Expression cassette and plasmid construction for Yeast Surface Display in *Saccharomyces cerevisiae*

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Abstract

Objectives Develop a Cell Surface Display system in *Saccharomyces cerevisiae*, based on the construction of an expression cassette for pYES2 plasmid.

Results The construction of an expression cassette containing the α-factor signal peptide and the C-terminal portion of the α-agglutinin protein was made and its sequence inserted into a plasmid named pYES2/gDαAgglutinin. The construction allows surface display of bovine herpesvirus type 5 (BoHV-5) glycoprotein D (gD) on *S. cerevisiae* BY4741 strain. Recombinant protein expression was confirmed by dot blot, and indirect immunofluorescence using monoclonal anti-histidine antibodies and polyclonal antibodies from mice experimentally vaccinated with a recombinant gD.

Conclusions These results demonstrate that the approach and plasmid used represent not only an effective system for immobilizing proteins on the yeast cell surface, as well as a platform for immunobiotics development.

Keywords *Saccharomyces cerevisiae* · Yeast Surface Display · Glycoprotein D · pYES2/ gDαAgglutinin

Introduction

Cell Surface Display is a technique developed for recombinant protein expression in heterologous systems, such as bacterial, insect and yeast cells (Bertrand et al. 2016). Its application allows immobilization of peptides, whole proteins, or small fractions of antibodies on the cell surface by binding with an anchor protein (Tanaka and Kondo 2015). The attached target retain activity and stability, allowing them to interact with specific molecules present in the extracellular environment (Ueda 2016). *Saccharomyces cerevisiae* is the most used yeast for Cell Surface Display, being used for different purposes such as: the production of bioethanol, chemicals synthesis, and more recently,
development of oral vaccines (Çelik and Çalık 2012; Parapouli et al. 2020).

The commercial availability of plasmid vectors for Yeast Surface Display (YSD) is generally based on options that depend of two main anchor proteins: a-agglutinin and α-agglutinin. The a-agglutinin anchoring system consists of its Aga1p and Aga2p subunits forming a protein complex, both being linked via two disulfide bonds, in which Aga1p subunit is responsible for cell wall attachment via GPI (Glycosylphosphatidylinositol) anchor and Aga2p is used to fuse target proteins (Kuroda and Ueda 2013). On the other hand, α-agglutinin system was developed based only on the C-terminal half of α-agglutinin, which is a fraction of the GPI-anchored cell wall protein able to immobilize fused proteins and expose them to the extracellular environment (Kuroda and Ueda 2013; Ueda 2016). The application of these systems are directly related to recombinant protein immobilization and their stability in YSD (Yang et al. 2019). Thus, new plasmids able to facilitate protein anchoring should be sought as possible alternative to implement this method. This techniques success depends on the choice of a plasmid, which should be able to allow the protein expression in the correct folding (similar to natural form) and requires an easy way to reproduce it (Routledge et al. 2016). YSD plasmid vectors are usually composed of a eukaryotic promoter, a multiple cloning site, selection markers, secretion factor, eukaryotic origin of replication (if not an integrative plasmid), and may contain tags to facilitate protein identification or purification (Çelik and Çalık 2012; Baghban et al. 2019). One of the alternatives for choosing the right plasmid for YSD is the construction of an expression cassette that has all the necessary components and allows the integration with conventional plasmids (Nasser et al. 2003).

Immunogenic potential of glycoprotein D (gD) of bovine herpesvirus type 5 (BoHV-5) has been studied by our group for some years, expressed recombinantly (rgD) using Pichia pastoris and Escherichia coli expression systems. In previous studies, our group have showed that rgD used as a vaccine antigen, was able to induce high levels of neutralizing antibodies titers in mice and cattle (Araujo et al. 2018). An ELISA test (Enzyme-Linked Immunosorbent Assay) composed of rgD demonstrated protein recognition by the serum of naturally infected animals (Dummer et al. 2016). Based on the knowledge using gD in our laboratory, we chose it as a model protein for the present work.

