Published in final edited form as:

Methods Mol Biol. 2015 ; 1319: 245–260. doi:10.1007/978-1-4939-2748-7_14.

Coupling Binding to Catalysis – Using Yeast Cell Surface Display to Select Enzymatic Activities

Keya Zhang^a, Karan Bhuripanyo^a, Yiyang Wang^b, and Jun Yin^b

^aDepartment of Chemistry, University of Chicago, 929 E57th Street, Chicago, IL 60637 (USA)

^bDepartment of Chemistry, Georgia State University, 50 Decatur Street SE, Atlanta, GA 30303 (USA)

Summary

We find yeast cell surface display can be used to engineer enzymes by selecting the enzyme library for high affinity binding to reaction intermediates. Here we cover key steps of enzyme engineering on the yeast cell surface including library design, construction, and selection based on magnetic and fluorescence activated cell sorting.

Keywords

Yeast surface enzyme display; enzyme library; enzyme substrate interaction; nonribosomal peptide synthetase

1. Introduction

Yeast cell surface display provides a high throughput platform for sorting out large libraries of proteins based on binding affinity (1). It has often been used to optimize protein-protein or protein-small molecule interactions, to improve protein folding and its expression, to map the binding epitopes of antibodies or receptors, and to screen for partner proteins in the cell. Diverse protein scaffolds such as immunoglobulin domain in antibodies and T-cell receptors, and leucine rich repeats in lamprey variable lymphocyte receptors can all be displayed on yeast cell surface to engineer their binding affinity and specificity (2–6). We recently demonstrated that the β -propeller domain of E3 ubiquitin ligase can be engineered by yeast cell surface display to recognize new target proteins (7).

Using yeast cell surface display to select for enzymatic activity is more challenging. Yeast selection commonly involves the binding of the protein library displayed on yeast surface to a ligand that is fluorescently labeled or immobilized on magnetic beads. Subsequently yeast cells that bind tightly with the ligand are selected by fluorescence-activated cell sorting (FACS) or by magnetic pull down. To select for enzyme catalysis, one needs to develop a strategy to couple catalytic turnover by yeast displayed proteins with the binding of fluorescent or magnetic ligand to the same yeast cell. In this way FACS or magnetic pull down can be used to enrich yeast cells displaying desired catalytic activity.

Creative methods have been developed to couple catalysis with fluorescent labeling of the yeast library. In one example, a fluorophore was conjugated to a mechanism-based inhibitor of the peroxidase enzyme to select for enzyme variants with enhanced enantioselectivity (8, 9). The peroxidase on the yeast cell surface catalyzed the oxidation of the inhibitor molecule that was transformed to a reactive specie linked to the fluorophore. Once it was formed, the reactive specie quickly conjugated with the cell surface proteins and became covalently attached to the yeast cells. Due to the short life of the reactive specie, most likely it would conjugate to the cells that generated them. In this way cells displaying peroxidase activity were labeled with fluorophore and selected by FACS.

We recently developed a method to use yeast cell display to engineer the adenylation domain of nonribosomal peptide synthetases (NRPS) (10). NRPS is the biosynthetic enzyme that produces natural product molecules of complex structures and important medicinal activities such as penicillin, vancomycin, and daptomycin (11, 12). The adenylation domains are part of the enzymatic assembly line of NRPS. They uptake building block molecules in the forms of amino acids or aryl acids for the synthesis of the final structure. We aim to engineer the substrate specificity of the adenylation domains so that nonnative substrates can be used by NRPS to produce “nonnative” natural product molecules. These new molecules may have different biological or medicinal activities from their native counterparts. We envision it would be much easier to generate the natural product analogues by engineered biosynthetic enzymes than by lengthy procedures of organic synthesis.

We developed a method to engineer the substrate specificities of adenylation domains by yeast cell surface display (10). We carried out the selection of the adenylation domain library on yeast cell surface based on the binding of the enzyme with a ligand we designed to mimic the reaction intermediate formed in the enzyme active site during catalysis. The ligand was conjugated with a fluorophore so that we could select for adenylation domains that bound tightly to the ligand by FACS. The selected adenylation domains were likely to catalyze the formation of the reaction intermediate and indeed we found that they had the desired catalytic activity. In this way we can couple catalysis with binding based yeast selection to engineer enzymatic activity.

Our design of the ligand was based on the formation of substrate-AMP conjugate **2** catalyzed by the adenylation domain (Fig. 1). The enzyme binds both the carboxylic acid substrate **1** and ATP and it first catalyzes the condensation of the two molecules to form a substrate-AMP intermediate **2**. This intermediate is highly reactive and it is subsequently attacked by a thiol group at the end of the phosphopantetheinyl (Ppant) arm of the neighboring carrier protein domain. This gives rise to the formation of the substrate-Ppant conjugate **3** through a thioester linkage. Next the substrate is incorporated into the natural product structure by cascade reactions catalyzed by other NRPS domains (10).

