# APPENDICES

## APPENDIX I

### YEAST CULTURE MEDIA

**YEPD (Yeast Extract Peptone Dextrose)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>1</td>
</tr>
<tr>
<td>Bacto peptone</td>
<td>2</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>2</td>
</tr>
<tr>
<td>Bacto agar (when required)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**YNB (Yeast Nitrogen Base medium)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Nitrogen Base (w/o aa, as)</td>
<td>0.67</td>
</tr>
<tr>
<td>Glucose</td>
<td>2</td>
</tr>
</tbody>
</table>

**Supplements (as required):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Sulfate</td>
<td>10 µM</td>
</tr>
<tr>
<td>L-Proline</td>
<td>200 µg/ml</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>L-canavanine</td>
<td>1 mM</td>
</tr>
<tr>
<td>L-azetidine-2-carboxylic acid</td>
<td>1 mM</td>
</tr>
<tr>
<td>Uracil</td>
<td>30 µg/ml</td>
</tr>
</tbody>
</table>

**SGM (Synthetic Growth Medium)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>0.5</td>
</tr>
<tr>
<td>Ammonium phosphate</td>
<td>0.3</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.3</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.025</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.025</td>
</tr>
<tr>
<td>d-Biotin</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**LB (Luria-Bertain Medium)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>1</td>
</tr>
<tr>
<td>Glucose</td>
<td>2</td>
</tr>
<tr>
<td>Tryptone</td>
<td>2</td>
</tr>
</tbody>
</table>

*Adjust pH to 7.2 - 7.4*
APPENDIX II

AMINO ACID TRANSPORT ASSAY

<table>
<thead>
<tr>
<th>Assay Mixture</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-citrate, pH 4.5</td>
<td>100 mM</td>
</tr>
<tr>
<td>Cell suspension</td>
<td>0.5 % (w/v)</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>200 µg/ml</td>
</tr>
<tr>
<td>L-[¹⁴C]-amino acid</td>
<td>variable</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Wash Buffer</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-citrate, pH 5.0</td>
<td>10 mM</td>
</tr>
<tr>
<td>L-[¹⁴C]-amino acid</td>
<td>same as in assay mixture</td>
</tr>
</tbody>
</table>

SCINTILLATION FLUIDS

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Cocktail O</th>
<th>Cocktail T</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPO¹</td>
<td>6.0 g</td>
<td>5.0 g</td>
</tr>
<tr>
<td>POPOP²</td>
<td>0.2 g</td>
<td>0.15 g</td>
</tr>
<tr>
<td>Toluene</td>
<td>1 litre</td>
<td>666 ml</td>
</tr>
<tr>
<td>Triton-X 100</td>
<td>-</td>
<td>332 ml</td>
</tr>
</tbody>
</table>

¹ PPO = (2,5-Diphenyloxazole)
² POPOP = (1,4-bis[5-Phenyl-2-oxazoly] benzene:2,2'-p-Phenylene bis[5-
phenyloxazole])
APPENDIX III

MEMBRANE PREPARATION

<table>
<thead>
<tr>
<th>HOMOGENISING MEDIUM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>250 mM</td>
</tr>
<tr>
<td>Tris-Cl pH 7.5</td>
<td>10 mM</td>
</tr>
<tr>
<td>PMSF (stock prepared in DMSO)</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

APPENDIX IV

PLASMA MEMBRANE SOLUBILISATION

<table>
<thead>
<tr>
<th>SOLUBILISATION BUFFER</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris-HCl, pH 7.5</td>
<td>50 μl</td>
</tr>
<tr>
<td>100 mM EDTA, pH 7.5</td>
<td>50 μl</td>
</tr>
<tr>
<td>100 mM PMSF</td>
<td>50 μl</td>
</tr>
<tr>
<td>Tween-40</td>
<td>35 μl</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>1 μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2814 μl</td>
</tr>
<tr>
<td><strong>FINAL VOLUME</strong></td>
<td><strong>3000 μl</strong></td>
</tr>
</tbody>
</table>
MODIFIED BRADFORD MICROASSAY
(For 2-10 μg protein in 200 μl final volume)

Reagents required:
1) K₃PO₄ 500 mM, pH 7.4
2) CaCl₂ 250 mM
3) Ethanol 100 % (v/v)
4) Bradford Dye Reagent

1. Make the final volume of protein samples to 200 μl (7.0 pH) using MQ water and add 10 μl K₃PO₄, 10 μl CaCl₂ & 1 ml ethanol. Centrifuge at 7000 X g for 1 min and discard the supernatant.