In this study, we first constructed an expression cassette using the C-terminal half of α-agglutinin anchor protein for YSD, in which several components were added for an efficient heterologous protein expression. It was constructed for pYES2 (Invitrogen) plasmid insertion, which allowed its use for transformation of S. cerevisiae BY4741. Glycoprotein D gene sequence was inserted on the cassette and protein expression was induced by GAL1 promoter, then rgD was immobilized on yeast surface by α-agglutinin and exposed to extracellular medium. We were able to identify rgD expressed and displayed on S. cerevisiae using Dot blot and Immunofluorescence techniques, what confirmed the effectiveness in establishment of YSD protocol.

**Material and methods**

**Construction of pYES2/gDαAgglutinin displaying vector**

Using Vector NTI Advance software (Invitrogen), the construction of gDαAgglutinin cassette was made aiming its expression on plasmid pYES2. The first cassette component added was the α-factor signal peptide sequence, which directs the processed protein for secretion into the extracellular medium. A sequence encoding 6 histidine amino acids (6XHis-tag) and a linker composed by Ser/Gly amino acids was also added. Finally, the sequence of the C-terminal half of α-agglutinin and the stop codon (TAA) were added to the construct.

The gene sequence of Bovine herpesvirus type 5 glycoprotein D was obtained from GenBank (accession number AAA67359.1). This protein is composed of transmembrane regions, cytoplasmic domain, signal sequence and extracellular domain. However, only the extracellular domain (a region composed of 311 amino acids) was selected and used in this construction. A sequence encoding 6 histidine amino acids (6XHis-tag) and a linker composed by Ser/Gly amino acids was also added. Finally, the sequence of the C-terminal half of α-agglutinin and the stop codon (TAA) were added to the construct.

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number MW556769. Nucleotide sequences added to the expression cassette are shown in Table 1. The final sequence was sent for synthesis by GenOne Company (Rio de Janeiro, Brazil), which also constructed the plasmid pYES2/gDαAgglutinin vector for YSD.

Strains, yeast transformation and cell culture

Escherichia coli TOP10F° {lacIq Tn10 (TetR)} mcrA, Δ(mrr-hsdRMS-merBC), Φ80lacZAM15, ΔlacX74, endA1, recA1, araD139, Δ(ara,leu) 7697, galU, galK, supG, recP (StrR) (Invitrogen), was used for transformation of the Plasmid pYES2/gDαAgglutinin. Briefly, were used CaCl₂ 100 mM, one colony of E. coli TOP10F° and 100 ng of pYES2/gDαAgglutinin or pYES2 plasmid. The sample was kept under an ice bath for 20 min, after that it was incubated for 1 min at 42 °C and, subsequently, 2 min ice bath again. Transformed E. coli were grown in liquid Luria Bertani (LB) medium with ampicillin 100 μg/mL, under agitation (150 rpm) for 16 h at 37 °C. Plasmid extraction was performed using alkaline lysis, described in Sambrook and Russel (2001).

The length comparison between plasmids pYES2 and pYES2/gDαAgglutinin was performed by electrophoresis in a 0.8% w/v agarose gel, on TBE buffer (Tris–Borate–EDTA).

Saccharomyces cerevisiae BY4741 strain (MATα his3-1 leu2 met15 ura3) was selected and transformed by electroporation following MicroPulser™ (BioRad) electroporator protocol. Different concentrations of both plasmids (100 ng, 1500 ng, 3000 ng and 6000 ng) were added to 40 μL of competent cells (1 × 10¹⁰ cells/mL) and subjected to electroporation in 0.2 cm cuvettes, with pulses of 5 ms and 1.5 kV voltage in the MicroPulser™ electroporator. After electroporation, the electroporated material was suspended in 1 mL of 1 M sorbitol, and 100 μL was plated on Sc-U agar medium [2 g/L agar, 2 g/L dextrose, 6.7 g/L yeast nitrogen base w/o amino acids and 1.9 g/L yeast synthetic dropout medium supplement (Sigma Aldrich)], being incubated over 72 h at 30 °C.