It has been reported that when the phosphate moiety in the substrate-AMP conjugate is replaced with a sulfamate group, the molecule is stable and it can tightly bind to the adenylation domain to inhibit the binding of free substrate and ATP, and thus inhibit enzyme catalysis (**5** in Fig. 1) (13). We thought we could take advantage of the high affinity binding of the enzymes with the substrate-adenosine monosulfamate (AMS) conjugate (**5**) for yeast

selection to engineer adenylation domain specificity (Fig. 2). We thus replaced the native substrate-AMS conjugate with a nonnative substrate and carried out yeast selection to identify mutants of the adenylation domain that would bind tightly to the nonnative substrate-AMS conjugates **9** and **10**. The mutant enzymes selected were able to recognize the nonnative substrates in their free form and catalyzed the condensation of the nonnative substrates with ATP to form substrate-AMP conjugates. In this way we were able to couple adenylation domain catalysis with yeast selection by binding the yeast library to the substrate-AMS probes **9** and **10**. In the probes we designed, we used a linker to attach biotin to the adenine base of AMS (**8**, Fig. 2). Once the yeast displayed enzyme was bound to the probe, the corresponding yeast cells were labeled with biotin that was next bound with a streptavidin-fluorophore conjugate. These cells were then selected by FACS.

We successfully used this method to engineer the substrate specificity of DhbE, an adenylation domain that activates 2,3-dihydroxybenzoic acid (DHB, **1**) for the synthesis of natural product bacillibactin (**10**). By yeast display, we identified DhbE mutants that prefer to recognize nonnative substrates 3-hydroxybenzoic acid (3-HBA, **6**) and 2-aminobenzoic acid (2-ABA, **7**) for the adenylation reaction. We hereby provide a detailed protocol for the yeast selection procedure including library design and construction, model selection and iterative rounds of selection to engineer enzymes with desired catalytic activities.

2. Materials

The materials needed for the library construction and yeast cell culturing are listed below. All aqueous solutions are prepared with deionized water from a commercial water purifier with a conductivity of 18 M Ω or higher. All media or solutions for cell cultures or yeast sorting are autoclaved or filter sterilized. All DNA oligonucleotides are ordered from commercial sources.

2.1. Materials for analyzing enzyme-substrate interactions

1. A computer with internet connection.
2. The Discovery Studio Visualizer program. It can be downloaded for free at <http://accelrys.com/>. Other programs such as PyMOL can also be used.

2.2. Materials for enzyme library construction

1. Taq DNA polymerase.
2. Restriction endonucleases.
3. QIAquick gel extraction kit (Qiagen) or other kits for purifying PCR amplified DNA fragments from the agarose gel.
4. XL1Blue electro competent cells.
5. LB agar plate supplemented with ampicillin (100 μ g/mL). Mix 25 g LB and 15 g agar in 1 L water. Autoclave the media with a liquid cycle for 20 minutes. After the solution is cooled down to around 50°C, add ampicillin to a final concentration of 100 μ g/mL. Mix melted LB-agar well and pour approximately 40 mL media to

each plate. Close the lid and leave the plates at room temperature to cool off. Inverse the plates and store the plates at 4°C after the agar solidifies.

6. pCTCON2 plasmid (2–4). This yeast display vector expresses the fusion protein of the enzyme to be displayed with the yeast protein Aga2p (Fig. 2). Upon disulfide bond formation between Aga2p and Aga1p in the yeast cell wall, the enzyme is anchored on yeast cell surface for ligand binding and selection.
7. DNA maxiprep kit (Qiagen).

