APPENDIX

APPENDIX A

MEDIA

Most of media used in this study are described by Yarrow (1998)

1. Yeast extract malt extract (YM) agar

Yeast extract	3.0	g
Malt extract	3.0	g
Peptone	5.0	g
Glucose	10.0	g
Agar	15.0	g
Distilled water	1000	ml
Sterilized at 121°C for 15 min		

2. Yeast extract malt extract (YM) broth

Yeast extract	3.0	g
Malt extract	3.0	g
Peptone	5.0	g
Glucose	10.0	g
Distilled water	1000	ml
Sterilized at 121°C for 15 min		

3. Yeast extract malt extract (YM) agar plates supplemented with chloramphinical (100 μg/l) and sodium propionate (0.2%).

Yeast extract	3.0	g
Malt extract	3.0	g
Peptone	5.0	g
Glucose	10.0	g
Chloramphenical	100	μg
Sodium propionate	2.0	g
Agar	15	g
Distilled water	1000	ml
Sterilized at 121°C for 15 min		

4. Yeast extract malt extract (YM) broth supplemented with chloramphinical (100 μg/l) and sodium propionate (0.2%).

Yeast extract	3.0	g
Malt extract	3.0	g
Peptone	5.0	g
Glucose	10.0	g
Chloramphenical	100	μg
Sodium propionate	2.0	g
Distilled water	1000	ml
Sterilized at 121°C for 15 min		

5. Yeast extract malt extract (YM) supplemented with 10% glycerol

Yeast extract	3.0	g
Malt extract	3.0	g
Peptone	5.0	g
Glucose	10.0	g
Glycerol	100	ml
Distilled water	1000	ml
Sterilized at 121°C for 15 min		

6. Yeast Peptone Dextrose (YPD)

Yeast extract	10.0	g
Peptone	10.0	g
Glucose	20.0	g
Glycerol	100.0	ml
Distilled water	1000	ml
Sterilized at 121°C for 15 min		

7. Acetate–GSH agar

Potassium acetate	15.0	g
Glucose	0.2	g
Yeast extract	0.1	g
Glutathione	10.0	mM
Agar	15.0	g
Distilled water	1000	ml

Sterilized at 121°C for 15 min

8. 5% malt extract agar

Malt extract	50	g
Agar	15	g
Distilled water	1000	ml

Sterilized at 115°C for 15 min

9. Stock carbon solution (10X)

Yeast Nitrogen Base (Difco)	6.7	g
Carbon compound	5.0	g
Distilled water	100	ml

Sterilized by filtration and stored in a freezer at -20°C until use

10. Stock nitrogen solution (10X)

Bacto Yeast Carbon Base (Difco)	11.7	g
Nitrogen compound*	x	g
Distilled water	100	ml

Sterilized by filtration and stored in a freezer at -20°C until use

* Nitrogen compound: $(NH_4)_2SO_4$ (0.5 g) or KNO_3 (0.78 g) or $NaNO_2$ (0.26 g) or ethylamine-HCl (0.64 g) or L-lysine-HCl (0.56 g) or cadaverine (0.68 g)

11. Yeast Carbon Base (YCB) medium:

1) Stock YCB solution (10X)

Bacto Yeast Carbon Base (Difco)	11.7	g
Distilled water	100	ml

Sterilized by filtration and stored in a freezer at -20°C until use

2) Ten fold of stock YCB solution (0.2 ml) was added to 13x100 mm test tube containing 1.8 ml sterilized distilled water

12. Yeast Nitrogen Base (YNB) medium

1) Stock YNB solution (10X)

Bacto Yeast Nitrogen Base (Difco)	6.7	g
Distilled water	100	ml

Sterilized by filtration and stored in a freezer at -20°C until use

2) Ten fold of stock YNB solution (0.2 ml) was added into 13 x 100 mm test tube containing 1.8 ml sterilized distilled water.

13. Carbon assimilation medium

Ten fold of stock carbon solution (0.2 ml) was added to 13 x 100 mm test tube containing 1.8 ml sterilized distilled water.

14. Nitrogen agar plate:

Ninety ml of 1.67 % agar solution was sterilized at 121°C for 15 min and left for cool down to 50-55°C, then 10 ml of stock nitrogen solution (10X) was added, mixed well and poured into the petri dishes.

15. Fermentation test medium

1) Basal medium

Yeast extract	4.5	g
Peptone	7.5	g
Bromothymol blue	small amount	
Distilled water	1000	ml

Fermentation basal medium (2 ml) was distributed into the cotton plugged test tubes (13x100 mm), insert Durham tube and sterilize at 121°C for 15 min. After cool down, add concentrate sugar, which was sterilized by membrane filtration to make the final concentration at 2% sugar (except 4% raffinose).

