Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Strains and plasmids

All *C. glabrata* strains and plasmids used in this study are listed in Tables 2.1 and 2.2, respectively.

Table 2.1: List of yeast and bacterial strains used in this study.

Yeast strain	Relevant genotype	Reference
BG2	Clinical isolate	Fidel *et al.*, 1996
YRK19	*ura3Δ::Tn903 G418^R*	Cormack & Falkow, 199
YRK20	*URA3*	De Las Penas *et al.*, 200
YRK83	*URA3 Cgyps1Δ::hph*	Kaur *et al.*, 2007
YRK84	*URA3 Cgyps7Δ::hph*	Kaur *et al.*, 2007
YRK85	*URA3 Cgyps1-11Δ::hph*	Kaur *et al.*, 2007
YRK86	*URA3 Cgyps1Δyps7Δ::hph*	Kaur *et al.*, 2007
YRK92	*URA3 CgypsCΔ(yps(3-6, 8-11)::hph*	Kaur *et al.*, 2007
YRK94	*URA3 Cgyps2Δ::hph*	Kaur *et al.*, 2007
YRK95	*URA3 Cgyps2Δyps(3-6, 8-11)Δ::hph*	Kaur *et al.*, 2007
YRK96	*URA3 Cgyps1Δ yps2Δ yps(3-6, 8-11)Δ::hph*	Kaur *et al.*, 2007
YRK97	*URA3 Cgyps2Δ yps (3-6, 8-11)Δ yps7Δ::hph*	Kaur *et al.*, 2007
YRK103	*ura3Δ::Tn903 G418^R Cgyps1-11Δ::hph*	Kaur *et al.*, 2007
YRK126	*URA3 Cgyps1Δ::hph/CgYPS1 (Clone 1)*	This study
YRK126-B	*URA3 Cgyps1Δ::hph/pRK74*	This study
YRK127	*URA3 Cgyps1Δ::hph/CgYPS1 (Clone 2)*	This study
YRK128	*URA3 Cgyps1-11Δ::hph/CgYPS1 (Clone 1)*	This study
YRK128-B	*URA3 Cgyps1-11Δ::hph/pRK74*	This study
YRK129	*URA3 Cgyps1-11Δ::hph/CgYPS1 (Clone 2)*	This study
YRK130	*URA3 Cg yps1-11Δ::hph/CgYPS7 (Clone 1)*	This study
YRK131	*URA3 Cg yps1-11Δ::hph/CgYPS7 (Clone 2)*	This study
YRK132	*URA3 Cg yps1-11Δ::hph/CgYPS7 (Clone 3)*	This study
YRK228	*ura3Δ::Tn903 G418^R Cgyps1Δ::hph*	Kaur *et al.*, 2007
YRK373	*ura3Δ::Tn903 G418^R Cgyps1Δyps7Δ::hph*	Kaur *et al.*, 2007
Tn*7* Transposon	*Tn7 R6Kγ ori URA3 npt (KmR)*	Castaño *et al.*, 2003
YRK688	*Cgstv1::*Tn*7*	This study
YRK689	*Cgvma13::*Tn*7*	This study
YRK690	*Cgypk9::*Tn*7*	This study
YRK735	*URA3 Cgyps1Δyps7Δ::hph/CgYPS1*	This study
YRK738	*URA3 Cgyps1Δyps7Δ::hph/CgYPS7*	This study
Bacterial strain	**Relevant genotype**	**Reference**
For maintenance of plasmid		
BRK2	*E. coli* DH5α *(Δ(argF-lac)U169 supE44 hsdR17 recA1 endA1gyrA96 thi-1 relA1(φ80lacZΔM15))*	
BRK74	DH5α /pRK74 Ampicillin Resistant (AmpR)	This study
BRK203	DH5α /pRK203 (AmpR)	This study
BRK204	DH5α /pRK204 (AmpR)	This study
BRK205	DH5α /pRK205 (AmpR)	This study
BRK206	DH5α /pRK206 (AmpR)	This study

For rescue of plasmid from *C. glabrata* Tn*7* insertion mutants		
BW23473	*E. coli* strain *(Δlac-169 robA1 creC510 hsdR514 ΔuidA::pir end A recA1)* carrying R6Kγ origin of replication that requires expression of the protein Π (the product of the *pir* gene) for replication.	Castaño *et al*., 2003

Table 2.2: List of plasmids used in this study.

Plasmid	Description	Reference
pRK74	A CEN-ARS plasmid (pGRB2.2) of *C. glabrata* carrying *S. cerevisiae URA3* as a selection marker. MCS sites are flanked by *S. cerevisiae PGK1* promoter at one end and by 3' UTR of *HIS3* at the other end.	Frieman *et al*., 2002
pRK203	*CgYPS1* cloned in the MCS of pRK74.	Kaur *et al*., 2007
pRK204	*CgYPS7* cloned in the Xba*I*-Xho*I* sites of pRK74. (Clone 1)	This study
pRK205	*CgYPS7* cloned in the Xba*I*-Xho*I* sites of pRK74.(Clone 2)	This study
pRK206	*CgYPS7* cloned in the Xba*I*-Xho*I* sites of pRK74.(Clone 3)	This study

2.1.2 Chemicals and antibodies

All chemicals were purchased from commercial sources. Media components for bacterial and yeast growth were obtained from BD (Becton, Dickinson and Company, USA). Other chemicals were purchased from Sigma-Aldrich Co., USA. Materials used in recombinant DNA experiments were primarily obtained from New England Biolabs, Invitrogen, Bangalore Genei and MBI Fermentas. SuperScript™ III first-strand synthesis system was purchased from Invitrogen. MESA GREEN qPCR MasterMix Plus for SYBR® Assay was purchased from Eurogenetec. Kits used for plasmid isolation, PCR product purification and DNA gel extraction were from Qiagen. Radioactive chemical, ortho- P^{32}-phosphoric acid, was procured from BRIT-Jonaki, CCMB, Hyderabad.

Anti-Pma1 polyclonal antibody raised against *S. cerevisiae* Pma1 was purchased from Santa Cruz Inc., USA. Anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) was purchased from Cell Signaling Technology, USA. Anti-CPY polyclonal antibody raised against *S. cerevisiae* CPY was procured from Thermo Scientific. Anti-Gapdh antibody raised against human Gapdh was purchased from Abcam. Secondary

antibodies, anti-mouse IgG and anti-rabbit IgG conjugated with horseradish peroxidase (HRP) were obtained from Cell Signaling Technology, USA.

2.1.3 Media

2.1.3.1 Bacterial medium

Luria Bertani (LB):

0.5% Yeast Extract

1% Tryptone

1% NaCl

LB-ampicillin and LB-kanamycin plates:

LB medium

50 µg/ml ampicillin

30 µg/ml kanamycin

Super Optimal Broth (SOB):

0.5% Yeast extract

2% Peptone

10 mM NaCl

2.5 mM KCl

10 mM $MgCl_2$

10 mM $MgSO_4$

2.1.3.2 Yeast medium

Yeast Extract-Peptone-Dextrose (YPD):

1% Yeast extract

2% Peptone

2% Dextrose

Yeast Nitrogen Base (YNB):

0.67% Yeast Nitrogen Base

2% Dextrose

Yeast Carbon Base (YCB):

1.17% Yeast Carbon Base

1% Dextrose

CAA:

0.67% Yeast Nitrogen Base

2% Dextrose

0.6% Casamino acids

Plates were made by adding 2% agar.

2.1.4 Buffers and solutions

2.1.4.1 Common buffers

Phosphate-Buffered Saline (PBS):

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

pH was adjusted to 7.3.

This was prepared as a 10 X stock solution and used at a 1 X concentration.

Tris-HCl buffer:

0.5 M Trizma Base

pH was adjusted to 7.6 using concentrated HCl.

This was prepared as a 10 X stock solution and used at a 1 X concentration.

Tris-EDTA (TE) buffer:

10 mM Tris-HCl (pH 8.0)

1 mM EDTA

Tris-Acetic acid EDTA (TAE) buffer:

40 mM Tris base

0.5 M EDTA

pH was adjusted to 8.5 with glacial acetic acid.

This was prepared as a 50 X stock solution and used at a 1 X concentration.

Tris-Borate EDTA (TBE) buffer:

90 mM Tris-borate

2 mM EDTA (pH 8.0)

pH was adjusted to 8.3 with HCl.

This was prepared as a 10 X stock solution and used at a 1 X concentration. Both TAE and TBE were used as standard gel electrophoresis buffers.

HEPES buffer:

This was used to prepare YNB medium of different pH.

1M HEPES

pH was adjusted to 7.5 with NaOH.

Buffer was filter-sterilized and stored in an amber-coloured bottle.

Citrate buffer (0.1M, pH 5.5):

4.7 volume of 0.1 M Citric acid

15.4 volume of 0.1 M Sodium citrate

2.1.4.2 Transformation-related solutions

INOUE transformation buffer:

For bacterial DH5α ultra-competent cells preparation

10 mM PIPES (free acid)

15 mM $CaCl_2.2H_2O$

250 mM KCl

55 mM $MnCl_2.4H_2O$

pH was adjusted to 6.7 with 1 N KOH.

$MnCl_2$ needs to be added separately, drop by drop with stirring, to the buffer. PIPES goes into solution when pH is greater than 6.7. The solution, after pH adjustment to 6.7 was filter-sterilized and stored at -20ºC.