2.3. Materials for model selection of the yeast cells

1. A flow cytometer. We used LSRII from BD Biosciences.
2. Yeast cell line EBY100 (2–4).
3. AMS probes conjugated with the native or nonnative substrates. The synthesis of the probes has been reported (10).
4. Mouse anti-HA antibody (Santa Cruz Biotechnology, sc-7392).
5. Chicken anti-c-myc antibody (Invitrogen, A-21281).
6. Goat anti-mouse antibody conjugated with Alexa Fluor 647 (Invitrogen, A-21235).
7. Goat anti-chicken antibody conjugated with Alexa Fluor 488 (Invitrogen A-11039).
8. Streptavidin conjugated with PE (Invitrogen, S-32350).
9. TBS with 0.1% BSA. Prepare a solution of TrisOH (25 mM), pH7.5, NaCl (150 mM) and bovine serum albumin (BSA) 0.1% (w/v). Filter sterilize the solution.
10. SDCAA media. Dissolve 20 g dextrose, 6.7 g Difco yeast nitrogen base without amino acids, 5g Bacto casaminoacids in 1 L H₂O with the addition of 50 mM sodium citrate and 20 mM citric acid. Filter sterilize the solution.
11. SGCAA media. Dissolve 20 g dextrose, 6.7 g Difco yeast nitrogen base without amino acids, 5g Bacto casaminoacids in 1 L H₂O with the addition of 38 mM Na₂HPO₄ and 20 mM NaH₂PO₄. Filter sterilize the solution.
12. -Trp plates. Dissolve 20 g agar, 20 g dextrose, 5 g (NH₄)₂SO₄, 1.7 g Difco yeast nitrogen base without amino acids, 1.3 g drop-out mix excluding the tryptophan amino acid in 1 L H₂O. Autoclave the media and cool the media to around 50°C. Pour the media into the plate. After the agar solidifies, store the plate at 4°C until use.

2.4. Materials for library transformation into the yeast cells

1. YPD media. Dissolve 20 g dextrose, 20 g peptone and 10 g yeast extract in 1 L deionized H₂O. Filter sterilize the media.
2. TE buffer. Prepare a solution of 100 mM TrisOH and 10 mM EDTA. Adjust pH to 8.0.
3. LiOAc-TE solution. Dissolve 100 mM lithium acetate in TE.

4. Single-stranded carrier DNA from salmon testes. Prepare a solution of 2 mg/mL carrier DNA. Dissolve the carrier DNA in TE buffer.
5. Polyethylene glycol (PEG) solution. Dissolve PEG 3350 to a final concentration of 40% (w/v) in LiOAc-TE.

2.5. Materials for yeast library selection

1. μ MACS Streptavidin Starting kit (Miltenyi Biotec).
2. Zymoprep II Yeast Plasmid Miniprep Kit (Zymo Research, D2004).
3. pET protein expression vectors (Novagen).

3. Methods

3.1. Designing the focused library of the enzyme

We find an effective way of engineering substrate recognition by the enzyme is to identify enzyme active site residues engaged in key interactions with the substrate and randomize these residues in the library. We then use a high throughput selection platform such as yeast cell surface display to identify enzymes with altered substrate specificity.

We use Discovery Studio Visualizer to analyze enzyme substrate interactions. The crystal structure of DhbE in complex with DHB and AMP has been reported (14). We thus use the program to analyze key interactions between DhbE and the hydroxyl groups of DHB to identify residues to be randomized.

1. Download the pdb file of the crystal structure of the enzyme-substrate complex from www.pdb.org.
2. Display the crystal structure with Discovery Studio Visualizer. If there are more than one asymmetric unit displayed, turn off the display of others and only leave one asymmetric unit displayed on the screen.
3. Display the protein structure in the ribbon model and the small molecule ligand in the ball-and-stick model.
4. Use “rotate” and “zoom-in” command buttons to find the best angle to show the interaction between the active site residues of the enzyme and the substrate molecule.
5. Identify the part of the native substrate that will be changed in the nonnative substrate and identify key residues of the enzyme that interact with the functional groups on the substrate to be changed. These residues can be randomized to generate new interactions between the enzyme and the nonnative substrates.

For DhbE engineering, we see the difference between the native substrate DHB and the nonnative substrate HBA is that the 2-OH group in DHB is missing in HBA. We thus identify four enzyme active site residues that interact with the 2-OH of DHB and randomize those in the library.

One useful function provided by the Discovery Studio Visualizer is the “show by radius” command. It allows the residues within a certain distance (5–7 Å) from a key atom on the substrate to be displayed. One can use this command to identify residues of a protein that are close to a specific atom in the ligand.

6. Use the distance function of the program to measure the distance between the substrate atom and the residue involved in the key interactions. Pay special attention to residues that are engaged in tight hydrogen bonding (~ 3 Å), hydrophobic packing (~ 3 –6 Å), electrostatic (salt bridge, ~ 2.5 Å), or π -stacking interactions.
7. Catalog the residues identified and rank their importance based on their interaction with the ligand. Assign importance based on the distance of the residue from the ligand and the types of interactions they are engaged in.
8. Identify 4–6 residues that are involved in the most important interactions with the nonnative part of the ligand. These residues can then be randomized in the library for yeast cell sorting.