Recombinant colonies of S. cerevisiae BY4741 were selected and cultured in liquid Sc-U medium with glucose during 20 h at 30 °C, under agitation (150 rpm). Upon reaching a biomass of 3 (O.D.₆₀₀nm), the culture was centrifuged (1,500×g during 5 min) in a DTR16000 centrifuge (DAIKI), washed three times and suspended in liquid Sc-U containing galactose for GAL1 promoter induction, in a final biomass of 0.4 D.O. at 600 nm. The induction step was maintained over 24 h in the same galactose-containing medium culture conditions, and later centrifuged (1,500×g for 5 min) to separate biomass. All stages of the expression process were also performed with a BY4741 sample transformed with pYES2 plasmid as a control culture.

Table 1 Components used to construct gDαAgglutinin expression cassette

| Component | Nucleotide sequence |
|-----------|---------------------|
| α-Factor secretion signal | ATGAGTTCACAGAATTCAGCTGCGCCTGATCTTGGTTACCTGAGCCGTCGACATTAAGACACCAAGGATGAAACTGCTGCAATCTCAGTCAAGAGAGGTCC |
| LacZ tag | CATACATCAAGACAGAGGAGTTCACAGAATTCAGCTGCGCCTGATCTTGGTTACCTGAGCCGTCGACATTAAGACACCAAGGATGAAACTGCTGCAATCTCAGTCAAGAGAGGTCC |
| μg/6 glycoprotein D (extracellular domain) | TGTACGACAGAGGAGTTCACAGAATTCAGCTGCGCCTGATCTTGGTTACCTGAGCCGTCGACATTAAGACACCAAGGATGAAACTGCTGCAATCTCAGTCAAGAGAGGTCC |
| Linker | TCAAGAGGAGTTCACAGAATTCAGCTGCGCCTGATCTTGGTTACCTGAGCCGTCGACATTAAGACACCAAGGATGAAACTGCTGCAATCTCAGTCAAGAGAGGTCC |
| Terminus half α-Agglutinin | TAA |

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The possibility of extracellular expression or non-attachment on yeast surface was evaluated through medium supernatant purification. The supernatant from \textit{S. cerevisiae} BY4741 culture expressing immobilized gD was subjected to purification by affinity chromatography using a 5 × 1 mL HisTrap™ column (GE Healthcare). Purified samples were recovered using AKTA™ wash buffer (2.34 g/L monobasic sodium phosphate, 29.2 g/L sodium chloride, 34 g/L imidazole) in different imidazole concentrations.

Detection of rgD expression on \textit{S. cerevisiae} BY4741 cell surface

\textit{Dot blot}

Biomass was diluted for a cell concentration of 10^7 cells/mL, then samples were sonicated with 6 s pulses at 3 s interval, on an ice bath for 10 min. It was collected 10 μL of each lysate, which were added over a nitrocellulose membrane (Hybond™ ECLTM, Amersham Biosciences) and incubated in a blocking buffer containing 5% w/v skim milk powder and PBS-T (phosphate buffered saline added of 0.5% v/v Tween™ 20) for 1 h at room temperature. Washing steps were performed between the next steps, using PBS-T. It was then incubated with anti-histidine monoclonal antibodies (Invitrogen) or anti-rgD mouse serum (1:3,000), for 1 h at 37°C. The membrane was washed and incubated (1 h at 37 °C) with horseradish peroxidase conjugated mouse IgG antibody (1:4,000 dilution) (Sigma Aldrich). Washing step was repeated and membranes were placed in the chromogenic substrate solution, containing 6 μg of DAB (3,3′-diaminobenzidine), 50 mM Tris–HCl, 0.3% nickel sulfate solution and 10 μL of hydrogen peroxide (H_2O_2). Biomass samples from the control culture BY4741 pYES2, BY4741 pYES2/gDαAgglutinin and their supernatants were tested against recognition by anti-histidine mAb, following the adaptation of the dot blot protocol published at Nizoli et al. (2009).