Reagents for yeast transformation:

1 M Lithium acetate (LiOAc)

50% Polyethylene glycol

10 mg/ml Carrier DNA

Dimethylsulfoxide (DMSO)

2.1.4.3 Genomic DNA and RNA isolation buffers

Genomic DNA isolation buffers

Buffer A:

50 mM Tris-HCl

10 mM EDTA

150 mM NaCl

1% Triton-X

1% SDS

Buffer B:

50 mM Tris-HCl (pH 7.5)

10 mM EDTA

1.1 M Sorbitol

50 mM β-mercaptoethanol (To be added just before use)

Buffer C:

100 mM Tris-HCl (pH 7.5)

10 mM EDTA

10% SDS

RNA isolation buffer

AE buffer:

3 M Sodium acetate

0.5 M EDTA (pH 8.0)

Phenol:Chloroform:Isoamyl Alcohol (25:24:1) solution:

25 volume of Phenol

24 volume of Chloroform

1 volume of Isoamyl alchol

DNA sample loading buffer:

0.25% Bromophenol blue

0.25% Xylene cyanol

15% Ficoll

2.1.4.4 Protein isolation and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

Whole cell lysis buffer (Homogenizing buffer):

50 mM Tris-HCl (pH 7.5)

2 mM EDTA

10 mM sodium fluoride*

1 mM sodium orthovanadate*

1 X protease inhibitor cocktail (Roche Cat # 04693159001) *

*To be added fresh before use.

SDS-PAGE

30% Acrylamide solution

29 g Acrylamide

1 g Bis-acrylamide

Dissolved in 100 ml H_2O.

10% Sodium Dodecyl Sulfate (SDS):

10 g SDS in 100 ml H_2O

Resolving gel mix (12%) (15 ml):

4.89 ml H_2O

6 ml 30% acrylamide:bisacrylamide (29:1) mix

3.8 ml 1.5 M Tris-HCl (pH 8.8)

150 µl 10% SDS

150 µl 10% APS

10 µl TEMED

Stacking gel mix (3 ml):

1.689 ml H_2O

500 µl 30% acrylamide:bisacrylamide (29:1) mix

380 µl 1 M Tris-HCl (pH 6.8)

30 µl 10% SDS

30 µl 10% APS

10 µl TEMED

SDS loading buffer:

130 mM Tris-HCl (pH 8.0)

20% (v/v) Glycerol

4.6% (w/v) SDS

0.02% Bromophenol blue

2% DTT

This was prepared as a 4 X stock solution and used at a 1 X concentration.

SDS-PAGE running buffer:

0.25 M Tris-HCl (pH 8.0)

1.92 M Glycine

1% SDS

This was prepared as a 10 X stock solution and used at a 1 X concentration.

Coomassie brilliant blue (CBB) staining solution:

50% Methanol

10% Acetic acid

0.1% Coomassie brilliant blue-R250

Western blot

Transfer buffer:

0.25 M Tris-HCl (pH 8.0)

1.92 M Glycine

1% SDS

This was prepared as a 10 X stock solution and used at a 1 X concentration.

1X Transfer buffer (1 litre):

200 ml of methanol

100 ml of 10 X transfer buffer

700 ml of water

Ponceau 3S staining solution:

0.25% Ponceau 3S

40% Methanol

15% Acetic acid

Tris-Borate Saline (TBS):

25 mM Tris

150 mM NaCl

pH was adjusted to 7.4 with HCl.

This was prepared as 10 X stock solution and used at 1 X concentration.

Blocking and wash buffers (PBS-T and TBS-T):

5% Fat-free milk

0.1% Tween-20

Volume was made to 100 ml either with 1 X PBS (PBS-T) or 1 X TBS (TBS-T).

2.1.4.5 Other buffers

Citric-Phosphate buffer:

0.5 M citric acid

0.5 M dibasic sodium phosphate

pH was adjusted to 5.0 with phosphoric acid and filter-sterilized.

MES/TEA buffer:

1 mM MES (2-(N-morpholino)ethanesulfonic acid)

pH was adjusted to pH 5.0 with TEA (triethanolamine).

Plasma membrane suspension buffer:

50 mM Tris-HCl (pH 7.5)

0.1 mM EDTA

0.1 mM Dithiothreitol

20% Glycerol

Polyphosphate extraction buffer:

50 mM HEPES (pH 7.2)

10 mM EDTA

0.1% SDS

1 M urea

Toluidine blue staining solution:

0.05% Toluidine blue

20% Methanol

2% Glycerol

Solution was prepared in H_2O.

Destaining solution for polyphosphate gels:

20% Methanol

2% Glycerol

Solution was prepared in H_2O.

Spheroplast buffer:

50 mM Potassium phosphate (pH 7.5)

0.6 M Sorbitol

0.2 X YPD medium

PS (PIPES-Sorbitol) buffer:

10 mM PIPES-KOH (pH 6.8)

200 mM Sorbitol

1 X protease inhibitor cocktail (Roche Cat # 04693159001)*

*To be added fresh before use.

2.1.5 Oligonucleotides

Oligonucleotides/primers used in this study were designed using either free online-tool Primer3 (http://frodo.wi.mit.edu/) or Gene Runner software (http://www.generunner.net/). Oligonucleotides used in this study were commercially

synthesized from MWG Biotech Pvt. Ltd., Bangalore. All primers used in this study are listed in Table 2.3.

Table 2.3: List of primers used in this study.

Primer name	Sequence (5' - 3')	Target gene
For *CgYPS7* cloning in pRK74		
OgRK173	AGAG*TCTAGA*ATGGCTACGCGTACGATGCTGTTTC	*CgYPS7*
OgRK174	CATA*CTCGAG*TTATATCACCATGGAGCCCAGGAGC	*CgYPS7*
For checking *CgYPS7* clone		
OgRK59	CACCAGTGACGCCAACGAGGC	*CgYPS7*
OgRK60	CCTTGCCCTCCAGGTCAACG	*CgYPS7*
For qPCR		
OgRK83	CGGGTCAAAGGCCATACCA	*CgYPS1*
OgRK62	CTTGGAGTGGTCGACGGCAC	*CgYPS1*
OgRK84	CTACAATCCTGCATTGAGTGA	*CgYPS2*
OgRK85	CCTGGCTCACCTTGTGGCAT	*CgYPS2*
OgRK65	CGACGACCCATCCCCAGGCTC	*CgYPS3*
OgRK66	ACTTAGCTCTTCATGGTAACG	*CgYPS3*
OgRK67	CAACTTGCTGCCAATGGCTC	*CgYPS4*
OgRK68	TCAATGTCATCTCTGGCTTGC	*CgYPS4*
OgRK69	GTACGCTATGTTCTCTGAAGA	*CgYPS5*
OgRK70	AACTGCTCTCCTGAGAAACAC	*CgYPS5*
OgRK71	CTATAAATTCTAACGGAACTACG	*CgYPS6*
OgRK72	GACTCTGCACCACTTGGGATG	*CgYPS6*
OgRK58	GCTACATCGCACGACACCAGC	*CgYPS7*
OgRK59	CACCAGTGACGCCAACGAGGC	*CgYPS7*
OgRK73	CGATGGTCCTTATGCTGGTAG	*CgYPS8*
OgRK74	GGCATCATTACTTGTATCCTC	*CgYPS8*
OgRK75	CTACTTTCAACTATTCTTCTAATGTC	*CgYPS9*
OgRK76	GTTAATACCATCAAGGGCAGAG	*CgYPS9*
OgRK77	CTCTTATGACACAGACATTAAGC	*CgYPS10*
OgRK78	CCGTTGTTTGGTGGAGCGACC	*CgYPS10*
OgRK79	GCAAAGTCTGAGCCAGTTGGAGC	*CgYPS11*
OgRK80	CGATCAAACTCAGAACAAG	*CgYPS11*
OgRK89	CTCCACCACTGCTGAAAGAG	*CgACT1*
OgRK90	GGTCAATACCAGCAGATTCTAG	*CgACT1*
For semi-quantitative RT-PCR		
OgRK545	GTGATGTTACTGTTCATTTTGATTATCCTG	*CgPMU2*
OgRK546	GATACATCGATCACAGTGTAAACTAG	*CgPMU2*
OgRK549	GTGTTCCCAACCAGATACAGATC	*CgPHO84*
OgRK550	CTGGGGCCAGCTTGGAGATGTC	*CgPHO84*
OgRK551	CGGGACCTGTCAAAGTCGA	*CgPHM1*
OgRK552	CACTTATAATTCTCAACCGTTTC	*CgPHM1*
OgRK557	CTGTGTGCATGAGCTCGACT	*CgPHM8*
OgRK558	GCAGTAGGTGATTCCGTCGAAC	*CgPHM8*
OgRK191	TTTCAGAGTGCCAACTGTCG	*CgGAPDH*
OgRK192	TGAAACAACAGCGTCCTCAG	*CgGAPDH*

For checking homologous recombination in identified Tn*7* insertion mutants.		
OgRK644	GATACTGACGTGGCACCGG	*CgFAB1*
OgRK645	TACCTCCACCTACAAGGCCC	*CgFAB1*
OgRK648	GGAGGAGCGCATGAAGATTG	*CgBST1*
OgRK649	GGTGACCGAGTGCAGTGACG	*CgBST1*
OGRK775	CAAATTGTATTGGGTTCCTCGGTGG	*CgSET2*
OGRK776	CGTTCTTCTTCCTTCTTGCGG	*CgSET2*
OGRK777	GAGCGGATCGAAAGAAGAGAAG	*CgERG4*
OGRK778	CCTTGCCGTACTTCTTCTCAC	*CgERG4*
OGRK779	GGACGTTGAGAGACAGACATTTAAG	*CgSLT2*
OGRK780	CTCCTATCCAGTCCAAACTCC	*CgSLT2*
OGRK783	GAACGTGGAAGAGAACGATGATTTG	*CgSAS1*
OGRK784	GTTCAAGTGTTTCAGGGTCGG	*CgSAS1*
OGRK785	GGCGTAACTGGTATAGGTGCCTC	*CgMNL1*
OGRK786	GCGTGGACACCACCTATCG	*CgMNL1*
OGRK789	GTTGGTGCATACATGCTGTTCAGG	*CgRPC31*
OGRK790	GCAGGCTCATCACCATAATCATC	*CgRPC31*
OGRK793	GGGTCTCAAACTAAGTGGTGGAC	*CgURB1*
OGRK794	GTAGGATCGGAAGCTAATATGCTC'	*CgURB1*
OGRK795	CCTATTGGAGGCTATTGTAGATC	*CgSGF73*
OGRK796	CCATGTTCGCACCTGGGGATCC	*CgSGF73*
OGRK797	GGAGATACAGAAGAAGATGACGATG	*CgDNA2*
OGRK798	CCTGAGACAGGTACATAACATCC	*CgDNA2*