3.2. Constructing the enzyme library

We use a special polymerase chain reaction (PCR) known as “gene splicing by overlap extension” to randomize a codon or a stretch of codons in the middle of a gene (15). Overlap extension typically involves the use of two pairs of primers and it consists of two steps (Fig. 3). In step 1, primers a and b, and c and d are paired respectively to amplify gene fragments AB and CD from the template DNA. Primers b and c are designed so that they have a 15–18 base pair overlap between them and they are oriented in opposite directions. As a result, amplified fragments AB and CD would have the same overlap as in primers b and c. AB and CD are purified and in step 2, and they are used for the assembly of the full length gene with primers a and d. The fully assembled fragment is then digested by restriction enzymes and ligated into the yeast display vector pCTCON2. As illustrated in Fig. 3, primer c contains the sequence of randomized codons. After the overlap extension PCR, the randomized codons are incorporated into the full length gene to generate a focused library of the gene. If multiple regions of the gene need to be randomized, overlap extension can be performed in tandem to introduce randomized sequence into a gene.

To avoid over representation of the wild type (wt) sequence at the randomized sites in the library, we always first construct a mutant template in which the codons to be randomized in the library are first replaced by the codon for Ala such as “GCA”. In this way the bias for primer annealing would not give rise to more wt clones in the library before selection. Sometimes the wt clone may have low activity with the nonnative substrate or ligand. If it over populates the library, the library may be converged to wt clones after selection.

1. Design PCR primers so that the overlapping region between primers b and c spans 15–18 bp. The melting temperature of the sequence should be between 45 and 60°C. Use “NNK” codon to replace the codon of the residues that are to be randomized. “N” is an equal mixture of nucleotides A, G, T, C. K is an equal

mixture of nucleotides G and T. The use of NNK codon minimizes the chance of incorporating stop codons at the randomized sites of the library.

2. Use site-directed mutagenesis to mutate the native codon to an Ala codon at the site of the residues to be randomized. The mutated gene with Ala codons is to be used as the template for the PCR amplification.
3. Use primers a and b to amplify PCR fragment AB from the Ala template. Use primers c and d to amplify PCR fragment CD from the Ala template.
4. Separate the amplified PCR fragments from the primer by agarose gel electrophoresis. Excise the band containing the amplified fragments from the gel. Extract the DNA by a gel extraction kit.
5. Set up an assembly PCR with fragments AB, CD and primers a and d. We typically use 0.1 µg of each of the DNA fragments as the templates and 10 µM of each of the primer in a 100 µL reaction. We use *Taq* polymerase for the PCR reaction.
6. Purify the assembled gene fragment by agarose gel electrophoresis. Extract the DNA from the gel by a gel extraction kit.
7. Digest the assembled fragment and ligate the fragment into a digested pCTCON2 vector.
8. Transform the ligation reaction into electro competent XL1Blue cells.
9. Titer the transformation efficiency. Also calculate the theoretical diversity of the library based on the number of residues being randomized. Preferably there should be more than 10^6 clones to cover a 4-residue randomized library and more than 10^7 clones to cover a 5-residue randomized library.
10. Plate out the transformed cells on the LB agar plate supplemented with ampicillin. After overnight incubation at 37°C, collect the cells by adding 1 mL sterilized LB media to each plate and scrape the cells off the plate with a sterilized spatula. Combine all the LB media containing the cells from the plate and pellet the cells by centrifugation.
11. Extract the plasmid DNA from the cell with a DNA maxiprep kit. Store the DNA library at -20°C until transformation into the yeast cell.

3.3. Optimizing yeast selection based on model selection

Before real selection of the enzyme library, model selection should be carried out to develop a selection protocol that is most efficient in enriching the catalytic active clones. We first grow yeast cell culture expressing the wt enzyme. We then double label the yeast cells with an anti-HA antibody and an anti-myc antibody (Fig. 2). After washing the cells, we label cells with secondary antibodies to bind distinct fluorophores to HA and myc tags. We then analyze the cells by flow cytometry. Such experiment are to be repeated to decide the best condition to achieve the best expression of the enzyme on the yeast cell surface.

We also bind the wt substrate-AMS probe **8** to the cell displaying the wt enzyme (Fig. 2). The anti-myc antibody is also added to bind to the myc tag fused to the enzyme.

Fluorophore labeled streptavidin and secondary antibody are added to attach fluorophores to the cells both expressing the enzyme and binding to the substrate-AMS probe. The number of double labeled cells can then be counted by flow cytometry. The major parameters to be optimized for best labeling efficiency are the density of the yeast cells in the labeling reaction, the concentrations of the probes, streptavidin, and the primary and secondary antibodies. Once the parameters are optimized for best selection efficiency, the selection of the yeast library can begin.