2. Add 10 μl MQ water and 1 ml ethanol to the pallet and vortex it.

3. Recentrifuge at 7000 X g for 1 min and again discard the supernatant.

4. Add 100 μl "Bradford Dye Reagent" to the pallet and leave the tubes undisturbed for 5 min.

5. Add 400 μl MQ water, mix the contents gently by inverting the tubes twice.

6. Transfer a portion of colored solution to the microcuvette (with minimal turbulence and air-bubble in the solution).

7. Measure the absorbance of solution in ELISA reader between 5th and 20th min of color development, at 595 nm.

Note: For blank, water or vehicle is carried through the same "calcium-phosphate precipitation".

Bradford Dye Reagent
Stock Solution: 100 mg of Coomassie Blue (CBB) was dissolved in 50 ml of 95 % ethanol. To this 100 ml of 85 % ortho-phosphoric acid was added and the final volume was made up to 200 ml with MQ water. The solution was stored in dark at 4 °C.
Working Solution: The stock solution was diluted in a ratio of 1:4 with MQ water and was filtered through Whatman No. 1 before use.
APPENDIX VI

SDS-POLY ACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

STOCK REAGENTS

A. Acrylamide/Bis (30%T, 2.67%C)

 Acrylamide 146 g
 N,N'-methylene-bis-acrylamide 4 g

B. 1.5 M Tris-HCl, pH 8.8 (RGB: Running Gel Buffer)

54.45 g Tris base
150 ml distilled water
Adjust to pH 8.8 with HCl, make up the volume to 300 ml with distilled water. Store at 4°C

C. 0.5 M Tris-HCl, pH 6.8 (SGB: Stacking Gel Buffer)

6 g Tris base
60 ml distilled water
Adjust to pH 6.8 with HCl, make up the volume to 300 ml with distilled water.
Store at 4°C

D. 10% (w/v) SDS

Dissolve 10 g SDS in 60 ml distilled water with gentle stirring, make up the volume to 100 ml.

E. 10% (w/v) ammonium persulphate

Dissolve 100 mg ammonium persulphate in 1 ml distilled water.

F. Sample Buffer

SDS reducing buffer: 62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-Mercaptoethanol

1. Distilled water 3.0 ml
2. 0.5 M Tris-HCl, pH 6.8 1.0 ml
3. Glycerol 1.6 ml
4. 10% SDS 1.6 ml
5. β-Mercaptoethanol 0.4 ml
6. 0.5% (w/v) bromophenol blue (in water) 0.4 ml
Total Volume 8.0 ml
Dilute the sample at least 1:4 with sample buffer. Heat at 95°C for 5 minutes.

G. 5X Electrode (Running) Buffer (1X = 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>45.0 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>216.0 g</td>
</tr>
<tr>
<td>SDS</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>

Make up the volume to 3 litres with distilled water, DO NOT ADJUST pH with acid or base. Store at 4°C, warm to 37°C before use if precipitation occurs. Dilute 300 ml 5X stock with 1.2 litres of distilled water for one electrophoretic run.

H. Preparation for 10% (10% T, 2.67% C) Gel

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Separating Gel (10%)</th>
<th>Stacking Gel (4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/bis (30%T, 2.67%C)</td>
<td>16.65 ml</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>20.00 ml</td>
<td>61.1 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8 (RGB)</td>
<td>12.50 ml</td>
<td></td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8 (SGB)</td>
<td>-</td>
<td>2.50 ml</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.50 ml</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulphate</td>
<td>0.25 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.025 ml</td>
<td>0.01 ml</td>
</tr>
<tr>
<td><strong>FINAL VOLUME</strong></td>
<td><strong>50 ml</strong></td>
<td><strong>10 ml</strong></td>
</tr>
</tbody>
</table>
## Silver Staining Protocol of PAGE

