on synthetic complete media lacking leucine.

3. Results and Discussion

3.1 Vector construction

The size of the undigested plasmid is 6.6kb by comparison with the molecular markers, which is consistent with documented reports. After removal of the multiple cloning site and AD fusion tag, the size of the plasmid is 6kb, which is as expected. Sequencing result confirms the fragment as the carboxyl -terminal region of Cwp2 by comparison with the gene database at National Centre for Biotechnology Information (NCBI). Figure 1 shows the site-directed mutagenesis strategy used in this work.

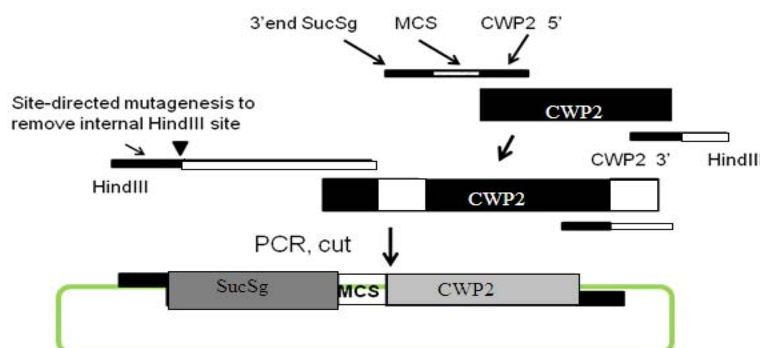

Figure 1. The schematic diagram of vector construction.

3.2 Vector ligation

Transformants obtained were subjected to restriction enzyme mapping to check if they are the desired plasmid and to eliminate unreacted pGAD424 or the recircularised vector backbone. Putative clones were sent for sequencing. Clones that were verified to contain the entire construct in the correct orientation were then sequenced again using primers external to the insert to verified that no cloning or PCR-induced mistakes has been introduced.. We have thus successfully recovered several clones of the new vector (pYDSM01).

3.3 Cloning and surface display of a bacterial amylase enzyme

The ORF of *B.subtilis* amylase, minus the start and stop codons with flanking restriction sites was successfully amplified and the sequences were verified by comparison with the NCBI database. The gene was cloned into the MCS of pYDSM01. DNA sequencing confirms the correct orientation reading frame of the insert. The resulting plasmid construct was transformed into a yeast host.

3.4 Preliminary screening of transformants

Yeast transformants were first selected for the presence of amylase activity using a simple starch hydrolysis assay. Amylase modifies starch by hydrolysing alpha-1, 4-glycosidic linkages, theoretically by attaching the amylase enzyme on the yeast surface, it will degrade starch into simple sugar. Fig. 2 shows an example of a 96-well microtiter plate results of transformants with amylase enzyme. Clear, or very light

purple wells can be seen at A7, C2 and C5. Altogether, 22 transformants which tested positive for starch hydrolysis were selected for further characterisation.

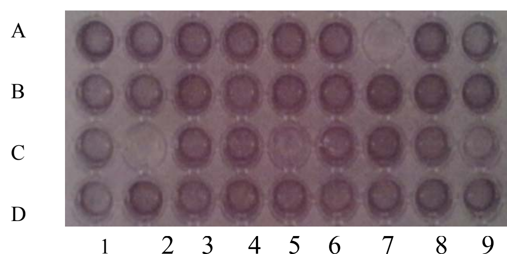

Figure 2. Screening for yeast transformants displaying amylase activity. Wells A1 to D1 contain yeast cells transformed with pGAD424 as controls. Wells C2, C5 and A7 shows reactions where the starch has been hydrolysed, indicating transformants with amylase activity. Ampicillin at 100ug/ml were added to all transformant cultures during the growth phase, to rule out bacterial contamination.

3.5 Glucose assay

Table 2 shows results from glucose assay of the amylase activity of the cell and supernatant fractions from selected transformants. The transformants were grown in YEPD, and centrifugation was used to separate the cell and supernatant. The cell pellet was washed and resuspended in PBS. Amylase activity was measured in three replicates for each transformant and the results averaged. Sample Y0 was the original yeast host transformed with the unmodified pGAD424 vector. No amylase activity was detected in either the cell or supernatant fractions. For transformants Y14 and Y22, amylase activity was detected only in the supernatant fractions. These represent transformants in which the amylase gene was successfully expressed and secreted out of the cell, but the fusion protein was not incorporated into the yeast cell wall. For transformants Y5, Y7 and Y9, the amylase activity was present mostly on the cell pellet. These represents transformants in which the recombinant construct was successfully expressed, secreted and anchored correctly on the cell wall. In one transformant (Y2), amylase activity was found on both the cell pellet and supernatant fractions. No activity was detected from transformant Y18, which showed positive results in the preliminary screening.

Table 2. Glucose amount relative to control.

Sample	**Glucose amount (%)**	
	Cell suspension	***Supernatant***
Y2	61.1	46.2
Y5	81.3	Not detected
Y7	30.0	Not detected
Y9	6.7	Not detected
Y14	Not detected	22.2
Y18	Not detected	Not detected
Y22	Not detected	26.3
Y0	Not detected	Not detected

The recovery of three types of transformants indicate that pYDSM01 is able to expressed a cloned gene fused to a signal sequence and a C-terminal tag that anchors to the cell wall of a yeast host. However, expression appears to be unstable in some transformants. Some transformants also failed to have the fusion protein displayed on the cell wall, although enzyme activity was detected, indicating successful expression. This variabilty expression and anchoring can be due to a number of factors e.g instability of the recombinant plasmid, variable promoter activity or premature truncation of the fusion protein resulting in lost of enzyme activity or the Cwp2 tag.