\textit{Indirect immunofluorescence assay}

Samples of BY4741 pYES2 and BY4741 pYES2/gDαAgglutinin cultures, both at a concentration of 10^7 cells/mL, were fixed (30 min at 30 °C) over indirect immunofluorescence slides. After that, slides were incubated in methanol at 4 °C for 10 min and then blocked with PBS + fetal bovine serum 10% v/v, for 30 min at 30 °C in a dark humid chamber. The primary antibody, anti-histidine (Invitrogen) or anti-rgD mouse serum (both 1:100 concentration) were applied and incubated for 2 h at 30 °C. After this step, slides were maintained for 18 h at 4 °C in a dark humid chamber. Secondary mAb anti-mouse IgG conjugated to FITC (fluorescein isothiocyanate) (Sigma Aldrich) was applied in a 1:80 dilution and incubated for 90 min at 30 °C. Antigen–antibody reaction was visualized using fluorescence microscope (Olympus BX51).

\textbf{Results}

\textit{pYES2/gDαAgglutinin plasmid construction for Yeast Surface Display}

Plasmid pYES2 had originally 5856 bp (Fig. 1a), while total length prediction for pYES2/gDαAgglutinin plasmid on Vector NTI Advance software (Invitrogen), after inserting the cassette in its sequence, was 7985 bp (Fig. 1b). Schematic representation of expression cassette components can be seen in Fig. 1c.

It was possible to verify by agarose gel electrophoresis that the synthetized plasmid presented approximately 7.9 kb, confirming the addition of ~2.0 kb to plasmid pYES2, corresponding to the insertion of gDαAgglutinin cassette (Fig. 2a). When the plasmid pYES2/gDαAgglutinin was digested using EcoRI restriction enzyme, a fragment of approximately 900 bp was observed, referring to the gD gene sequence included in the expression cassette (Fig. 2b).

Confirmation of recombinant protein expression immobilized by YSD in \textit{S. cerevisiae}

Protocols applied for transformation and culture of \textit{S. cerevisiae} BY4741 had their effectiveness proven with the yeasts culture on Sc-U agar, where only transformed cells were able to grow (Fig. S1). Biomass and supernatant samples from BY4741 pYES2 control culture and BY4741 pYES2/gDαAgglutinin culture were tested against their recognition by mAb anti-histidine, which recognizes the 6xHis tag added to the expression cassette construction.
lysate of BY4741 pYES2/gDαAgglutinin culture revealed reaction positivity, demonstrating effectiveness on cassette construction and recombinant protein expression. Thus, in addition to presenting recombinant gD on yeast cells, the glycoprotein is also found in the culture supernatant (Fig. 3a). Dot blot was applied for a quick and easy screening of recombinant protein expression, in which was possible...
to observe rgD recognition in BY4741 pYES2/gDβAgglutinin cells by polyclonal antibodies from mice immunized with purified rgD (produced by Dummer et al. 2009, in *P. pastoris*) (Fig. 3b).

Detection of rgD by indirect immunofluorescence was performed using the same antibodies describe in dot blot, however a 1:100 dilution was used as a primary antibodies. FITC fluorescence was observed only in cell surface of clones that had pYES2/gDβ-Agglutinin plasmid, while control samples resulted in absence of fluorescence detection (Fig. 4), therefore recognition of recombinant protein displayed in *S. cerevisiae* BY4741 by immunofluorescence confirmed that YSD was successfully executed.

**Fig. 3** Confirmation of gD expression by *S. cerevisiae*. a Dot blot test performed using anti-histidine antibodies. 1 BY4741 pYES2 culture, 2 BY4741 pYes2/gDβAgglutinin, 3 BY4741 pYes2/gDβAgglutinin Supernatant purified, 4 Reaction positive controls. b Dot blot using anti-rgD mice sera. 1 BY4741 pYES2, 2 BY4741 pYes2/gDβAgglutinin, 3 purified rgD expressed in *Pichia pastoris* (positive control)

**Discussion**

Recombinant protein expression on *S. cerevisiae* surface is a methodology that has been consolidated in basic and applied research, arousing more and more interest in improving the technique (Jeong et al. 2019; Lei et al. 2020). Choosing eukaryotic cells as expression platforms proves to be attractive because there is the ability to express proteins with the original fold, an important factor when there is a need for the maintenance of conformational epitopes in recombinant protein structure. Thus, the choice of *S. cerevisiae* is justified for this work, in which the glycoprotein D of BoHV-5 requires the correct assembly to attain its structure, similar to when it is expressed by the virus, and important for vaccines and diagnostic tests development.