1. Transform yeast cell EYB100 with the yeast display plasmid pCTCON2 harboring the wtDhbE gene. Streak the transformation reaction on a -Trp plate. Incubate the plate for two days at 30 °C.
2. After incubation scrape the yeast cells to inoculate a 5 mL SDCAA culture. Incubate the culture in a shaker at 30°C to reach an initial optical density of 0.5 at 600 nm (OD₆₀₀).
3. Pellet the cells by centrifugation at 3,000 rpm for 5 min. To induce enzyme expression, resuspend the cells in 5 mL SGCAA media. Allow the cell culture to shake at 20°C for 16–24 hours.
4. To analyze the display of the enzyme on the surface of yeast cells, measure the OD₆₀₀ of the yeast cells and take 10⁶ cells from the culture and add it to 0.1 mL TBS with 0.1% BSA. OD₆₀₀ of 1 corresponds to 3×10^7 yeast cells/mL.

Add mouse anti-HA antibody and chicken anti-c-myc antibody as primary antibodies. The final concentration of the primary antibodies is 10 µg/mL. Incubate the cell with antibodies for 45 minutes at 4 °C.

5. Wash the cells twice with 0.1% BSA in TBS. Stain the cells with 5 µg/mL goat anti-mouse antibody conjugated with Alexa Fluor 647 and goat anti-chicken antibody conjugated with Alexa Fluor 488 in 0.1 mL 0.1% BSA in TBS. Shield the tubes of the labeling reaction from light and incubate the reaction mixture at 4 °C for 30 min.
6. After incubation wash the cells twice with 0.1% BSA in TBS. Analyze the washed cells on a flow cytometer to count the number of cells that are labeled with both fluorophores. Cells are also analyzed from control labeling reactions in which primary antibodies are either excluded from the reaction or cells are only labeled with primary and secondary antibodies that bind to one of the affinity tags.
7. Record the results and optimize the expression and labeling conditions to get the most cell to be labeled with both fluorophores. In this way one can be confident that the cells are expressing maximum amount of the enzymes on the cell surface. See Fig. 4A for a typical result on analyzing enzyme display on the cell surface by flow cytometry.
8. To assay the function of the displayed enzyme on the yeast cell surface, set up the binding of wt substrate-AMS probe **8** with cells displaying the enzyme. To a 100 µL solution containing 10⁶ cells, add 10 nM probe **8** and 10 µg/mL chicken anti-c-myc antibody. Incubate the labeling reaction for 45 min at 4°C.

9. Wash the cells twice with 0.1% BSA in TBS. Resuspend the cells in 100 μ L BSA-TBS buffer. Add 5 μ g/mL streptavidin conjugated with phycoerythrin (PE) and 5 μ g/mL goat anti-chicken antibody conjugated with Alexa Fluor 488.
10. Analyze the labeling reaction by flow cytometry. Record the results and optimize the concentration of various primary and secondary reagents to achieve the maximum percentage of the number of the yeast cells doubly labelled with fluorophores. See Fig. 4B for a typical result.

3.4. Displaying the enzyme library on yeast cell surface

To carry out yeast selection, the library plasmid need to be first transformed into the yeast cells. A high efficiency in library DNA transformation is necessary to guarantee a good coverage of the library.

1. For large scale transformation of the library DNA, EYB100 yeast cell is first cultured in 200 mL YPD media at 30°C to an OD₆₀₀ of 0.5. Pellet the cells by centrifugation at 3,500 rpm for 5 minutes. Remove the media.
2. Resuspend the cells in 20 mL TE buffer. Centrifuge again and remove the supernatant.
3. Resuspend the cells in 20 mL LiOAc-TE. Pellete the cells by centrifugation and remove the supernatant.
4. Resuspend the cell in 0.8 mL LiOAc-TE.
5. Set up a transformation reaction containing pCTCON2 library DNA (1 μ g), single strand carrier DNA (2 μ L), yeast competent cells (25 μ L) and PEG 3350 solution (300 μ L) (see Note 1).
6. Incubate the transformation reaction at 30°C for 1 hour and then at 42°C for 20 minutes.
7. Pellet the cells in each transformation by centrifugation at 13,000 rpm for 30 seconds. Resuspend each pellet in 20 μ L SDCCA. Combine all the cell suspension and add the cell to 1 L SDCAA. Culture the cells in a 30°C shaker for two days until the culture reaches an OD₆₀₀ above 5.
8. To store the yeast library, take 20 mL of the cell culture and add glycerol to a final concentration of 15%. Aliquot the culture and store the tubes in a -80°C freezer.

3.5. Selecting the enzyme library by yeast cell sorting

The initial yeast library is induced to display the enzyme on the cell surface. After confirming the efficient display of the library clones, the yeast library is bound to the probe

¹A pilot transformation reaction can be set up to test the transformation efficiency first and calculate how many transformations need to be done to cover the plasmid library. To titer the transformation efficiency, dilute 10 μ L of the transformation reaction by 10-, 100- and 1000-fold in SDCAA. Plate out 100 μ L of the diluted cells on Trp- plate. Incubate the plates in a 30°C incubator for two days. Based on the number of colonies on the plate, calculate the transformation efficiency. We find we need to do 30 transformations to cover a plasmid library of 10⁶ in size.