2.2 Methods

2.2.1 Microbiological techniques

2.2.1.1 Strains and culture conditions

C. glabrata strains were maintained either on rich YPD or synthetically-defined YNB medium. *C. glabrata* cells were routinely cultured at 30˚C with shaking at 200 revolutions per min (rpm) unless otherwise mentioned. For growth experiments, *C. glabrata* strains were freshly revived on YPD medium from glycerol stocks. *Escherichia coli* DH5α bacterial strain was used for plasmid transformation and propagation purposes and maintained on LB medium. *E. coli* BW23473 bacterial strain was used to rescue Tn*7* transposon cassette from *C. glabrata* Tn*7* insertion mutants and maintained on LB medium. Bacterial strains harboring plasmids were maintained on LB agar plates supplemented with appropriate antibiotics. For plasmid isolation purpose, bacterial strains were grown overnight in liquid LB broth containing appropriate antibiotics at 37˚C with shaking at 200 rpm. For preparation of the solid medium, 2% bacto-agar was added to the medium and autoclaved. To prepare medium of different pH, YNB medium was either buffered with citrate or HEPES buffer (100

mM final concentration) and pH was adjusted to the desired value by addition of HCl or NaOH. Medium was sterilized by autoclaving. YNB agar plates of different pH were prepared by mixing equal volume of separately autoclaved 4% bacto-agar solution and 2X varied pH-adjusted-YNB liquid medium .

All routine sterilization of medium and solutions was either carried out by autoclaving at 121˚C for 15-20 min at high pressure condition (15 psi) or filtration with 0.2 µm polyvinylidene fluoride (PVDF) membrane filter unit (Millex®-GV, Millipore).

Both yeast and bacterial strains were stored as frozen 15% glycerol stock at -80˚C for extended lifetime.

2.2.1.2 Growth assay and measurement of generation time

For growth analysis of *C. glabrata* strains, a single colony from YPD or YNB agar medium was inoculated in appropriate liquid medium and incubated at 30˚C with shaking at 200 rpm for 14-16 h. This overnight grown culture was used to inoculate test medium to an initial OD_{600} of 0.1 to 0.3. Optical density/Absorbance of the cell suspension was measured using Ultraspec 2100 pro UV/visible spectrophotometer (Amersham Biosciences) at 600 nm at regular time-intervals up to a period of 96 h. Absorbance values were plotted with respect to time. Generation time of yeast strains was calculated from the logarithmic (log) phase of cell growth. Growth profiles between 4 (t_1) and 8 h (t_2) time interval were considered for calculation of generation time using following formula.

$$\text{Generation time } (G) = (t_2 - t_1) \times \{\log (2)/ [\log (B_f/B_i)]\}$$

G = Generation time in h

t_1 = Initial time point taken for analysis

t_2 = Final time point taken for analysis

B_f = Number of cells at time t_2 (calculated on the basis of OD_{600} values, wherein 1 OD_{600} of *C. glabrata* corresponds to 2 X 10^7 cells.)

B_i = Number of cells at time t_1 (calculated as mentioned above)

Several yeast strains used in this study were analysed for their susceptibility to various chemical compounds, drugs and metal ions. For this purpose, stock solutions were

prepared in appropriate solvents, sterilized by autoclaving or filtration and stored at appropriate temperature.

2.2.1.3 Yeast cell viability assessment *via* colony forming unit (CFU) assay

Yeast cell viability was measured by plating appropriate dilutions of cell culture on YPD plates at various time intervals during growth. Cell suspension was diluted in 1X PBS. YPD plates were incubated at 30˚C for 2-3 days and total colony forming units (CFUs) were calculated by counting the number of colonies that appeared on YPD plates and dividing that number by an appropriate dilution factor.

2.2.1.4 Serial dilution spot assay

C. glabrata strains were grown overnight either in YPD or YNB liquid medium at 30˚C with shaking at 200 rpm. Cells were harvested and suspended in 1X PBS to a final OD_{600} of 1.0. Five 10-fold serial dilutions of cell suspension were prepared in PBS and 3-4 µl was spotted on YPD/YNB plates containing various test compounds using a multi-channel pipette. Plates were incubated at 30˚C and growth profiles were recorded after 2-4 days.

2.2.1.5 Cultivation of logarithmic-phase cell culture

All experiments in this study were performed with log-phase cells unless otherwise mentioned. For obtaining log-phase cells, overnight YNB- or YPD medium-grown yeast cells were re-inoculated in fresh YNB or YPD medium to an initial OD_{600} of 0.1-0.2. Cells were incubated at 30˚C with shaking at 200 rpm till the OD_{600} reached to 0.4-0.6 OD. After incubation, log-phase cells were collected by centrifugation at 4,000 rpm for 3 min, washed once with the same medium and used for further analysis.

2.2.1.6 *E. coli* DH5α ultra-competent cells preparation

A single colony of *E. coli* DH5-α strain was inoculated in 10 ml LB medium and incubated at 37˚C for overnight. 4 ml of this overnight culture was inoculated in 2 lt SOB medium and incubated at 18˚C till the OD_{600} reaches to 0.5. Cells were harvested by centrifugation at 2,500 g for 10 min at 4˚C and washed gently in 80 ml ice-cold Inoue transformation buffer. Cells were collected by centrifugation at 2,500 g for 10 min at 4˚C and gently resuspended in 20 ml ice-cold Inoue transformation buffer. To this cell suspension, 1.5 ml sterile DMSO was added and swirled gently. Cell

suspension was kept on ice for 10 min and 50 µl volume was aliquoted to chilled sterile microcentrifuge tubes. Cells were immediately snap-frozen in liquid nitrogen and stored at -80˚C.

2.2.1.7 Bacterial transformation

E. coli DH5α ultra-competent cells were transformed with plasmid DNA by heat shock at 42˚C for 90 sec as described previously in Molecular Cloning-A Laboratory Manual (Sambrook and Russell, 2001). Bacterial transformants were selected on LB agar medium containing appropriate antibiotics. Transformants obtained were colony purified on LB plates containing antibiotics. Presence of the desired insert was first verified by colony PCR followed by PCR using extracted plasmid DNA as template.

2.2.1.8 Bacterial plasmid isolation

5-10 ml saturated bacterial culture harboring the desired plasmid was harvested at 5,000 g for 3 min. Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen, USA) or GenElute™ HP Plasmid Miniprep kit (Sigma-Aldrich, USA) as per manufacturer's instructions.

2.2.1.9 Yeast transformation using lithium acetate (LiOAc) strategy

C. glabrata yeast cells were grown overnight in 5 ml YPD medium at 30˚C. An aliquot from the overnight culture was inoculated in 10 ml fresh YPD medium to an initial OD of 0.1. Cells were incubated at 30˚C till the culture OD_{600} was between 0.4 and 0.6. Cells were harvested in a sterile 50 ml centrifuge tube and washed twice with sterile *Milli-Q* (MQ) water. Washed cells were suspended in 100 µl of 100 mM LiOAc, mixed thoroughly and transferred to a sterile 1.5 ml microcentrifuge tube. A transformation mix containing 240 µl polyethylene glycol (PEG) (50% *(w/v)*), 36 µl LiOAc (1 M), 25 µl ultrapure single-stranded salmon sperm DNA (2 mg/ml) (Clonetech) was added to 50 µl cell suspension. 50 µl transforming DNA (1 µg circular plasmid DNA) was added to the above suspension. Whole mixture was vortexed gently and incubated at 30˚C for 45 min. 43 µl DMSO was added to the tube and incubated at 42˚C for 15 min. Cells were collected after centrifugation at 5,000 rpm for 1 min and suspended in minimal medium containing 0.6% Bacto-Casamino acid. Transformation mixture was plated on CAA plates and transformants were selected for uracil prototrophy.

2.2.2 Molecular biology techniques

2.2.2.1 Yeast genomic DNA isolation: Based on the subsequent use, DNA from *C. glabrata* cells was extracted using three different methodologies.

2.2.2.1.1 Protocol I (Quick genomic DNA isolation)

This quick extraction method was used to isolate genomic DNA which was used as template to amplify gene of interest or to verify the knock-out. *C. glabrata* cells were grown overnight to saturation in 10 ml YPD medium at 30˚C. Cells were harvested at 4,000 rpm for 5 min, resuspended in 400 µl Buffer A containing 50 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, 1% Triton X-100 and 1% SDS and were transferred to a 2 ml microcentrifuge tube. Equal volume of phenol-chloroform solution was added to the above suspension followed by vortexing for 2-3 min and incubation at 42˚C for 30 min with continuous agitation at 800 rpm on thermo mixer (Eppendorf). Cell debris was removed by centrifugation at 12,000 rpm for 5 min and aqueous fraction (~ 350 µl) was transferred to a new 2 ml microcentrifuge tube. 0.3 µl RNase Cocktail™ (Ambion® # AM2286) containing RNase A (500 U⁄ml) and RNase T1 (20,000 U⁄ml) was added and tubes were incubated at 37˚C for 30 min. DNA was precipitated with 2.5 volumes of chilled ethanol and 1/10th volume of 3 M sodium acetate (pH 5.2). DNA pellet was washed with chilled 70% ethanol and semi-dried under air. Pellet was suspended in 100 µl TE (10 mM Tris-HCl and 1 mM EDTA; pH 8.0) and stored at -20˚C. DNA concentration was determined by recording absorbance at 280 nm in Nanodrop (Nanodrop ND-1000, Thermo Scientific).