Plasmid pYES2 allows the intracellular expression of recombinant proteins in *S. cerevisiae*, however if
the objective is its use in YSD method, there is a need to add new components that enable the recombinant protein to enter the secretory pathway and subsequent immobilization on cell surface. When digesting pYES2/gDαAgglutinin using EcoRI restriction enzyme, a fragment of approximately 900 bp was observed, encoding to gD gene sequence. This fragment was not observed in pYES2 control plasmid, confirming that the plasmid can be used for expression of other proteins, as long as the gene sequence should be flanked by EcoRI sites and its insertion in the region destined for gD.

Lezzi et al. (2012) expressed the Agaricus bisporus tyrosinase intracellularly, and Chen et al. (2011) observed, encoding to enzyme, a fragment of approximately 900 bp was immobilized on cell surface. When digesting protein to enter the secretory pathway and subsequent to add new components that enable the recombinant protein expression analysis. It was identified by different techniques for recombinant protein conformation, codon optimization for expression of target protein conformation, facilitating the molecular stability and protein function (Reddy Chichili et al. 2013). One may suggest that its presence can be a promising tool to increase the access to the immobilized protein (Tanaka and Kondo 2015).

In our study, we observed that the codon optimization leads to a higher concentration and more effective expression of GFP protein. The choice for yeast usual codons provides greater and easier expression of target protein (Sharp and Cowe 1991), what suggests a facilitated and favored expression of immobilized gD in S. cerevisiae and its recognition by mice antibodies immunized with rgD.

The designed cassette had a linker connecting the C-terminal portion of α-agglutinin to gD, consisting of a flexible structure with no significant changes in target protein conformation, facilitating the molecular stability and protein function (Reddy Chichili et al. 2013). One may suggest that its presence can be a promising tool to increase the access to the immobilized protein (Tanaka and Kondo 2015). In the present study, since there are no purification tags in pYES2 original composition, the sequence that codes for 6-histidines amino acids was added as a cassette component. This insertion proved to be effective, since mAb anti-histidine was able to recognize the recombinant protein on yeast surface, being demonstrated by dot blot (Fig. 3) and Immunofluorescence (Fig. 4). Dot blot revealed that rgD could be identified on biomass sample, as well as in the culture supernatant (Fig. 3a, b). This fact was also observed by Baptista (2013), who when immobilizing α-amylase, detected its presence in the culture supernatant. This is usually related to protein debris that detaches from the cell wall, protein units that do not adequately attach to the cell surface and protein proteolysis (Harnpicharanchai et al. 2010; Baptista 2013). Even though a recombinant protein expression to the extracellular medium was not the objective in this work, supernatant purification was performed as a control step for YSD, in case rgD was not found on S. cerevisiae surface.

Indirect immunofluorescence assay was used to confirm the rgD expression by YSD technology. It is important highlight that there were no considerable impacts on molecule structure with 6xHis tag insertion and rgD immobilization, since C-terminal portion of α-agglutinin was able to immobilize rgD on S. cerevisiae surface and thereby displaying it for an easy recognition by specific antibodies generated in mice immunized with soluble “free” rgD (Santos et al. 2018) (Fig. 4b). Recombinant protein detection through dot blot and immunofluorescence using serum
from these mice suggests that epitopes remained on displayed gD, which demonstrates the potential of *S. cerevisiae* BY4741 pYES2/gDAgglutinin yeast for future immunobiological applications.

In conclusion, gDAgglutinin expression cassette was constructed in silico and inserted on pYES2 plasmid, being able to conduct the target protein rgD to the surface of *S. cerevisiae* BY4741 strain. Finally, we can conclude that the construction of pYES2-target protein-αAgglutinin opens a new perspective as a Yeast Surface Display tool to be used in the most diverse biotechnological areas.

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**Declarations**

**Conflict of interest**  Authors declare that they have no conflict of interest.

**Ethical approval**  This article does not contain any studies with animals performed by any of the authors.

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