In parallel to the library transformation, a control reaction should also be set up with the exclusion of the plasmid DNA. This is to assay if there is anything in the yeast competent cell or transformation reagents that may contaminate the library.

and selected by FACS or magnetic beads. The selected yeast cells are cultured again for subsequent rounds of selection. When the library is converged, the genes of the selected mutants are cloned into a protein expression vector to express the enzyme variants and assay their catalytic activities.

3.5.1. Initial selection by magnetic bead-based affinity capture

- 1 Inoculate the initial yeast cell library in 10 mL SDCAA supplemented with ampicillin (100 µg/mL) and kanamycin (50 µg/mL). Culture the cell at 30°C to an OD₆₀₀ between 1 and 2.
- 2 Pellet the cells by centrifugation and resuspend the cells in 10 mL SGCAA to induce the expression of the enzyme library. Incubate the cell culture in a 20°C shaker for 20 hours.
- 3 Characterize the display of the enzyme library on the cell surface following step 4 in section 3.3. If the display is good, the yeast cells can be used for library selection.
- 4 In the first round of selection we use a kit for magnetic activated cell sorting (MACS). Dilute 10⁸ yeast cells in 0.6 mL TBS supplemented with 0.1% BSA. Add 3 µM biotin conjugated probe (**9** or **10**) to the cell suspension. Incubate the tube for 45 minutes at 4°C on a rotating platform to ensure thorough mixing.
- 5 Pellet the cells by centrifugation. Resuspend the cells in 1 mL TBS with 0.1% BSA. Repeat this process twice to remove the unbound probes in the solution. After wash, resuspend the cells in 800 µL TBS with 0.1% BSA.
- 6 Add 90 µL streptavidin-coated microbeads to the cell suspension. Incubate the tube at 4°C for 30 minutes with gentle rocking. After incubation, add the cells and the microbeads to 30 mL TBS with 0.1% BAS. Mix the suspension thoroughly and pellet the beads and the cells by centrifugation.
- 7 Remove the supernatant. Resuspend the beads and the cells in 0.5 mL TBS with 0.1% BSA. Collect the magnetic beads and the yeast cells bound to the beads by the magnetic stand following the manufacturer's protocol.
- 8 Elute the cells captured by the beads into SDCAA (5 mL) supplemented with ampicillin (100 µg/mL) and kanamycin (50 µg/mL). Culture the cells at 30°C for overnight. After overnight culturing of the yeast cells from the first round of selection, follow steps 1 and 2 to induce the cells to express the enzyme library for the second round of selection.

3.5.2. Enrich full length-displayed enzyme—Below we use a second round of selection to enrich yeast cells that display full length enzymes on the cell surface (see Note 2). We bind both the anti-HA and the anti-myc antibody to the cell and collect the cells that have strong display of both the HA and myc tags. In this way we can eliminate the cells displaying truncated enzymes to increase the efficiency of subsequent rounds of selection.

- 9 After induction, add mouse anti-HA antibody (10 µg/mL) and chicken anti-c-myc antibody to 10^7 library cells in 0.5 mL TBS with 0.1% BSA. Incubate the cells with the antibody for 45 minutes at 4°C. Shield the cells from light during incubation.
- 10 Wash the cells as in step 5. Resuspend the cells in 0.5 mL TBS with 0.1% BSA. Add goat anti-mouse antibody-Alexa Fluor 647 (5 µg/ml) and goat anti-chicken antibody-Alexa Fluor 488 (5 µg/mL). Incubate the cells with antibodies for 45 minutes at 4°C. Shield the cells from light during incubation.
- 11 Wash the cells twice with TBS with 0.1% BSA. Collect the cells within top 15–20% brightness for both fluorophores by FACS. Culture the collected cells overnight in a 30°C shaker in SDCAA supplemented with ampicillin (100 µg/mL) and kanamycin (50 µg/mL).

3.5.3. Selection by FACS—For subsequent rounds of selections, FACS is used to identify cells both expressing the enzyme fused to the myc tag and binding to the probe-biotin conjugate.