2.2.2.1.2 Protocol II (Glass bead lysis method)

In this method of genomic DNA extraction, yeast cells were lysed by mechanical disruption with glass beads. Briefly, yeast cells were harvested after overnight growth in YPD medium, resuspended in 500 µl water and transferred to a 2 ml microcentrifuge tube. Cells were pelleted down at 10,000 rpm for 1 min. Resulting supernatant was discarded and the pellet was resuspended in 500 µl Buffer A. The tube was incubated at 65˚C for 15 min. After incubation, 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and 0.5 gm of acid-washed glass beads (Sigma # G8772) were added to the tube. Cells were lysed by three cycles of high speed vortexing with intermittent ice breaks for 45 sec and pelleted down at 12,000 rpm for 3 min at 4˚C. Uppermost aqueous phase was transferred to a 2 ml microcentrifuge tube, 500 µl of

phenol:chloroform:isoamyl alcohol (25:24:1) was added to the tube and mixed thoroughly. Aqueous phase was collected after centrifugation at 12,000 rpm for 3 min and was transferred to a new 2 ml microcentrifuge tube. 1 ml absolute ethanol was added to the aqueous phase and DNA was precipitated by centrifugation at 12,000 rpm for 8 min at 4˚C. DNA pellet was washed with chilled 70% ethanol and dried under air. DNA pellet was resuspended in 50 µl TE containing 0.3 µl of RNase Cocktail™ (Ambion® # AM2286) and incubated at 50˚C for 20 min. 200 µl additional TE was added to the above suspension and DNA was stored at -20˚C.

2.2.2.1.3 Protocol III (Spheroplast lysis method)

The method was used for isolation of good quality genomic DNA that was used to map Tn*7* insertion in *C. glabrata* mutants. Briefly, 10 ml saturated yeast culture was harvested, resuspended in 1 ml sterile water and transferred to a 2 ml microcentrifuge tube. Cells were pelleted down by centrifugation at 4,000 rpm for 5 min. Supernatant was discarded and the pellet was resuspended in 500 µl freshly prepared solution containing 100 mM EDTA and 5% β-mercaptoethanol and incubated at 42˚C for 10 min. After incubation, cells were spun down at 5,000 rpm for 1 min and resuspended in 500 µl freshly-prepared Buffer B. One tip full of lyticase (Sigma # L4025) was added and cell suspension was incubated at 37˚C for 1 h. Following incubation, cell suspension was spun down at 6,000 rpm to recover spheroplasts. Spheroplasts were gently resuspended in 500 µl Buffer C and DNA was twice extracted with 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) solution. Aqueous layer was collected in a new 2 ml microcentrifuge tube and DNA was precipitated with 1 ml ethanol and 1/10th volume of 3 M sodium acetate (pH 5.2) by centrifugation at 13,000 rpm for 5 min. Pellet was resuspended in 200 µl TE containing 0.3 µl of RNase Cocktail™ and incubated at 37˚C for 30 min. After incubation, 300 µl additional TE was added and DNA was re-precipitated with ethanol and 3 M sodium acetate as described above. Pellet was washed with 70% ethanol and dried under air. DNA pellet was finally suspended in 100 µl TE and stored at -20˚C.

2.2.2.2 Total RNA isolation

All solutions were made in RNase free diethylpyrocarbonate (DEPC) water. Microcentrifuge tubes and tips employed for RNA work were autoclaved twice and kept at 70˚C for overnight before use. RNaseZap® (Ambion) was sprayed on non-

autoclavable plastic items to remove RNase contamination. RNA was isolated from *C. glabrata* cells using hot phenol extraction strategy. Log-phase cells well harvested at 5,000 g for 5 min at 4˚C, resuspended in 1 ml ice-cold DEPC water and transferred to a 2 ml microcentrifuge tube. Cells were spun down at 6,000 g for 3 min at 4˚C and resuspended in 350 µl AE solution. 50 µl SDS and 400 µl acid phenol were added to the above tube and mixed well by vortexing. Tubes were incubated at 65˚C for 15 min with continuous mixing. After incubation, tubes were kept on ice for 5 min and centrifuged at 12,000 rpm for 5 min at 4˚C. Aqueous phase was collected and re-extracted with an equal volume of cholroform. Total RNA was precipitated at -20˚C with $1/10^{th}$ volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of ice-cold 100% ethanol and collected by centrifugation at 12,000 rpm for 5 min at 4˚C. RNA pellet was washed with ice-cold 70% ethanol and resuspended in 100 µl commercially available DEPC-treated water (Sigma # 95284). RNA concentration was measured by recording absorbance at 260 nm. Purity of RNA sample was checked by A_{260nm}/A_{280nm} ratio where ratio of >1.8 was considered as good quality RNA. RNA integrity was checked by gel electrophoresis on 8% agarose gel made in DEPC-treated TAE buffer.

2.2.2.3 Complementary DNA (cDNA) synthesis

1 µg good quality RNA was treated with DNase I (amplification grade, Invitrogen) to remove DNA contamination and used for complementary DNA (cDNA) synthesis using reverse transcriptase enzyme and oligo-dT primers. SuperScript® III First-Strand Synthesis System (Invitrogen) was used to carry out cDNA synthesis reaction according to the manufacturer's instructions. cDNA was stored at -20˚C.

2.2.2.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

To determine the expression level of a specific gene, quantitative real-time polymerase chain reaction (qRT-PCR/qPCR) was performed on cDNA using gene specific primers. Primers for qPCR were designed in such a way so as to get amplification products in a size range of 150 to 300 bp. Optimal primer and cDNA concentrations were standardized and qPCR was performed in ABI Prism 7000/7500 Real time PCR Machine (Applied Biosystems). Briefly, 0.4 µl cDNA was mixed with 0.1 to 0.2 picomoles of gene specific forward and reverse primers and 10 µl 2X MESA GREEN qPCR™ Mastermix Plus containing SYBR green dye (Eurogentec) in a well of a 96-well PCR plate (Axygen). SYBR green is a dye that specifically binds to double

stranded DNA. Final reaction volume was adjusted to 20 µl with DEPC-treated water and amplification reaction was carried out using these parameters: initial denaturation at 95˚C for 5 min followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 55˚C-57˚C for 30 sec, elongation at 72˚C for 40 sec and final extension at 72˚C for 10 min. Transcript levels were quantified with an end-point value known as C_t (cycle threshold). The C_t defines the number of PCR cycles required for the fluorescent signal of SYBR green dye to cross more than the background level. The C_t value is inversely proportional to the amount of nucleic acid product. C_t values were obtained during exponential phase of amplification and used for calculation of relative-fold change in gene expression after normalization to C_t values of either housekeeping gene *ACT1* (gene encoding actin) or *TDH3* (gene encoding Gapdh) with the help of the following formula.

$$\text{Fold change in expression} = 2^{-\Delta\Delta Ct}$$

$\Delta\Delta C_t = \Delta C_t$ treated - ΔC_t untreated

ΔC_t treated = C_t value for gene of interest under test/treated condition - C_t value for internal control gene (*ACT1/TDH3*) under test/treated condition

ΔC_t untreated = C_t value for gene of interest under untreated condition - C_t value for internal control (*ACT1/TDH3*) gene under untreated condition

2.2.2.5 Cloning of *CgYPS7* gene

C. glabrata CgYPS7 ORF was cloned in a self-replicating pGRB2.2 plasmid which contains *C. glabrata* CEN-ARS, *S. cerevisiae URA3* gene, *S. cerevisiae PGK1* promoter and *C. glabrata HIS3*-3′ untranslated region. For cloning *CgYPS7* in pGRB2.2, *CgYPS7* ORF (1.764 kb) was PCR-amplified from the wild-type genomic DNA with high fidelity Platinum Pfx DNA polymerase using primers carrying restriction sites for XbaI and XhoI. The 1.764 kb amplified PCR product was purified with QIAquick PCR purification kit (Qiagen # 28104), digested with XbaI and XhoI and cloned in the pGRB2.2 plasmid at XbaI–XhoI sites in the multiple cloning site (MCS) region downstream of the *PGK1* promoter. Positive clones were verified by PCR, sequencing and complementation analyses of *Cgyps7*Δ mutant. Yeast transformants obtained by lithium acetate method were selected on plates lacking uracil

and colony purified on CAA plate. 15% glycerol stocks were made for two independent transformants and stored at -80˚C.

2.2.2.6 Screening of *C. glabrata* Tn*7* insertion mutants

C. glabrata Tn*7* insertion mutant library was screened for reduced growth in YNB-pH 2.0 medium. This mutant library, composed of 9,134 Tn*7* insertion mutants, is arrayed in 96-well microtitre plates (Castaño *et al.*, 2003). 2 µl of each mutant strain was inoculated in 120 µl YNB medium and grown overnight at 30˚C in an incubator with constant shaking at 120 rpm. Overnight grown cultures were 120-fold diluted with 1X PBS in a 96 well block and transferred, using a 96-well pin replicator, to YNB and YNB-pH 2.0 medium. Plates were incubated at 30°C and mutant phenotypes were recorded after 3-4 days.