<table>
<thead>
<tr>
<th>Step</th>
<th>Substrate</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fixing Gel</td>
<td>Ethanol</td>
<td>40.0 %</td>
</tr>
<tr>
<td></td>
<td>Acetic Acid</td>
<td>12.0 %</td>
</tr>
<tr>
<td></td>
<td>Formaldehyde (37 %)</td>
<td>0.5 ml/l</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>&gt; 1 h (three changes)</td>
</tr>
<tr>
<td>2. Washing of Gel</td>
<td>Ethanol</td>
<td>50.0 %</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>10 min (three changes)</td>
</tr>
<tr>
<td>3. Pretreatment of Gel</td>
<td>Sodium thiosulphate</td>
<td>0.2 g/l</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>1 min</td>
</tr>
<tr>
<td>4. Rinsing of Gel</td>
<td>Water</td>
<td>100 ml</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>20 s (three changes)</td>
</tr>
<tr>
<td>5. Impregnate the Gel</td>
<td>Silver nitrate</td>
<td>2.0 g/l</td>
</tr>
<tr>
<td></td>
<td>Formaldehyde (37 %)</td>
<td>0.75 ml/l</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>20 min</td>
</tr>
<tr>
<td>6. Rinsing of Gel</td>
<td>Water</td>
<td>100 ml</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>10 s (two changes)</td>
</tr>
<tr>
<td>7. Development of Gel</td>
<td>Sodium carbonate</td>
<td>60.0 g/l</td>
</tr>
<tr>
<td></td>
<td>Formaldehyde (37 %)</td>
<td>0.5 ml/l</td>
</tr>
<tr>
<td></td>
<td>Sodium thiosulphate</td>
<td>0.004 g/l</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>10 min</td>
</tr>
<tr>
<td>8. Stop the Development Reaction</td>
<td>Ethanol</td>
<td>40.0 %</td>
</tr>
<tr>
<td></td>
<td>Acetic acid</td>
<td>12.0 %</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>10 min</td>
</tr>
<tr>
<td>9. Washing of Gel</td>
<td>Methanol</td>
<td>50.0 %</td>
</tr>
</tbody>
</table>

**Drying the gel:** Shake the gel for 30 min in 30 % methanol followed by 3 % glycerol for an additional 30 min and dry the gel between cellophane paper in gel drier.
APPENDIX VIII

RECONSTITUTION OF PROLINE PERMEASE INTO PROTEOLIPOSOMES

SOLUTIONS:

1. Dialysis Buffer (DB1) 1 litre
   - Tris-citrate, pH 5.0 50 mM
   - K$_2$SO$_4$ 50 mM
   - MgSO$_4$ 10 mM

2. Dialysis Buffer (DB2) 1 litre
   - Tris-citrate, pH 5.0 50 mM
   - Na$_2$SO$_4$ 50 mM
   - MgSO$_4$ 10 mM

3. Tris-citrate, pH 5.0, 1 M
4. MgSO$_4$, 5 mM
5. Radioactive [¹⁴C]-L-proline stock solution = 1 mM
6. Valinomycin stock solution = 100 μM, working conc. = 10 μM
7. Wash buffer
   - Tris-citrate 10 mM, pH 5.0
   - L-arginine 10 μM

8. Phosphatidylcholine 20 mg/ml in ethanol
9. n-Octyl-β-D-glucoside 40 mg in 1.0 ml of phosphatidylcholine (PC) solution (from step 8, above)