- 12 After induction of the cells to express the enzyme library, add biotin-conjugated probe (1 µM) and chicken anti-c-myc antibody to 10^7 cells in TBS with 0.1% BSA. Incubate the binding reaction at 4°C for 30 minutes. Wash the cells as in step 5.
- 13 Resuspend the cells in 0.5 mL TBS with 0.1% BSA. Add streptavidin-PE (5 µg/mL) and goat anti-chicken antibody-Alexa Fluor 488 (5 µg/mL). Incubate the cells with the secondary antibodies for 30 minutes at 4°C. Shield the cells from light during incubation.
- 14 Wash the cells as in step 5 and resuspend the cells in TBS with 0.1% of BSA. Use FACS to collect top 1% of the cell in the library that are most brightly labeled with both fluorophores. Subject the cells from round 3 selection to another round of probe and anti-c-myc antibody binding and FACS (see Note 3).

After iterative rounds of selection, the pCTCON2 plasmids in the selected cell need to be sequenced to see if the yeast library is converged. Individual clones of the selected enzyme variants should also be expressed to assay if they have the desired catalytic properties see Note 4).

²Sometimes we found iterative rounds of yeast selection gave rise to the accumulation of yeast cells displaying truncated enzyme variants. The truncated clones may exhibit significant nonspecific interactions with streptavidin-PE or the secondary antibodies conjugated with fluorophores. To avoid the selection of truncated clones, we implement a “expression-based” FACS sorting after the first round bead selection to remove cells missing either the HA or the Myc tag at the N- or C- terminus of the displayed protein. For this purpose, the yeast library was first stained with mouse anti-HA monoclonal antibody and chicken anti-c-Myc antibody, and then stained with a mixture of goat anti-mouse antibody-Alexa Fluor 647 and goat anti-chicken antibody-Alexa Fluor 488 conjugates (Fig. 2). For expression based selection we collect top 15–20% brightest double-positive cells for the next round of sorting based on binding to the probes mimicking reaction intermediates.

³To increase the selection stringency from round to round, gradually decrease the concentration of the probe and the amount of yeast cell input for the selection. Also narrow the sorting gate to allow the enrichment of only the brightest cells labeled with both the biotin-probe conjugate and anti-myc antibody. Typically a total of 5–6 rounds of selection can be performed to converge the library to a few mutant clones.

- 15 Grow the cells from cell sorting in 5 mL SDCAA to an OD₆₀₀ of 0.5. Use Zymoprep II yeast plasmid miniprep kit to extract pCTCON2 plasmid DNA from the yeast cells. Transform the plasmid into XL1Blue electro competent cells. Sequence the gene of the enzyme in the plasmid. Align the sequence of the selected clones to see if the randomized residues in the library are converged after the selection.
- 16 If library is converged to a few clones after selection, subclone the gene of the representative enzyme variant from the pCTCON2 vector to a protein expression vector such as the pET vectors for expression of the enzyme variants in *E. coli*. Express the enzyme variants and characterize their catalytic activity (see Note 5).

Acknowledgments

This work was supported by a National Science Foundation CAREER award (1057092) and a National Institute of Health grant 1R01GM104498 to J.Y.

References

1. Gai SA, Wittrup KD. Yeast surface display for protein engineering and characterization. *Curr Opin Struct Biol.* 2007; 17:467–473. [PubMed: 17870469]
2. Boder ET, Wittrup KD. Yeast surface display for directed evolution of protein expression, affinity, and stability. *Methods Enzymol.* 2000; 328:430–444. [PubMed: 11075358]
3. Chao G, Lau WL, Hackel BJ, Sazinsky SL, Lippow SM, Wittrup KD. Isolating and engineering human antibodies using yeast surface display. *Nature protocols.* 2006; 1:755–768. [PubMed: 17406305]
4. Colby DW, Kellogg BA, Graff CP, Yeung YA, Swers JS, Wittrup KD. Engineering antibody affinity by yeast surface display. *Methods Enzymol.* 2004; 388:348–358. [PubMed: 15289082]
5. Miller, KD.; Pefaur, NB.; Baird, CL. Construction and screening of antigen targeted immune yeast surface display antibody libraries. In: Paul Robinson, J., managing editor, et al., editors. *Current protocols in cytometry.* Vol. Chapter 4. 2008. p. 7
6. Tasumi S, Velikovsky CA, Xu G, Gai SA, Wittrup KD, Flajnik MF, Mariuzza RA, Pancer Z. High-affinity lamprey VLRA and VLRB monoclonal antibodies. *Proceedings of the National Academy of Sciences of the United States of America.* 2009; 106:12891–12896. [PubMed: 19625627]
7. Zhang K, Li H, Bhuripanyo K, Zhao B, Zheng N, Yin J. Engineering new protein-protein interactions on the beta-propeller fold by yeast cell surface display. *ChemBioChem.* 2013; 14:426–430. [PubMed: 23401304]
8. Antipov E, Cho AE, Wittrup KD, Klivanov AM. Highly L and D enantioselective variants of horseradish peroxidase discovered by an ultrahigh-throughput selection method. *Proceedings of the National Academy of Sciences of the United States of America.* 2008; 105:17694–17699. [PubMed: 19004779]

⁴Our experience teaches us that it is very important to follow the quality of the library in between rounds of selection. It is better to identify a defective round of selection and repeat it rather than allowing the truncated, nonspecific binding, or contaminating clones to accumulate in the library. To check the library quality, we collect the cells after sorting and culture them in SDCAA. We then extract the pCTCON2 DNA from the cultured cells and transform the plasmid DNA into *E. coli* XL1Blue cells. We plate out the transformation reaction to get single colonies and perform colony PCR with gene specific primers to make sure that the majority clones in the library have the full length gene of the target protein.