2.2.2.7 Tn*7* insertion mutant rescue and gene identification

Identification of disrupted locus in Tn*7* insertion mutants was carried out as described previously (Kaur *et al.*, 2004). Disrupted locus in each mutant is physically marked with a mini Tn*7* transposon derivative containing conditional origin of replication R6Kγ (facilitates Tn*7* recovery), *S. cerevisiae URA3* and *Klebsiella pneumoniae hph* gene (confers resistance to hygromycin B) (Castaño *et al.*, 2003). Briefly, genomic DNA was isolated from overnight grown Tn*7* insertion mutants using spheroplast lysis method. After RNAse treatment, 10 µg DNA was either digested with Mfe1 or SpeI restriction enzyme as the Tn*7* cassette lacks these enzyme sites. Following overnight digestion, DNA was precipitated with 1 ml ethanol and 1/10th volume of 3 M sodium acetate (pH 5.2). DNA pellet was washed twice with ice-cold 70% ethanol, air dried and resuspended in sterile MQ water. DNA was recircularized with T4 DNA ligase. Resultant circular plasmid contains the Tn*7* cassette flanked on either side by the gene, it has disrupted in the genome of *C. glabrata*. This circular plasmid DNA was transformed in *E. coli* BW23473 strain, which contains protein Π (the product of the pir gene) required by R6Kγ *ori* for replication. Transformation of circularized DNA in *E. coli* BW23473 electrocompetent cells was performed as described below. Plasmids from selected transformants were isolated and sequenced with outward primers from Tn*7* right and left ends to sequence the disrupted gene fragment. For identification of

the disrupted gene, BLAST N of the sequences from rescued plasmids was performed against *C. glabrata* Genolevures database (http://www.genolevures.org/blast.html).

2.2.2.8 Electro-competent cell preparation

A single colony of *E. coli* BW23473 strain from a freshly-streaked LB plate was inoculated in 50 ml LB medium. Culture was incubated overnight at 37°C with shaking at 200 rpm. 25 ml of the overnight-grown BW23473 culture was transferred to 500 ml pre-warmed LB medium and incubated at 37°C till the OD_{600} reached to 0.4. After incubation, cultures were transferred to an ice-water bath and centrifuge at 1,000 g for 15 min at 4°C. Cells were washed twice with 500 ml ice-cold water, thrice with 250 ml ice-cold 10% glycerol solution and resuspended in 1 ml 10% glycerol solution. Cell suspension was normalized to final cell density of 3 x 10^{10} cells/ml and dispensed in 50 µl volume into sterile ice-cold microcentrifuge tubes. Aliquots were snap frozen in liquid nitrogen and stored at -70˚C for further use.

2.2.2.9 *E.coli* BW23473 transformation by electroporation

E. coli BW23473 electro-competent cell aliquots were thawed on ice and mixed with 1-2 µl of plasmid DNA. Mixture was pulsed with the Gene Pulser® electroporation apparatus (Bio-Rad) at 1800 Volts with 25 µF and 200 Ω current in a chilled 0.1 cm electroporation cuvette (Bio-Rad). Immediately after successful pulsing, 1 ml LB medium was added to the cuvette and suspension was transferred to a 1.5 ml sterile centrifuge tube. Cells were incubated at 37°C for 1 h with shaking and further plated on LB plates containing kanamycin (30 µg/ml). Positive colonies were inoculated in LB liquid medium containing kanamycin (30 µg/ml) for plasmid isolation.

2.2.2.10 Microarray analysis

Log-phase wild-type and *Cgyps1*Δ cells were grown in YNB and YNB-pH 2.0 medium. After 1 h incubation, yeast cells were collected, washed and were stored in RNAlater at -80°C. These frozen samples were sent to Genotypic Technology Ltd., Bangalore (http://www.genotypic.co.in) which provides services of global gene analysis on Agilent platform. A 8x15k GE array comprised of 60mer oligonucleotides for a total of *C. glabrata* 5503 genes was used wherein average number of replicates for each probe was three. Labeling was done in single color and data is the average of two

hybridizations from biological replicates for each sample. Data was extracted with Feature Extraction software v 10.5 (Agilent) and normalized with GeneSpring GX v 11.0.1 (Agilent) software using the recommended Percentile shift Normalization to 75th percentile. Raw Data sets for this study are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24267.

2.2.3 Biochemical techniques

2.2.3.1 Protein extraction

Log-phase yeast cell cultures were harvested and total protein was extracted by lysing yeast cells using glass beads. Briefly, 10 ml log-phase yeast cultures grown in appropriate medium were harvested, washed once with ice-cold water and suspended in 250 µl homogenizing buffer containing 1 mM phenylmethylsulfonylfluoride (inhibits serine proteases), 10 mM sodium fluoride (inhibit Ser/Thr and acid phosphatases), 1 mM sodium orthovanadate (inhibits Tyr and alkaline phosphatases) and 1X concentration of protease inhibitor cocktail (Roche Cat # 04693159001). Cells were lysed with glass beads by vortexing five times at high speed for 1 min with intermittent 1 min ice breaks. Unbroken cells and cell debris were removed by centrifugation at 1,000 g for 5 min at 4˚C. Cell lysate was collected and protein was quantified using bicinchoninic acid (BCA) protein assay kit (Thermo Scientific # 23227) as per supplier's instructions.

2.2.3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS- PAGE was performed as described previously (Laemilli, 1970). 10-40 µg protein samples were mixed with 4X SDS loading buffer and either incubated at 50˚C or 90˚C for 10 min. Denatured samples were loaded either on 8% or 10% SDS-PAGE gel and run in Tris-Glycine-SDS gel running buffer at 70-100 Volts for 2-3 h in a Mini-PROTEAN® 3 electrophoresis unit (Bio-Rad). After electrophoresis, gels were either visualized by coomassie brilliant blue (CBB) staining or processed for western blotting as described below.

2.2.3.3 Western blot analysis

Total proteins resolved by SDS-PAGE were transferred to PVDF nylon membrane by Western blotting using a Bio-Rad Mini Trans-Blot electrophoretic transfer unit in Tris-glycine transfer buffer at 4˚C either at 100 Volts for 3 hr or 30 Volts for overnight.

Following transfer, membrane was either stained with Ponceau reagent for checking the efficiency of transfer or directly processed for protein detection using protein-specific antibodies. For immunoblotting, membranes were blocked with 5% *(w/v)* non-fat milk solution either in PBS-T or TBS-T for 2 h at RT and probed with primary antibodies against the target proteins.

For detection of CgPma1, membranes were probed with 1:1000 dilution of polyclonal anti-Pma1 antibody raised against *S. cerevisiae* Pma1 (Santa Cruz # sc-33735) in PBS-T with 5% *(w/v)* fat-free milk for overnight at 4°C.

For detection of phosphorylated form of CgSlt2, immunoblotting analysis was done with an anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) primary antibody raised against human p44 MAPK (Cell signaling technology # 4370S) at a dilution of 1:6000 in TBS-T with 5% *(w/v)* fat-free milk for overnight at 4°C.

For detection of CgCPY, membrane was incubated with polyclonal anti-CPY antibody raised against *S. cerevisiae* CPY (Thermo Scientific # PA 1-27244) at a dilution of 1:10,000 in TBS-T with 5% *(w/v)* fat-free milk for overnight at 4°C.

For CgGapdh detection, anti-Gapdh primary antibody raised against human Gapdh (Abcam # ab22555) at a dilution of 1:7000 was used in TBS-T with 5% *(w/v)* fat-free milk.

Secondary antibodies conjugated with horseradish peroxidase (HRP) enzyme were used in 1:10,000 dilution to detect the immune-reactivity of primary antibodies with the help of ECL plus Western blotting system (GE Healthcare) as per manufacturer's instructions.

2.2.3.4 Measurement of intracellular pH (pH_i)

Intracellular pH (pH_i) in yeast cells was determined using fluorescent 5,(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes) as described previously (Bracey *et al.* 1998). For pH_i probe estimation, YNB medium-grown log-phase cells were inoculated in YNB, YNB-pH 2.0 or YNB medium supplemented with acetic acid and incubated at 30˚C for different time points. Log-phase *C. glabrata* cells were harvested and washed twice with 50 mM citric-phosphate (CP) buffer (pH 4.0). Washed cells were resuspended in 1 ml 50 mM CP buffer to an

OD_{600} of 0.5 and transferred to a 1.5 ml microcentrifuge tube. Probe loading was carried out by adding freshly-prepared CFDA-SE solution (0.01 M stock in DMSO) to cell suspension to a final concentration of 160 µM. Cell suspension was mixed on vortex mixer for 10 sec and incubated at 37˚C for 1 h with shaking at 300 rpm on thermo mixer. Cells were harvested, washed twice with 1 ml 50 mM CP buffer to remove unloaded probe, resuspended in 250 µl CP buffer and were incubated at 30˚C for 30 min with shaking to recover from the stress induced during probe loading. After incubation, fluorescent intensity was determined with spectrofluorophotometer (Varioskan flash-3001, Thermo Scientific) by excitation at 430 nm (pH-independent) and 490 nm (pH-dependent) with emission at 525 nm. Background fluorescence of the probe was removed by subtracting the fluorescence intensity of the probe in CP buffer from the fluorescence intensity of the probe-loaded cells.

2.2.3.5 *In vivo* intracellular pH calibration curve

For determining the intracellular pH from fluorescence intensity values of CFDA-SE-loaded cells, an *in vivo* calibration curve was prepared between fluorescent intensity and pre-adjusted environmental pH values. Briefly, CFDA-SE-loaded wild-type *C. glabrata* cells were incubated with 0.5 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma # C2759) at 30˚C for 10 min in 50 mM CP buffer adjusted to different pH values ranging from 4.0 to 7.5, with an interval of 0.5 unit. CCCP is an ionophore which dissipates the plasma membrane pH gradient, thus, rendering the intracellular pH similar to the extracellular pH. Fluorescent intensities were determined and a calibration curve was plotted between the ratio of intensity at 490 to 430 nm *versus* pH. A polynomial distribution of fluorescent intensity signal and pH was observed for CFDA-SE probe (Figure 2.1) and the graph equation was used to determine the intracellular pH of *C. glabrata* cells.

