

Development of an Efficient Transformation System for Halotolerant Yeast *Debaryomyces hansenii* CBS767

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[Abstract] *Debaryomyces hansenii* is one of the most osmotolerant and halotolerant yeasts. Further, its association with traditional cheese and meat products imparting special flavors to these products project this yeast with enormous biotechnological potential in the agrofood sector. However, lack of an efficient transformation system in *D. hansenii* still direct the complementation based assay in *S. cerevisiae* mutants for functional analysis of *D. hansenii* genes. Here, we have described the development of an efficient transformation system for *D. hansenii* that is based on a histidine auxotrophic recipient strain, DBH9 (generated by UV induced random mutagenesis), and the DhHIS4 gene as the selectable marker. Moreover, the same method has also been employed for gene disruption in *D. hansenii* by homologous recombination.

Keywords: *Debaryomyces hansenii*, Transformation, Gene disruption, Halotolerant yeast, Electroporation, Auxotroph

[Background] *Debaryomyces hansenii* CBS767 is one of the most osmotolerant and halotolerant yeasts and can tolerate salinity levels up to 4 M NaCl, whereas growth of *Saccharomyces cerevisiae* is inhibited when salinity reaches above 1.7 M NaCl. *Debaryomyces hansenii* is frequently associated with traditional cheese and meat products imparting special flavors to these products. Therefore, this yeast is not only an excellent model to study salt tolerance mechanisms in eukaryotic cells but it also has enormous biotechnological potential in the agrofood sector. However, lack of efficient transformation system and gene disruption tools required for gene manipulation in *D. hansenii* is a big lacuna behind the molecular analysis of its halotolerant feature. Ricaurte and Govind (1999) developed an *ura3* mutant based transformation system for *D. hansenii*; however this system exhibited low-efficiency. Despite the development of transformation system for *Candida famata* (Voronovsky *et al.*, 2002), an anamorph of *D. hansenii*, functional studies of the genes from *D. hansenii* are still based on complementation of *S. cerevisiae* mutants. Our transformation system is based on a histidine auxotrophic strain, DBH9 and the DhHIS4 gene as the selectable marker. The same method has also been demonstrated for gene disruption in *D. hansenii* by homologous recombination (Minhas *et al.*, 2009).

Materials and Reagents

1. Pipettes (Thermo Fisher Scientific, catalog numbers: 4652080, 4652050, 4652020, 4652010)
2. Flask (Borosil, catalog numbers: 4980009, 4980012, 4980016, 4980021)
3. Graduated cylinder (Borosil, catalog number: 3021029)
4. Centrifuge tube (Oak Ridge, catalog number: 541040)
5. Safe-Lock tubes 2.0 ml (Eppendorf, catalog number: 0030120094)
6. Disposable Petri plates (Tarson, catalog number: 460090)
7. Pipette tips (Tarson, catalog numbers: 521000, 521010, 521020)
8. 0.2 μ m syringe filter (Millipore, catalog number: SLGP033RS)
9. 0.2 cm Electroporation cuvette (BioRad, catalog number: 1652086)
10. DBH9: *D. hansenii* strain CBS767 (MTCC 3461) $\Delta dhhs4$ (Original manuscript, Minhas *et al.*, 2009)
11. Plasmid pDH4 (for cloning details refers to materials and methods section in Minhas *et al.*, 2009)
12. Sodium phosphate, dibasic (Merck, catalog number: 567550)
13. Sodium phosphate, monobasic (Merck, catalog number: 567549)
14. DL-Dithiothreitol (DL- DTT) (Sigma, catalog number: 43815)
15. Glycerol (Sigma-Aldrich, catalog number: G9012)
16. Sucrose (Sigma, catalog number: S8501)
17. Glucose (Himedia, catalog number: MB037)
18. Agar (Himedia, catalog number: GRM026)
19. YPD broth (BD Difco, catalog number: DF0428-17-5)
20. Yeast minimal media-SD base (Clontech, catalog number: 630411)
21. Sterile water (Lab Pure-Series Model-Analytic UV)
22. YPD broth medium (see Recipes)
23. SD minimal agar medium with 0.3 M sucrose (see Recipes)
24. YPD agar medium (see Recipes)
25. 1 M sodium phosphate buffer, pH 7.5 (see Recipes)
26. 1 M D L- DTT (see Recipes)
27. 1 M Sucrose (see Recipes)

Equipments

1. Incubator, New Brunswick™ Innova® 44 (Eppendorf, model: M1282-0002)
2. UV-VIS spectrophotometer (Shimadzu, model: UV 1900)
3. Microcentrifuge (Eppendorf, model: 5415R)
4. Centrifuge (REMI, model: CPR-24Plus)
5. Water bath (Julabo, model: PURA 4)
6. Gene Pulser (BioRad, model: GenePulserXcell)

7. Vortex (DLAB, model: MX-S)
8. Laminar air flow (Ocean life science corp, model: OLSC 113-4)
9. L- spreader (Himedia, model: PW1085)
10. Autoclave

Software

1. TBLASTN search tool on NCBI website
(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome)

Procedure

Notes:

- a. Perform all steps involving microbial culture under sterile environment in laminar air flow.
- b. Use autoclaved water in all the steps.