A TYPICAL REACTION MIXTURE

<table>
<thead>
<tr>
<th>ADDITIVES</th>
<th>VOLUME (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reconstituted vesicles</td>
<td>100</td>
</tr>
<tr>
<td>Tris-citrate 1 M, pH 5.0</td>
<td>50</td>
</tr>
<tr>
<td>MgSO$_4$, 50 mM</td>
<td>100</td>
</tr>
<tr>
<td>Valinomycin 100 μM</td>
<td>10</td>
</tr>
<tr>
<td>Water</td>
<td>640</td>
</tr>
<tr>
<td>Hot (100 μM)</td>
<td>100</td>
</tr>
<tr>
<td><strong>FINAL VOLUME</strong></td>
<td><strong>1000</strong></td>
</tr>
</tbody>
</table>
INDEX

y-aminobutyric acid transporter (Uga4p) ... 20
active transport .................................. 4, 41
amino acid ........................................ 1, 4, 18, 20, 32, 43, 103, 104
primary ........................................... 3
secondary ......................................... 4
affinity chromatography
proline permease purification ................. 63
amino acid permease
transcriptional regulation ...................... 37
amino acid transport ............................ 32
repression-depression cycle .................... 32
regulation ........................................ 33
ammonia repression ............................ 18, 19
Amino Acid Transport
and ER Chaperone & Sensor Proteins ...... 37
Nitrogen Catabolic Regulation (NCR) .... 34
ammonium repressing condition (NCR) .... 130
anti-porter .......................................... 4
anti-porters ....................................... 8
arginine permease 20, 37, 42, 45, 57, 59, 63, 64,
73, 76, 77, 82, 87, 90, 94, 101, 139, 143, 157,
158
binding specificity ................................ 94
arginine permease regulation
phosphorylation .................................. 20
arginine transport ............................... 20
asparagine & glutamine permease ............ 23, 63
basic amino acid permease (Can1p) ......... 20
binding kinetics of Purified permeases
fluorimetric method ............................. 88
bidirectional kinetics
proline transport and ............................ 70
C. albicans ......................................... 11, 28, 32
amino acid permeases ............................ 103
and Candida (Candidosis) ................. 28, 35, 49, 62
cloning of AAP and ......................... 104
gene isolation .................................... 103
genomic library ................................... 104
inducible proline transport system ........... 32
nutrient transport systems 17, 19, 30, 32, 33,
41, 49, 61, 62, 104, 110
oligopeptide transport, OPT1 .................. 34
proline transporter gene of .................... 133
peptide transporter, Cap1PR2 ................. 34
Candida genomic library ....................... 59
Candida .......................................... 30
chaperonin proteins ............................ 134
chemiosmotic coupling .......................... 3
cispentacin ....................................... 30, 33
complemented growth
NCR and ......................................... 130
complemented transport
NCR and ......................................... 130
coe-reconstituted system
mutual interaction studies ...................... 87
coe-reconstitution
L-arginine accumulation ....................... 82
L-proline accumulation ......................... 82
Crude Membrane isolation ..................... 50
cytochalasin E .................................... 126
cytoskeleton
organization of .................................... 126
dansylated amino acids .......................... 47, 58, 59
detergent
critical micelle concentration (CMC) ........ 86
detergent removal
SM2 biobeads ..................................... 51
ultrafiltration method .......................... 51
diffusion ........................................... 3, 6, 24
facilitated ........................................ 2, 8, 35
equilibrium dialysis
disadvantages and limitations of ............. 100
loss of protein activity ......................... 100
protein-ligand interaction ..................... 88
thermal denaturation of permease .......... 94
limitations of .................................. 89
ER/Golgi sorting ................................ 135
ER/Golgi quality control machinery .......... 135
eukaryotic transporters ......................... 17
expression vector
Yep352, pYeUra3 ................................ 59
fluorescence method
advantages of .................................... 89
reusability of purified permease ............. 97
fluorescence quenching
permease-ligand interactions and .......... 101
fluorescence spectroscopy
ligand: macromolecules interactions ........ 100
polarity of the ligands binding sites ..... 100
protein-ligand interaction .................... 88
fluorescence study
artificial probes and ............................ 89
fluorescence-quench method
protein reusability and ......................... 102
flux ............................................... 2, 4
functional complementation .................. 104
Functional complementation .................. 133
GABA Transport ................................. 20
GAP1 activity
anal and anal mutations and .................. 37
GAP1 and PUT4
Nitrogen Catabolic Regulation (NCR) ....... 14
Gel Electrophoresis ........................................... 52
General Amino Acid Transport ......................... 18
Generation time measurement ........................ 49
germ tube inducer
L-proline ......................................................... 32, 63, 85
glucose sensor proteins
Snf3p and Rgt2p ............................................... 24