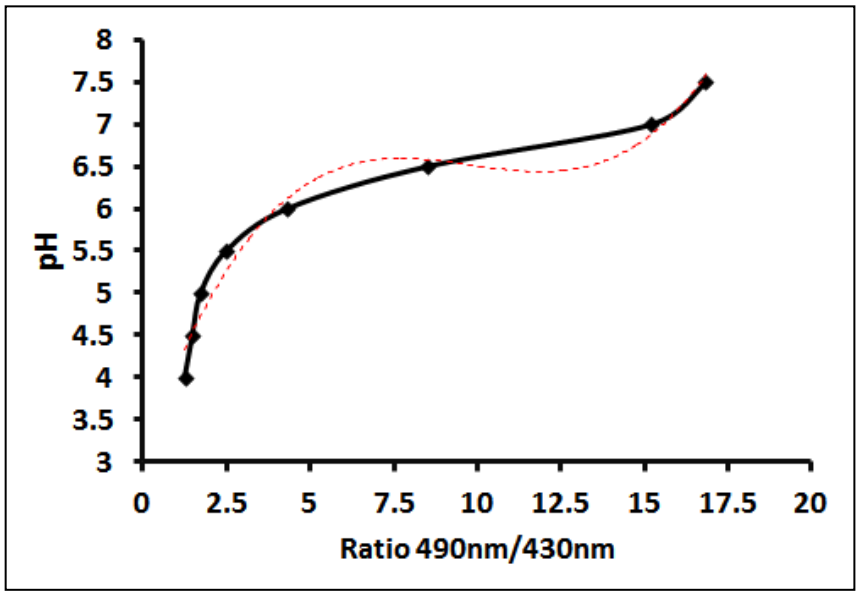

Figure 2.1: pH_i calibration curve plotted between ratio of the fluorescence intensity recorded at 490 nm to that at 430 nm excitation in CFDA-SE-loaded wild-type cells *versus* pH of the surrounding medium. Trendline (red dotted line) shows the polynomial distribution of the curve.

2.2.3.6 Determination of intracellular reactive oxygen species (ROS) levels

Intracellular reactive oxygen species (ROS) levels in yeast cells were determined using fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma # D6883). Cellular esterases remove the diacetate groups of the DCFH-DA and produce DCFH which gets readily oxidized to highly fluorescent product 2′,7′-dichlorofluorescein (DCF) by intracellular ROS. The fluorescent intensity of DCF corresponds to the amount of intracellular ROS present in the cell. Cells grown under different environmental conditions were harvested, washed once with tissue-culture grade phosphate-buffered saline (PBS) and resuspended in PBS to the final cell density of 1 OD. Freshly-prepared DCFH-DA (0.01 M stock in DMSO) was added to the cell suspension to a final concentration of 100 µM. Cell suspension was mixed and incubated at 30˚C for 30 min. After incubation, cells were washed 2-3 times with 1 ml PBS and then resuspended in 200 µl PBS. Fluorescence intensity values were recorded using spectrofluorophotometer (Varioskan flash-3001, Thermo Scientific) with excitation and emission at 488 and 530 nm, respectively. Fluorescence intensity values obtained from probe-loaded cells were subtracted from the fluorescence intensity values obtained from cells-alone samples to remove background fluorescence.

2.2.3.7 Measurement of intracellular calcium levels

Log-phase cells grown in YPD medium containing or lacking $CaCl_2$ and FK506 were collected, PBS-washed and loaded with ratiometric, high affinity, membrane-permeable calcium indicator, Fura-2 AM (10 μM; Sigma # 47989). After 30 min incubation at 30°C, labelled cells were washed thrice with cold PBS, suspended in PBS and fluorescence was recorded at 505 nm with dual excitation at 340 and 380 nm. The ratio of fluorescence intensities between 340 and 380 nm, representing Ca-bound and Ca-free Fura-2 molecules, respectively, reflected free intracellular calcium concentrations.

2.2.3.8 Measurement of plasma membrane H^+-ATPase activity

2.2.3.8.1 Whole cell acidification assay

To assess the activity of plasma membrane proton pump, CgPma1, in cells grown in different external pH environment, whole cell acidification assay was carried out. This assay is a measurement of glucose-responsive proton pump activity in live cells and is based on a decrease in the pH of a weakly-buffered solution upon extrusion of H^+ ions from the cell. The amount of change in the pH of the medium represents a crude measurement of the activity of functional plasma membrane proton pump in live cells. Whole cell acidification assay was conducted with cells grown in YNB pH 5.5 and YNB pH 2.0 medium as described previously (Martinez-Munoz and Kane, 2008) with slight modifications. After growth at 30°C for 2 h, cells were harvested, washed and resuspended (1.5-3.0 mg wet weight/ml) in 15 ml MES/TEA (1mM; pH 5.0) buffer. Cell suspension was kept at 25°C with continuous agitation. Extracellular pH of the buffer solution was recorded at 1 min interval for 20 min with the help of a pH meter (BT-600, Boeco Germany). To activate plasma membrane proton pumping, glucose and KCl were added to a final concentration of 40 mM after 3 and 8 min incubation, respectively. Plasma membrane proton pump activity was plotted as a change in the pH of the extracellular solution *versus* time.

2.2.3.8.2 Total membrane preparation

Isolation of total membrane fractions from *C. glabrata* strains were carried out as described previously (Fernandes *et al*., 1998). Cells grown to log-phase under different environmental conditions were harvested, washed and suspended to a final density of 20 OD_{600} cells in 1 ml solution containing 100 mM Tris (pH 10.7), 5 mM EDTA, 2 mM

dithiothreitol and 1X protease inhibitor cocktail. Cell suspension was rapidly frozen at -80˚C, thawed and lysed with 0.5 mm acid-washed glass beads in a homogenizer (FastPrep®-24, MP Biomedicals) at maximum speed of 60 sec five times. Homogenate was diluted with 5 ml Tris-HCl (0.1M; pH 8.0) solution containing 0.33 M sucrose, 5 mM EDTA and 2 mM dithiothreitol and centrifuged at 1,000 g for 3 min at 4˚C. Supernatant was collected and centrifuged again at 3,000 g for 5 min at 4˚C to remove unbroken cells. The resulting supernatant was centrifuged at 19,000 g for 45 min at 4˚C to obtain total membrane fraction. Total membrane pellet was resuspended in 100 µl membrane suspension buffer and stored at -80˚C till further use. Total protein concentration in the membrane fraction was estimated using BCA protein assay kit (Thermo Scientific, US) with bovine serum albumin (BSA) used as a standard.

2.2.3.8.3 Plasma membrane H^+-ATPase activity assay

Plasma membrane H^+-ATPase activity was measured in the total membrane fraction as described previously (Nakamura *et al*., 2001). 5 µg total membrane fraction was incubated at 30˚C in 120 µl reaction buffer containing 10 mM $MgSO_4$ and 50 mM KCl in 50 mM MES (pH 5.7) with 5 mM adenosine tri-phosphate (ATP). To eliminate possible contribution of residual ATPases, *viz*., vacuolar ATPases, mitochondrial ATPases or non-specific phosphatases, 50 mM KNO_3, 5 mM NaN_3 and 0.2 mM ammonium molybdate were used, respectively, in the assay mixture. Reaction was stopped after 30 min by adding 130 µl stop-developing solution containing 1% *(w/v*) SDS, 0.6 M H_2SO_4, 1.2% *(w/v)* ammonium molybdate and 1.6% *(w/v*) ascorbic acid. Amount of inorganic phosphate (P_i) liberated was measured at $A750_{nm}$ after 10 min incubation at room temperature. A standard curve prepared with 0-50 µmoles of KH_2PO_4 was used for determination of total P_i amount. ATPase activity of the plasma membrane was expressed in micromoles of P_i released per milligram protein per min. ATPase activity was also determined in the presence of plasma membrane H^+-ATPase inhibitor diethylstilbestrol (DES, Sigma # D4628), wherein total membrane fraction was incubated with 0.2 mM DES for 5 min, prior to the enzymatic measurement.

2.2.3.9 Phosphate starvation of yeast cells

For phosphate starvation, yeast cells grown to log-phase in YNB medium were harvested, washed with water, transferred to either regular YNB or YNB medium lacking phosphate and were grown for 16 h at 30˚C. Cells cultured in YNB medium

were maintained in log-phase by continuous passaging in fresh YNB medium every 4 h.

2.2.3.10 Polyphosphate extraction

Polyphosphates from yeast cells were extracted with phenol-chloroform solution as described previously (Neef and Kladde, 2003). Cells grown either overnight or to logarithmic phase in YPD medium were harvested by centrifugation at 1,700 rpm for 5 min. Cells were washed with 10 ml sterile MQ water and resuspended in ice-cold 500 µl 20% trichloro acetic acid (TCA) solution to the final cell density of 100 OD. Cell suspension was transferred to a 1.5 ml microcentrifuge tube. After incubation at room temperature for 5 min, cells were harvested by centrifugation at 12,000 g for 10 min at 4˚C and resuspended in 250-350 µl polyphosphate extraction buffer. Equal volume of phenol-chloroform (25:24) was added to the microcentrifuge tube and aqueous phase was extracted by centrifugation at 12,000 g for 8 min at room temperature. Top aqueous layer was collected with a 200 µl tip. Aqueous layer extraction was repeated once more after removal of DNA with chloroform. After centrifugation, aqueous phase containing RNA and polyphosphates was collected, RNA was quantified at $A260_{nm}$ and stored at -20˚C.

2.2.3.11 Quantitative analysis of polyphosphates

Total cellular polyphosphates were quantified *via* polyacrylamide-Tris borate gel electrophoresis (PAGE-TBE). Briefly, PAGE was performed with Tris-borate buffer (pH 8.3) to determine the quantity and the type of polyphosphates extracted from yeast strains. Equal amount of total RNA (20 to 100 µg) was loaded on 34% PAGE-TBE gel (~ 22 cm long, 16 cm wide and 0.8 mm thick) and electrophoresed at 500 Volts for 20-24 h in cold-room till the marker dye bromophenol blue (BPB) had migrated 15-16 cm away from the well. After electrophoresis, total polyphosphates were visualized by staining the gel with 0.05% toluidine blue staining solution followed by destaining. Polyphosphates were observed both as a smear in the top most portion of the gel as well as discreet bands of long chain polyphosphates and short chain polyphosphates in middle and bottom half of the gel, respectively. Polyphosphate band intensity in the gel was quantified using ImageJ software (http://rsbweb.nih.gov/ij/) and relative amounts of long chain and short chain polyphosphates in *C. glabrata* cells were calculated.