16. Gelatin liquefaction

Gelatin	100	g
Glucose	5.0	g
Bacto Yeast Nitrogen Base	6.7	g
Distilled water	1000	ml

Sterilized at 121°C for 15min

The medium in tubes was allowed to gel with the tubes in a vertical position

17. Vitamin requirement test medium

1) Vitamin requirement basal medium

Glucose	10.0	g
Vitamin-free cassamino acids (Difco)	5.0	g
$KH_2PO_4.7H_2O$	1	g
$MgSO_4$	0.5	g
NaCl	0.1	g
$CaCl_2$	0.1	g
Distilled water	1000	ml

Adjust pH = 5.5

Sterilized at 121°C for 15min

2) Vitamin stock solutions (100X) are prepared as follows:

- Biotin: 0.02 mg Biotin was dissolved in 100 ml distilled water, to make the final concentration at 2 μg/ml.
- Ca-Pantothenate: 4 mg Ca-Pantothenate was dissolved in 100 ml distilled water, to make the final concentration at 400 μg/ml.
- Folic acid: 0.02 mg Folic acid was dissolved in 100 ml distilled water, to make the final concentration at 2 μg/ml.
- Inositol: 20 mg Inositol was dissolved in 100 ml distilled water, to make the final concentration at 2,000 μg/ml.
- Niacin: 4 mg Niacin was dissolved in 100 ml distilled water, to make the final concentration at 400 μg/ml.
- ρ-Aminobenzoic acid (PABA): 2 mg PABA was dissolved in 100 ml distilled water, to make the final concentration at 200 μg/ml.
- Pyridoxine-HCl: 4 mg Pyridoxine-HCl was dissolved in 100 ml distilled water, to make the final concentration at 400 μg/ml.
- Riboflavin: 2 mg Riboflavin was dissolved in 100 ml distilled water, to make the final concentration at 200 μg/ml.
- Thimine-HCL: 4 mg Thimine-HCl was dissolved in 100 ml distilled water, to make the final concentration at 400 μg/ml.

3) Active test medium

One hundred folds of vitamin solution were diluted with 9 volumes of the basal medium and sterilized by membrane filtration. The composition of vitamins in each medium is shown in Table 32. A mount of 0.2 ml of sterilized 10X vitamin mixture was added aseptically into cotton plugged tube containing 1.8 ml of the sterilized basal medium then incubated at 25°C for 3 days at room temperature to confirm the sterility.

Appendix Table A1 Vitamin requirement test

Vitamin	Medium No.											Final concentration (μg/ml)
	1	2	3	4	5	6	7	8	9	10	11	
Biotin	X	X	✓	✓	✓	✓	✓	✓	✓	✓	✓	2
Pantothenate	X	✓	X	✓	✓	✓	✓	✓	✓	✓	✓	400
Folic acid	X	✓	✓	X	✓	✓	✓	✓	✓	✓	✓	2
Inositol	X	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	2000
Niacin	X	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	400
PABA	X	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	200
Pyridoxine	X	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	400
Riboflavin	X	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	200
Thiamine	X	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	400

X: No add 10X vitamin solution.

✓: Add 10X vitamin solution.

Remark: Medium No.1: Negative control (with out vitamin); No.2: Biotin requirement; No.3: Pantothenate requirement; No.4: Folic acid requirement; No.5: Inositol requirement; No.6: Niacin requirement; No.7: PABA requirement; No.8: Pyridoxine requirement; No.9: Riboflavin requirement; No.10: Thiamine requirement and No.11: Positive control.

18. Lipase test medium

Fresh beef suet was melted and filtrated, then sterilized at 121°C for 15 min. Molten fat (0.5 ml) was pored into a slightly warmed sterilized petri dish and spread over the bottom and surplus fat was removed. Preti dish was moved into a refrigerator and left for 1-2 hr. Molten YM agar was cooled to about 40°C, and then poured over the fat.

19. Acid formation from glucose

Glucose	50.0	g
Calcium carbonate	5.0	g
Yeast extract	5.0	g
Agar	20.0	g
Distilled water	1000	ml

Sterilized at 121°C for 15min

Tubes was cooled to around 45°C and agitated gently to resuspend the chalk and then slanted or pored to plate.

20. Cycloheximide resistant

1) Basal medium

Cycloheximide 1 g in acetone	2.5	ml
Bacto Yeast Nitrogen Base (Difco)	6.7	g
Distilled water	100	ml

Sterilized by filtration

2) Active medium

Amount of 0.2 ml of 10 fold basal medium was added to 13 x 100 mm test tube containing 1.8 ml sterilized distilled water.