Growth Rate Measurement ................................ 49
haxose transport
S. cerevisiae ..................................................... 22
hexose transporters
low-affinity .................................................... 24
histidine permease ........................................... 23, 37, 63, 163
histidine transporter .......................................... 21
Isolation of Genomic DNA ................................. 62
L-proline transport in intact cell ....................... 50
leaky liposomes ............................................... 72
lipid extraction (total) ....................................... 55
liposomal reconstitution ................................... 72
liposomes ....................................................... 56, 57, 58
Liposomes preparation ...................................... 56
L-proline transport
in PTH1 transformants ..................................... 116
lysine permease .............................................. 21, 23, 33, 37, 63, 104
lysine transport systems
Can1p, and Lyp1p ............................................. 21
major facilitator superfamily (MSP) ...................... 24
mediated transport ........................................... 1, 2
membrane permeability ..................................... 117
membrane vesicle
advantages of .................................................. 42
methionine transport ....................................... 21
methioninesulfoxide (metSO) .............................. 21
molecular cloning
yeast AAP and ............................................... 133
nitrogen permease reactivator (NPR1) ................. 19
non-ionic detergents
Tween-40 and 8-OG .......................................... 64
non-ionic detergents and CMC ......................... 65
non-mediated transport ..................................... 2
Npi1p, Npi2p and Npr1p
Nitrogen catabolic repression (NCR) and ......... 36
osmotic-osmotic coupling .................................. 4
passive diffusion ............................................. 2
passive flux .................................................... 1
permeability coefficient .................................... 4
permease: ligand binding stoichiometry ............ 66
permease inhibition studies .............................. 55
permease binding assay
equilibrium dialysis ....................................... 53
permease reconstitution
removal of detergent and .............................. 72
plasma membrane
isolation and purification ................................ 51
Percoll™ gradient ........................................... 51
Plasmid DNA Preparation of E. coli .................... 61
prevacular compartimentation
of mutated permeases ...................................... 139
post-transport compartimentalization ............... 42
pre-vacular targeting ........................................ 139
proline permease 19, 23, 33, 44, 45, 52, 53, 57,
63, 64, 65, 66, 70, 72, 73, 76, 77, 82, 85, 86,
87, 89, 90, 94, 100, 101, 104, 134, 154, 164
binding specificity .......................................... 70, 94
competitive inhibitor of .................................. 70
co-reconstitution with arginine permease 73, 76
half saturation constant (Ks) ......................... 86
reconstitution of ............................................. 72
reusability and .............................................. 89
sulfation and purification ............................... 51
proline permease, binding activity
dansylated amino acids .................................. 58
proline transport ............................................. 19
directionality and proteoliposomes .................... 57
Proline Transporter Helper (PTH)
chromosomal localization .............................. 110
Proline transporter C. albicans
cloning and DNA manipulations ....................... 59
Proline Uptake
in reconstituted proteoliposomes ..................... 57
protein estimation .......................................... 52
Bradford method .......................................... 65, 85
interference by detergents ................................ 66
Modified Bradford Micro-assay ....................... 66, 86
Warburg & Christian method ......................... 65, 85
proteoliposomes
tightness and non-leakiness of ....................... 87
Proteoliposomes ............................................. 87
See liposomes
proton ionophore
CCCP ......................................................... 126
Protoplasts Preparation .................................. 62
PTH1 transformants
transport activity ......................................... 123
PTH1 and PTH2
CCAA T-box ................................................... 108
Chromosomal localization ............................. 110
functional expression studies of ..................... 127
putative promoter elements in ........................ 109
putative response element ................................ 109
substrate specificity ....................................... 117
TATA-motifs ............................................... 108
PTH1 and PTH2 proteins
hydropathy plots .......................................... 110
protein sequence analysis of ......................... 110
PTH1d and PTH2d
effect of energy inhibitors ............................. 126
L-proline uptake and .................................. 133
Pth1p and Pth2p
as regulatory proteins .................................. 135
retained transport activity ............................. 130

176