2.2.3.12 Phosphate uptake assay

Cells grown overnight in YNB medium were inoculated in fresh YNB medium and incubated at 30˚C with shaking at 200 rpm. Cells were harvested when the cell density reached to an OD_{600} of 0.6-0.8. Cells were consecutively washed with sterile MQ water and YNB without phosphate (YNB-P_i) medium. Washed cells were inoculated either in YNB or YNB-P_i medium to the initial OD_{600} of 0.1. Cells were incubated at 30˚C for 3-4 h, harvested and resuspended in 100 µl YNB-P_i medium. Radioactive P^{32}-labelled *o*-phosphoric acid (Jonaki # LCP 32) was added to the cell suspension to a final concentration of 1 µCi/ml and cells were incubated for 30 min. For determining phosphate uptake, a 10-12 µl cell suspension aliquot, after every 5 min, was removed and kept on ice. To this cell suspension, 500 µl ice-cold YNB-P_i medium was added and cells were harvested by centrifugation at 5,000 g for 5 min at 4˚C. These cells were washed with ice-cold YNB-P_i medium thrice and resuspended in 100 µl PBS (1X). 10-20 µl of this cell suspension was added to 5 ml scintillation fluid and β-decay counts were measured in a scintillation counter (Tri-Carb 2910 TR Liquid Scintillation Analyzer, PerkinElmer). Scintillation counts were normalized to total cell number and plotted with respect to time. Total phosphate uptake was expressed as P^{32} c.p.m/OD_{600} cells where c.p.m refers to counts per min.

2.2.3.13 Determination of acid phosphatase activity

Quantitative measurement of periplasmic acid phosphatase activity in phosphate-starved *C. glabrata* cells was performed as mentioned previously (Orkwis *et al.*, 2010). A total of 0.5 OD_{600} YNB-grown and phosphate-starved cells were collected, washed thrice with cold water and once with cold 0.1 M sodium acetate buffer (pH 4.2). Washed cells were resuspended in 500 µl sodium acetate (0.1 M) and incubated at 30˚C with constant stirring. After 10 min incubation, 500 µl freshly-prepared solution of 20 mM *p*-nitrophenyl phosphate in 0.1 M sodium acetate (pH 4.2) was added to the cell suspension. Enzymatic activity was stopped after incubation at 25˚C for 20 min by addition of 250 µl sodium carbonate (1 M) to the reaction mix. Resultant colour change was measured by monitoring absorbance at 400 nm. Acid phosphatase activity was expressed as a ratio of OD_{400} to OD_{600} to normalize against cell density.

2.2.3.14 Estimation of trehalose, glycogen and ATP levels

2.2.3.14.1 Estimation of trehalose content

Trehalose from *C. glabrata* cells was extracted by trichloro acetic acid (TCA) solution as described previously (Lillie *et al.,* 1980). Cells grown in YPD medium were collected at different time points of growth and washed thrice with ice-cold sterile water. Cells were immediately stored at -20˚C till further use. For trehalose isolation, 10-20 OD_{600} cells were thawed in 500 µl TCA (0.5 M) solution on ice and incubated at room temperature for 1 h. Supernatant fraction was collected by sedimenting cells at 14,000 rpm for 5 min at 4˚C. TCA extraction was repeated with cells once more and the resulting supernatant was mixed with the earlier fraction. Extracted trehalose was measured by phenol-sulphuric acid method of carbohydrate determination with commercially available purified trehalose (Becton, Dickinson and Co.) as a standard. Total trehalose content was normalized to the cell density and expressed as µg/2 x 10^7 cells.

2.2.3.14.2 Estimation of glycogen levels

Estimation of total glycogen in cells was performed as described previously (Parrou et al., 1997) with slight modifications. Briefly, YPD medium-grown *C. glabrata* cells were harvested, washed once with 1 ml ice-cold water and resuspended in 250 µl sodium carbonate (0.25 M) solution. After incubation at 95˚C for 4 h in water bath with occasional stirring, cell suspension was cooled and pH of the suspension was adjusted to 5.2 by adding 150 µl 1 M acetic acid. To this suspension, 600 µl 0.2 M sodium acetate was added and cell suspension was incubated with 1-2 U/ml of α-amyloglucosidase from *A. niger* (Sigma # A7420) at 57˚C for overnight with constant agitation. Resultant glucose liberated by α-amyloglucosidase digestion was collected in the supernatant fraction and quantified by phenol-sulphuric acid method of carbohydrate determination. For quantification, commercially available purified glucose was used as a standard and total glycogen in cells was expressed as µg/2 x 10^7 cells to normalize against cell density.

2.2.3.14.3 Determination of intracellular ATP levels

ATP concentration in yeast cells was measured by luminometric luciferase-luciferin based assay using ATP bioluminescent kit (Sigma # FLAA). Briefly, log-phase yeast

cells were collected and washed with chilled sterile water. 1 OD_{600} cells were resuspended in 20 µl chilled 10% TCA solution containing 8 mM EDTA (pH 8.0) and incubated at room temperature for 15-20 min. Following incubation, cell suspension was centrifuged at 12,000 rpm for 5 min at 4˚C and supernatant was transferred to a fresh 1.5 ml microcentrifuge tube. 10 µl of this supernatant fraction was diluted 75-fold with ATP assay mix dilution buffer provided with the kit. 50 µl of diluted suspension was added to an equal volume of ATP assay mix (Sigma # FLAAM) which contained firefly luciferase and luciferin with $MgSO_4$, EDTA, DTT and BSA in Tricine buffer. Luminescence was measured in luminometer (Varioskan flash-3001, Thermo Scientific). Total ATP was quantified using purified ATP as the standard and expressed in moles/OD cells.

2.2.3.15 Staining of yeast vacuoles with FM4-64

Vacuolar morphology of *C. glabrata* cells was examined by staining vacuoles with FM4-64 (Molecular Probes, Invitrogen). FM4-64 is a lipophilic dye that exhibits long wavelength red fluorescence when bound to lipids. FM4-64 binds to the plasma membrane and follows the endocytic pathway to reach the vacuole (Vida and Emr, 1995). Log-phase, YPD medium-grown cells were harvested and washed with 1X PBS. 1 OD cells were resuspended in 50 µl YPD medium containing 30 µM FM4-64 and incubated at 30˚C for 30-45 min. After incubation, cells were washed thrice with YPD medium and resuspended in 100 µl of the same medium. Cells were observed under confocal laser scanning microscope (Zeiss LSM 510 Meta) with 63X objective lens, 2.5X final zoom, pinhole set at 108 µm and emission filter set to LP 565_{nm} to capture fluorescence image. Along with the fluorescence image, a phase contrast image was also captured for each sample.

2.2.3.16 Measurement of vacuole pH

Vacuole pH in yeast cells was determined as described previously (Padilla-López and Pearce, 2006). Briefly, log-phase, YPD medium-grown yeast cells were harvested and suspended in 200 µl YPD medium containing 50 µM 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Invitrogen # B1150) to the final cell density of 4 x 10^7 cells. Cells were incubated at 30℃ for 30 min at room temperature followed by three washes with YPD medium. Washed cells were resuspended in 1 ml YPD medium and 200 µl cell suspension was used for recording

fluorescence by excitation at 440 (pH-independent) and 490 nm (pH-dependent) with emission at 535 nm. Ratio of fluorescence intensity at 490 to 440 nm was used to calculate the vacuolar pH. Background fluorescence was removed by subtracting the fluorescence intensity values of cells without BCECF-AM from the fluorescence intensity values of the probe-loaded cells.

2.2.3.17 *In vivo* vacuolar pH calibration curve

A calibration curve of fluorescence intensity values *versus* pH was prepared for BCECF-AM-loaded *wt* cells by incubating cells in YPD medium containing 50 mM MES, 50 mM HEPES, 50 mM KCl, 50 mM NaCl, 0.2 M ammonium acetate, 10 mM NaN_3, 10 mM 2-deoxyglucose and 5 µM carbonyl cyanide m-chlorophenylhydrazone, titrated to five different pH values in the range of 4.0-8.0. Fluorescence intensity values were measured by excitation at 440 and 490 nm with emission at 535 nm and a graph was plotted between the ratio of intensity at 490 to 440 nm *versus* pH. Similar to pH_i calibration curve, a polynomial distribution of fluorescent intensity signal and pH was observed for BCECF-AM probe.

2.2.3.18 Vacuolar H^+-ATPase activity measurement

2.2.3.18.1 Crude vacuolar membrane extraction

Crude fractionation of total membranes was carried out *via* differential centrifugation as described previously (Morano and Klionsky, 1994) with slight modifications. Cells grown to log-phase in YPD medium were collected, washed, normalized to 10 OD_{600} and resuspended in 1 ml spheroplast buffer containing 1-2 mg of zymolyase 20T (MP Biomedicals). Following incubation at 30˚C for 30-45 min, spherolplasts were collected by centrifugation at 800 g for 3 min at 4˚C and resuspended in 1 ml ice-cold Tris-EDTA (pH 7.5). Spheroplasts were lysed with 100 µl 0.5 mm glass beads on a vortex mixer with 10 sec pulse given thrice with intermittent ice-breaks. Cell suspension was centrifuged at 800 g for 5 min at 4˚C to pellet unbroken spheroplasts down and the supernatant was centrifuged at 15,000 g for 5 min at 4˚C to obtain the membrane fraction pellet. Pellet was washed once with ice-cold Tris-EDTA (pH 7.5), resuspended in 50 µl of the same buffer and stored at -20˚C till further use. Protein concentration of pellet fraction was estimated using BCA protein assay kit with BSA as the standard.