3) 50% glucose medium

This medium prepared by dissolving 13 g of agar in 1% solution of yeast extract, and then 500 g of glucose was added and sterilized at 110°C, for 10 min.

REAGENTS

1. Lugol's solution

Iodine	1	g
Potassium iodide	2	g
Demineralized water	300	ml

2. Lysis buffer

Tris (pH 8.0)	100	mM
EDTA (pH 8.0)	30	mM
Sodium dodecyl sulfate (SDS)	0.5	%

3. 10X SSC

NaCl	1.5	M
Trisodium citrate	0.15	M
Adjust pH = 7.0		

4. 1/15 M phosphate buffer with 0.1 M EDTA (pH 7.5)

K_2HPO_4 (1/15 M)	86.6	ml
KH_2PO_4 (1/15 M)	13.4	ml
Mix and adjust pH =7.5		
Add EDTA	0.1	M

5. Acetate-EDTA

Sodium acetate (pH 8.0)	3.0	M
EDTA (pH 8.0)	0.5	M

6. TBE buffer

Tris-borate	90.0	mM
EDTA (pH 8.0)	1.0	mM

7. SCE

Sorbitol	1.0	M
Sodium citrate	0.1	M
EDTA (pH 7.0)	0.006	M

Sterilized at 121°C for 15 min

8. Zymolyase solution (1 ml)

Zymolyase 100T	3	mg
2-mercaptoethanol	0.1	ml
SCE	0.9	ml

9. 10X TE buffer

Tris-HCl	0.1	M
EDTA (pH 8.0)	0.01	M

Sterilized at 121°C for 15 min

10. RNase solution

RNase	5	mg/ml
Tris-HCl (pH 7.5)	10	mM
NaCl	15	mM

Boil for 15 min and cooled solution was stored at -20°C

11. Proteinase K solution

Proteinase K	20	mg
Demineralized water	1	ml

Stored at –20°C

12. Tris-saturated phenol

Phenol	250	ml
Chloroform	240	ml
Isoamyl alcohol	10	ml

Equilibrated with Tris-HCl (pH 8.0)

13. PBS

$Na_2HPO_4.12H_2O$	8	mM
KH_2PO_4	1.5	mM
NaCl	137	mM
KCl	2.7	mM
pH 7.2		
Sterilized at 121°C for 15 min		

14. PBSM1, 2

$Na_2HPO_4.12H_2O$	8	mM
KH_2PO_4	1.5	mM
NaCl	137	mM
KCl	2.7	mM
$MgCl_2$	0.1	M
pH 7.2		
Prepare before use and do not autoclave		

15. Photobiotin solution

Photobiotin acetate	1	mg
Purified water	1	ml
(Prepared in 1.5ml Eppendorf tube, and vigorous shaking)		
Tris-HCl	0.1	M
EDTA (pH 9.0)	1	mM

16. Pre-hybridization solution (10 ml)

Deionized formamide	5.0	ml
20X SSC	1.0	ml
50X Denhardt solution	1.0	ml
10 mg/ml Salmon DNA (Type III, Sigma)	0.1	ml
Sterilized distilled water	2.9	ml

17. 20X SSC

NaCl	3.0	M
Trisodium citrate	0.3	M
Adjust pH = 7.0		

18. 0.1 mg/ml salmon DNA

Salmon DNA	10	mg
TE	1	ml

Denature by heating at 100°C for 5 min, then rapidly cooling in ice water

19. Hybridization solution (10 ml)

Deionized formamide	5.0	ml
20X SSC	1.0	ml
50X Denhardt solution	1.0	ml
10 mg/ml Salmon DNA (Type III, Sigma)	0.1	ml
50% Dextran sulphate sodium salt	1.0	ml
Denatured photobiotin labeled DNA	5.0	ml
Sterilized distilled water	2.9	ml

20. PBS-BSA-Triton solution

Triton X-100	0.1	ml
PBS	100	ml
Autoclave at 121°C for 15 min		
Add bovine serum albumin (Fraction V) (after autoclave)	0.5	g

21. SABG solution

Streptavidin-β-galactosidase	1	μl
PBS-BSA-Triton solution	1	ml

22. MUF-Gal solution (0.1 mg/ml)

4-methylumbelliferyl-β-galactopyranoside	0.1	mg
DMSO	25	μl
PBSM2	975	μl

23. TE buffer (pH 9.0)

Tris-HCl (pH 9.0)	0.1	M
EDTA (pH 8.0)	1.0	mM
Adjust pH = 9.0		

APPENDIX B