1. Streak DBH9 strain on an YPD agar plate from frozen glycerol stock (-70 °C).
2. Incubate streaked plate at 25 °C in an incubator for 3-4 days or till colonies appear.
Note: Always start with freshly streaked culture for enhanced transformation efficiency.
3. Inoculate a single colony of DBH9 from the plate in 10 ml YPD broth in a flask and incubate for appx. 24 h at 25 °C at 200 rpm for growth (saturated culture).
4. Check the absorbance of the culture (after 10 times dilution in water) using spectrophotometer at 600 nm.
5. Re-inoculate 120 µl of above grown saturated culture (if O.D. of culture is 3.0) into 30 ml of YPD broth in a flask (so that an initial A_{600nm} of 0.045 is achieved).
Note: The volume of re-inoculation may vary with O.D. of culture.
6. Grow the re-inoculated culture at 25 °C at 200 rpm for ~16 h till the A_{600nm} reached 2.6-2.8 (appx. 4.3×10^7 cells ml⁻¹).
7. Confirm the absorbance using spectrophotometer.
Note: A drastic reduction in the number of transformants has been observed when the A_{600nm} of the harvested cells was > 2.8.
8. Harvest cells from the liquid culture by centrifugation in a centrifuge at 4,500 x g using centrifuge tube for 10 min at room temperature (RT).
9. Discard the flow-through.
10. Re-suspend cells pellet in 6 ml of 50 mM sodium phosphate buffer, pH 7.5 (containing 25 mM DL-Dithiothreitol).
Note: Vortex the cell suspension at low rpm for 5-10 s for re-suspension.
11. Incubate cells suspension in a 30 °C water bath (pre-warm) for 15 min.

12. Again harvest cells from the suspension by centrifugation at 4,500 x *g* using centrifuge tube for 10 min at RT.
13. Discard the flow-through.
14. Wash the cells by re-suspending cell pellet in 15 ml sterile water and harvest the cell pellet by centrifugation at 4,500 x *g* for 10 min at RT.
15. Discard the flow-through.
16. Repeat Steps 14-15.
17. Re-suspend the cell pellet in 1.0 ml ice-cold 1 M sucrose and transfer the entire chilled cell suspension to pre-chilled 2.0 ml safe-lock tube.
18. Harvest the cell pellet again by centrifugation at 4,500 x *g* for 10 min at 4 °C in a microcentrifuge.
19. Discard the flow-through.
20. Finally to the cell pellet, add 175 µl ice-cold 1 M sucrose to make the final volume of chilled cell suspension to 200 µl in a safe-lock tube.
21. Uniformly re-suspend the cells by pipetting.
22. To this chilled cell suspension, add 0.25-1.5 µg DNA (pDH4 plasmid) and transfer the entire mixture to 0.2 cm electroporation cuvette.
23. Electroporate the mixture using Gene Pulser at a setting of 2.3 kV, 50 mF and 100 Ω.
Note: Perform Steps 17-22 strictly on ice.
24. To the electroporation cuvette, immediately add 800 µl of 1 M sucrose solution (stored at room temperature).
25. Spread 200 µl of the cells transformed with pDH4 on SD minimal medium (containing 0.3 M sucrose) plates using L-spreader.
26. Incubate these plates at 25 °C for 3-4 days for appearance of transformants positive for histidine prototrophy (as replicating vector pDH4 has *DhHIS4* gene as the selection marker).
Note: We haven't used DhHIS4 as the selection marker in other yeast lines.
27. An extra sample is always used as a control where no DNA will be added.
28. Around 3,500 colonies would appear on the plate transformed with 0.25 µg of pDH4 plasmid DNA. No colony will appear in control transformation without DNA.

Data analysis

For details, refers to Minhas *et al.* (2009).

Recipes

1. YPD agar medium
 - a. Add 5.0 g of YPD broth powder in 250 ml flask
 - b. Add 2.0 g of agar powder

- c. Dissolve the medium completely in 100 ml sterile water and sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min
2. YPD broth medium
 - a. Suspend 5.0 g of YPD broth medium in 100 ml distilled water in 250 ml flask
 - b. Dissolve the medium completely and sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min
3. SD minimal agar media with 0.3 M sucrose (100 ml)
 - a. Add 0.67 g Yeast minimal media-SD base to 80 ml of distilled water in a 250 ml flask
 - b. Add 2.0 g glucose, 2.0 g agar and 1.02 g sucrose to it
 - c. Mix the solution uniformly
 - d. Make up the final volume to 100 ml with distilled water
4. 1 M Sodium phosphate buffer, pH 7.5
 - a. Add 20.209 g of sodium phosphate dibasic to 800 ml of distilled water in a graduated cylinder (1000 ml capacity) to prepare 0.0754 M sodium phosphate dibasic solution
 - b. Add 3.394 g of sodium phosphate monobasic (0.0246 M) to the above solution
 - c. Mix the solution uniformly and adjust the final pH to 7.5 using HCl or NaOH (if required)
 - d. Make up the final volume to 1 L with distilled water
5. 1 M DL-DTT

Note: Always use freshly prepared DL-DTT solution.

 - a. Add 0.154 g of DL-DTT to 1.0 ml of sterile dH₂O in a safe lock tube
 - b. Dissolve completely by pipetting and sterilize the stock through a 0.2 µm syringe filter
6. 1.0 M Sucrose
 - a. Add 34.23 g of sucrose to 80 ml of sterile dH₂O in 250 ml flask. Dissolve completely
 - b. Make up the final volume to 100 ml and sterilize by autoclaving as directed above

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Competing interests

Authors have no competing interest.

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