2.2.3.18.2 Purified vacuole membrane isolation

Vacuole membranes were isolated with slight modifications of Cabrera's method (Cabrera *et. al.,* 2008). Log-phase, YPD medium-grown cells were inoculated in 1 lt YPD medium to an initial OD_{600} of 0.1. Cells were incubated at 30˚C with shaking at 200 rpm till the cell density reached to OD_{600} of 0.8-1.0. Cells were harvested by centrifugation at 5,000 g and washed once with 30 ml 2% ice-cold glucose solution. Cells were incubated in 15 ml solution containing glycine-NaOH (50 mM; pH 10) and DTT (2 mM) at 30˚C for 10 min. After incubation, cells were normalized to a density of 1000 OD_{600} and resuspended in 15 ml spheroplasting buffer containing 10-15 mg of zymolyase 20T. Cells were incubated at 30˚C for 45-60 min or till the spheroplasting was completed. Spheroplasts were collected by centrifugation at 4,500 rpm for 5 min at 4˚C, washed gently with 15 ml 1.2 M sorbitol solution and resuspended in 3.5 ml 15% ficoll solution made in PS buffer containing 1X protease inhibitor cocktail. This suspension was homogenized on ice with 20-25 strokes in a loose-fitting Dounce homogenizer. Homogenate was transferred to an ice-cold, ultra-clear Beckman ultracentrifuge tube, overlaid with a gradient of 3 ml 8% ficoll solution, 2.5 ml 4% ficoll solution and 2.5 ml PS buffer lacking ficoll and centrifuged at 1,10,000 g (30,000 rpm) for 90 min at 4˚C in a pre-cooled Beckman ultracentrifuge with SW41-Ti swinging bucket rotor. Centrifugation was carried out with slow acceleration and deceleration settings. White creamy vacuole membrane layer was collected from the interface of 0 and 4% ficoll gradient without mixing the layers. Total protein concentration in the vacuole fraction was estimated using BCA protein assay kit as described earlier.

2.2.3.18.3 Vacuolar H^+-ATPase activity measurement

Vacuolar membrane H^+-ATPase activity was measured in both crude membrane fraction and purified vacuolar membrane fraction as described previously (Woolford *et al.,* 1990). Activity in the crude membrane fractions was carried out with 2.5-10 µg protein in 50 µl assay buffer (5 mM $MgCl_2$, 25 mM MES/Tris-HCl (pH 6.9) and 25 mM KCl). For activity in the purified vacuolar membrane fraction, a total of 300 µl reaction mix was set up with of 2.5-10 µg protein samples. Residual activities from other ATPases such as mitochondrial ATPases, plasma membrane H^+-ATPase and phosphatases were inhibited by adding 2 mM NaN_3, 200 µM $NaVO_4$ and 0.2 mM

ammonium molybdate, respectively, to the assay buffer. For specific inhibition of vacuolar membrane H^+-ATPase activity, vacuolar membrane fractions were incubated with 1-2.5 µM bafilomycin for 5 min prior to the activity assay. ATPase activity was initiated by adding ATP to the assay buffer to a final concentration of 5 mM and incubating the reaction at 30˚C for 30-60 min. Reaction was stopped by adding an equal volume of a stop-developing solution (1% *(w/v)* SDS, 0.6 M H_2SO_4, 1.2% *(w/v)* ammonium molybdate and 1.6% *(w/v)* ascorbic acid). Amount of inorganic phosphate (P_i) liberated was measured at $A750_{nm}$ after 10 min incubation at room temperature. Standard curve prepared with 0-50 micromoles of KH_2PO_4 was used for the determination of total P_i. The ATPase activity of the vacuolar membrane H^+-ATPase was expressed in micromoles of P_i released per milligram protein per min.

2.2.3.19 Carboxypeptidase Y (CPY) activity assay

CPY activity was measured as described previously (Jones, 2002). A 2.5 mg/ml stock solution of CPY-specific substrate *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTPNA, prepared in dimethyl formamide) was diluted 5 times with 0.1 M Tris-HCl (pH 7.5). 100 µl diluted substrate solution was added to a 96-well plate containing 25 µl cell suspension (5 x 10^7 cells). After 18 h of incubation at 37˚C, plate contents were clarified by centrifugation and colour formation was quantified by absorbance at 405 nm. Background absorbance measured using BTPNA-free cell cultures was subtracted from BTPNA-loaded cell cultures and absorbance values were normalized to total number of viable cells to enumerate total cellular CPY activity.

2.2.3.20 Carboxypeptidase Y secretion assay

Cells grown to log-phase in YPD medium were spotted on CAA medium and overlaid with a nitrocellulose filter. Cells were allowed to grow at 30˚C for 18-20 h. After incubation, the filter was washed with water to remove cells and membrane-bound CPY was detected by immunoblotting with polyclonal anti-CPY antibody (Thermo Scientific) at a dilution of 1:15,000.

2.2.3.21 Cell wall isolation, zymolyase digestion assay and β-glucan estimation.

2.2.3.21.1 Crude cell wall isolation

Yeast cell wall was isolated as described previously (De Groot *et al.*, 2004). Briefly, cells grown under different environmental conditions were harvested at 5,000 g for 5

min. Cells were normalized to equal OD_{600}, resuspended in 1 ml 50 mM Tris-HCl (pH 7.5) and transferred to 2 ml microcentrifuge tubes. Cells were lysed with glass beads in a homogenizer (FastPrep®-24, MP Biomedicals) as described earlier. Broken cells were washed from glass beads with 500 µl Tris-HCl (50 mM, pH 7.5) and pelleted down at 15,000 g for 10 min to obtain all cell wall and membrane content. Pellet was then boiled for 10 min in 1 ml Tris-HCl (50 mM; pH 7.5) solution containing 2% SDS. SDS-extractable material (mannoproteins) was saved and remaining pellet was boiled again in 500 µl Tris-HCl (50 mM; pH 7.5) buffer containing 2% SDS. Cell wall was collected by centrifugation at 15,000 g for 10 min, washed twice with 1 ml water and resuspended in 100 µl 67 mM potassium phosphate buffer. This washed cell wall material was used for β-glucan estimation as described below.

2.2.3.21.2 Total β-glucan estimation

Cell wall β-glucan measurement was carried out as described previously with some modifications (Kapteyn *et. al.*, 2001). Briefly, cell wall fractions were washed multiple times with 1 N NaCl. Washed cell walls were boiled twice in 50 mM Tris-HCl (pH 7.8) containing 2% SDS, 100 mM Na-EDTA and 40 mM β-mercaptoethanol for 5 min to remove non-covalently linked proteins and other contaminants. SDS-treated cell wall fraction was collected and rinsed thrice with water. For β-glucan isolation, cell walls were extracted three times, each for 1 h, in 0.5 ml 3% NaOH at 75˚C and centrifuged at 1,200 g. All 3% NaOH supernatant fractions were saved for isolation of mannan as described below. 3% NaOH-extractable cell wall pellet was neutralized twice in 100 mM Tris-HCl (pH 7.5) and once in 10 mM Tris-HCl (pH 7.5) and digested with 5 mg/ml zymolyase-20T in 10 mM Tris-HCl (pH 7.5) for 14-16 h at 37˚C. This treatment liberates approximately 90-95% glucose into the supernatant. Total glucan content in the cell wall was measured by estimating glucose from both the solubilised supernatant and zymolyase-20T insoluble pellet fractions with phenol-sulphuric acid carbohydrate estimation method using purified glucose as the standard.

2.2.3.21.3 Total mannan estimation

Total mannan from 3% NaOH-extractable supernatant of cell wall was precipitated by Benedict's solution. Reducing sugars (mostly mannan) from alkali-extractable supernatant react with copper (II) sulphate present in Benedict's solution and forms red copper (I) oxide precipitate. Briefly, equal volume of Benedict's solution was added to 3% NaOH-extractable cell wall supernatant fraction and heated at 99˚C for 10 min.

Resultant precipitate was dissolved in 3 N HCl and reprecipitated in methanol:acetic acid (8:1) solution. Following 16 h incubation at room temperature, the precipitate was washed with methanol:acetic acid (8:1) solution till green colour of the supernatant disappeared. Finally, pellet was washed thrice with methanol and air dried. Dried pellet was resuspended in 0.5 N HCl and total mannan content was quantified with phenol-sulphuric acid carbohydrate estimation method as described earlier. Commercially available purified glucose was used as the standard.

2.2.3.21.4 Zymolyase digestion assay

Log-phase yeast cells were collected, washed and suspended in 10 mM Tris-HCl (pH 7.5) containing 50 mg/ml zymolyase-20T. Cell suspension was incubated at room temperature and absorbance was monitored at 600 nm every 10 min interval. Initial absorbance of the cultures at 0 min was normalized to 100% and the graph was plotted as % decrease in the absorbance with respect to time.

2.2.3.22 Electron Microscopy

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed at the Electron Microscope Facility, RUSKA LABs, Acharya N. G. Ranga Agricultural University, Hyderabad.

2.2.3.22.1 Scanning electron microscopy

For SEM, *C. glabrata* cells were fixed for 24 h in 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2) at 4˚C, post-fixed in 2% aqueous osmium tetroxide for 4 h and dehydrated. After drying to critical point, mounted samples were coated with a thin layer of gold for 3 min using an automated sputter coater and visualized by SEM (JEOL-JSM 5600).

2.2.3.22.2 Transmission electron microscopy

For TEM, *C. glabrata* cells were digested with zymolyase 20T for 3 h at 30˚C, centrifuged at 1,000 g and washed with YPD medium. Cell fixation was performed as described for SEM and dehydrated samples were embedded in araldite 6005 resin. After complete polymerization at 80˚C for 72 h, ultra-thin (50-70 nm) sections were prepared with a glass knife on Leica Ultra cut (UCT-GA-D/E-1/00) microtome and mounted on copper grids. Aqueous uranyl acetate-stained and Reynolds lead citrate-counterstained samples were viewed under Hitachi H-7500 transmission electron microscope.