⁵Once the library selection is complete and the clones are tested for activity, a secondary library can be constructed to further increase the binding affinity or the catalytic activity of the selected clones. In the secondary library, more residues in or around the enzyme active site can be randomized to fine tune the interaction of the protein with the target ligand. In our mind there is no “final solution” to a protein engineering project. The timing to stop library construction and selection depends on if the clone selected from the library can serve the function that it is needed for.

9. Lipovsek D, Antipov E, Armstrong KA, Olsen MJ, Klibanov AM, Tidor B, Wittup KD. Selection of horseradish peroxidase variants with enhanced enantioselectivity by yeast surface display. *Chem Biol.* 2007; 14:1176–1185. [PubMed: 17961829]
10. Zhang K, Nelson KM, Bhuripanyo K, Grimes KD, Zhao B, Aldrich CC, Yin J. Engineering the substrate specificity of the DhbE adenylation domain by yeast cell surface display. *Chem Biol.* 2013; 20:92–101. [PubMed: 23352143]
11. Cane DE, Walsh CT, Khosla C. Harnessing the biosynthetic code: combinations, permutations, and mutations. *Science.* 1998; 282:63–68. [PubMed: 9756477]
12. Clardy J, Walsh C. Lessons from natural molecules. *Nature.* 2004; 432:829–837. [PubMed: 15602548]
13. Somu RV, Boshoff H, Qiao C, Bennett EM, Barry CE 3rd, Aldrich CC. Rationally designed nucleoside antibiotics that inhibit siderophore biosynthesis of *Mycobacterium tuberculosis*. *J Med Chem.* 2006; 49:31–34. [PubMed: 16392788]
14. May JJ, Kessler N, Marahiel MA, Stubbs MT. Crystal structure of DhbE, an archetype for aryl acid activating domains of modular nonribosomal peptide synthetases. *Proc Natl Acad Sci U S A.* 2002; 99:12120–12125. [PubMed: 12221282]
15. Horton RM, Ho SN, Pullen JK, Hunt HD, Cai Z, Pease LR. Gene splicing by overlap extension. *Methods Enzymol.* 1993; 217:270–279. [PubMed: 8474334]

Fig. 1.

The reaction catalyzed by the adenylation domain of NRPS enzyme DhbE.

Dihydroxybenzoic acid (DHB, **1**) and salicylic acid (SA, **4**) are the native substrates of DhbE. **2** is the substrate-AMP intermediate bound to the enzyme active site. Once it is formed, the enzyme catalyzes the transfer of the substrate to a thiol group on the carrier protein to form a thioester conjugate **3**. SA-AMS conjugate **5** has been found to mimic **2** and it can bind tightly to the enzyme active site. 3-hydroxybenzoic acid (3-HBA, **6**) and 2-aminobenzoic acid (2-ABA, **7**) are the nonnative substrates to be recognized by the engineered adenylation domains.

Yeast selection scheme for the identification of adenylation domains with alternative substrate specificity. The native substrate SA is conjugated to adenosine through a sulfamate moiety in the SA-AMS conjugate in **8**. Nonnative substrates 3-HBA and 2-ABA are also conjugated to AMS in **9** and **10**. In the probe molecules **8**, **9** and **10**, biotin are linked to the substrate-AMS conjugates to enable yeast cell sorting.

Fig. 3. Scheme of overlap extension PCR. a, b, c, and d are the primers. Red region designates randomized codons in the primer or in the amplified PCR fragments. AB and CD are the DNA fragments amplified by the primer pairs a and b, and primers c and d, respectively.

Fig. 4.

Flow cytometry analysis of the yeast cells displaying the wt DhbE enzyme. (A) Analysis of the displaying of the enzyme on cell surface. Cell labeling by Alexa Fluor 488 and 647 reports the display of the myc and HA tag fused to the enzyme. (B) Analysis of enzyme binding to the substrate-AMS conjugate. The intensity of PE fluorescence measures the binding of the AMS conjugate to the enzyme on cell surface. Alexa Fluor 488 is to report the display of myc tag fused to the enzyme displayed on the cell surface.