It is also possible that we tried to expressed bacterial enzyme, which may not be fully functional in the yeast host cell. The yeast strain used in the experiments contain a meiosis-specific glucoamylase gene, and has been tested extensively for background amylase activity, which was found to be absent. We are at

present attempting to expressed this gene in pYDSM01. Construction of a yeast strain with stable amylase activity will have good potential in industrial biotechnology [2].

4. Conclusion

We have thus far successfully built a new vector pYDSM01 that contains the backbone of an *E.coli*-yeast shuttle vector. We inserted a construct that will allow cloning of a gene protein. This gene protein, the alpha amylase protein of *B.subtillis* has been successfully incorporated into the newly built vector. Through functionality experiments, expression of the resulting protein was found to be located at the cell wall.

5. Acknowledgement

The authors would like to thank the RMI, UiTM for a short term grant and the NSF, MOSTI for a scholarship to Nadzarah A.Wahab.

6. References

[1] M.P. Schreuder, S. Brekelmans, H. van den Ende and F. M. Klis, Construction of a starch-utilizing yeast by cell surface engineering. *Yeast.* 1993, **9** :399 – 409

[2] T. Murai, M. Ueda, M. Yamamura, H. Atomi, Y. Shibasaki, N. Kamasawa, M. Osumi, T. Amachi and A. Tanaka. Construction of a starch – utilizing yeast by cell surface Engineerin. *Appl Environ Microbiol*. 1997a, **63**: 1362 – 66

[3] T. Murai et al., Genetic immobilization of cellulase on the cell surface of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol*. 1997b, **48** :499 – 503

[4] T. Murai, M. Ueda, T. Kawaguchi, M. Arai and A. Tanaka. Assimilation of cellooligosaccharides by a sell Surface- engineered yeast expressing β- glucosidase and carboxymethycellulase from *Aspergillus aculeatus*. *Appl Environ Microbiol.* 1998**, 64:**4857 – 61

[5] Y. Fujita et al., Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes. *Appl Environ Microbiol* 2002, **68**:5136-5141.

[6] T. Matsumoto, M. Ito, H. Fukuda and A. Kondo. Enantioselective transesterification using lipase –displaying yeast whole-cell biocatalyst. *Appl Microbiol Biotechnol* 2004, **64**: 481-485

[7] K. Kuroda and M. Ueda. Bioadsorption of cadmium ion by cell surface-engineered yeasts displaying metallothionein and hexa-His. *Appl Microbiol Biotechnol* 2003, **63**: 182-186

[8] K. Kuroda, S. Shibasaki, M. Ueda and A. Tanaka. Cell surface-engineered yeast displaying a histidine oligpeptide(hexa-His) has enhanced adsorption of and tolerance to heavy metal ions. *Appl Microbiol Biotechnol.* 2001, **57**: 697-701

[9] E. J. Routhledge and J. P. Sumpter. Structural features of alkylphenolic chemicals associated with estrogenic activity. *J Biol Chem* 1997, **272**:3280-3288

[10] S. Shibasaki, M. Ueda, K. Ye, K. Shimizu, N. Kamasawa, M. Osumi and A. Tanaka. Creation of cell-surfaced-engineered yeast that display different fluorescent proteins in response to the glucose concentration”. *Appl Microbiol Biotechnol.* 2001, **57**:528-533

[11] Y. Nakamura, S. Shibasaki, M. Ueda, A. Tanaka and H. Fukuda. Development of novel whole-cell immunoadsorbents by yeast surface display of the IgG-binding domain. *Appl Microbiol Biotechnol.* 2001, **57** :500-505

[12] Y. Lin, , et al. Display of a functional hetero-oligomeric catalytic antibody on the yeast cell surface. *Appl Microbiol Biotechnol* 2003, **62**:226-232

[13] W.Zou, M. Ueda and A. Tanaka. Screening of a molecule endowing *Saccharomyces cerevisiae* with n –nonane-tolerance from a combinatorial random protein library. *Appl Microbiol Biotechnol* 2002, **58:**806-812

[14] E.T. Boder and K.D. Wittrup, Yeast surface display for screening Combinatorial polypeptide libraries. *Nat Biotechnol.* 1997, **15**, pp. 553 -7

[15] S. Shiraga, M. Kawakami, M.Ishiguro and M. Ueda. Enhanced reactivity of *Rhizopus oryzae* lipase displayed on yeast cell surfaces in organic solvents: potential as a whole-cell biocatalyst in organic solvents. *Appl Environ Micronbiol* 2005, **71**:4335-4338

[16] J. M. Van Der Vaart, R.T.Biesebeke,J. W. Chapman, H.Y. Toschka, F.M. Klis,T.Verrips. Comparison of cell wall proteins of *Saccharomyces cerevisiae* as anchors for cell surface expression of heterologous proteins. *Appl. Env Microbiol.* 1997, **63**: 615-620