MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 *Yarrowia lipolytica* strains

Table 7: ***Yarrowia lipolytica*** strains used in this study.

Strain	Genotype	Source
ATCC 8661 (Laboratory strain no. M240)	Wild type.	K.H.Tan, Meat Industry Research Institute of New Zealand. P.O. Box 617, Hamilton, New Zealand.
S-22 (IFO no. 1545/Laboratory strain no. M243)	High citric acid producer with low level of isocitric acid (aconitate hydratase deficient).	Shun-Ichi Akiyama, Research Development Division. Takeda Chemical Industries Ltd. 532 Juso, Osaka, Japan.
F21	Derived from mutagenesis of ATCC 8661.	From this study.
F21A	NTG mutagenesis of F21. High citric acid producer in high concentration NBD palm olein.	From this study.
INAG 33120	A *his1*	Dr. M. Chasles, Institute de la Recherche Agronomique, 78850 Thiverval-Grignon, France.
INAG 33044	B *leu2 lys5 ade1 xpr2*	As above.
ATCC 32338	A *ade1*	American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, U.S.A. 20852.
ATCC 32339	B *trp1*	As above.

2.2 *Escherichia coli* strains

Plasmidless *E. coli* **HB101** *F-Δ(mcr(-mrr)leu suppE44 ara14 galK2 lacY1 proA2 rpsL20 (Str^r) xyl-5 mtl-1 recA13 and* **JM109***(F' traD36 lacI^q Δ(lacZ) M15 proAB/recA1 endA1 gyrA96 (Nal^r) thi hsd R17 (r_k^- -m_k) suEA4 e(14$^-$)(mcrA$^-$) relA1Δ(lac-proAB)* were supplied by New England Biolabs, Inc. 32 Tozer Road, Beverly, MA 01915-5599 U. S. A.

2.3 Plasmids

Plasmids p1NA62 was a gift from Prof. C.M. Gaillardin and A.M. Ribet, Institut National de la Recherche Agronomique, 78850 Thiveral-Grignon, France. Whereas, plasmid pINA230 and pINA214 were supplied by B.Y. Treton, I.N.R.A.Thiverval-Grignon, France.

2.4 Materials

All chemicals used were of Analar grade or of the highest grade available commercially, obtained from BDH chemical Ltd., England, Sigma Chemical Co., U.S.A.; Oxoid Ltd. England, Fluka Biochemika, Switzerland; Hopkin and Williams Ltd. England; Pharmachia, Sweden and Difco Laboratories, U.S.A.

Ampicillin used was obtained from Boehringer Mannheim, Germany and tetracycline used was supplied by Sigma Chemical Co., U.S.A.

Bacto-agar, Tryptone, Yeast extract and Bacto-peptone for bacterial or yeast culture media were obtained from Oxoid Ltd., England.

All amino acids and tricarboxylic acid used were from Sigma Chemical Co., U.S.A.

Agarose powder was from Pharmachia, Sweden. Boric acid, Ethylenediaminetetra-acetic acid, *di*-Ammonium orthophosphate, *di*-Ammonium nitrate, *di*-Ammonium ferrous sulphate, *di*-Potassium hydrogen orthophosphate, *di*-Sodium hydrogen orthophosphate anhydrous, Potassium dihydrogen orthophosphate, Glucose, Potasium acetate, Bromocresol green, Ammonium chloride, Sodium acetate, Magnesium sulphate 7-hydrate, Sodium chloride, Sodium hydroxide, Glycerol, Sodium thiosulphate, Ethyl acetate, Polyvinyl alcohol, Acetone, Ethanol, Phenopthalein, Magnesium chloride hexahydrate, Bromophenol, Sodium dodecyl sulphate, Mecaptoethanol, Phenol , Sodium citrate and Chloroform were from BDH chemicals, Ltd., England.

Ammonium sulphate was from Hopkin and Williams, Ltd., England.

Yeast nitrogen base was from Difco Laboratories, U.S.A. Sodium carbonate was from Fluka Biochemica, Switzerland.

Bovine serum albumin, 5,5'-dithio-bis (2-nitro benzoic acid), Thiamine HCl, Sodium monofluoracetate, Sodium succinate, Malic acid, Oxalacetic acid, α-ketoglutaric acid, isocitrate acid, N-methyl-N'-nitro-N-nitrosoguanidine, Olive oil, Trizma base, Tris-HCl, Oxaloacetate acid, Acetyl-CoA, Cis-aconitic acid, Phenylhydrazine, Cysteine HCl, Glyoxylic acid, Diphosphopyridine nucleotide, Ethidium bromide, Ficoll (type 4000), threo-D_s(+)isocitric acid, citric acid (Both for HPLC) and Folin Ciocalteu's reagent were from Sigma. Enzymes used in this study are listed in Table 8.

Table 8: Enzymes used in this study

Enzyme	Source
Novozyme	SC
Proteinase K	SC
RNase A (DNA free)	SC
T_4 DNA ligase	NEB
Restriction endonuclease	
Bam HI	NEB
Pvu I	AI
Sau 3AI	AI
Pst I	AI
Not I	AI

AI - Amersham International plc. SC - Sigma chemical Co. NEB - New England Biolabs., Inc.

GENECLEAN II supplied by Bio 101, Inc., P. O. Box 2284, La Jolla, C. A. 92038-2284.

NBD palm olein kindly supplied by United Plantation Berhad, Perak, Malaysia.

Surfactant (Sucrose palmitic) supplied by Mitshubishi-Kasei food Corporation, 13-3, Ginza 5Chome, Chuo-ku, Tokyo, Japan.

2.5 Storage and maintenance of bacterial and yeast strains

All the bacterial and yeast cultures were stored in glycerol by mixing 1 volume of 80% sterile glycerol and three volumes of bacterial or yeast liquid culture in LB or YEPD medium (where relevant with the appropriate antibiotic or amino acid added). The glycerol cultures were kept at -20°C.

E. coli strains harbouring plasmids pINA62, pINA230 and pINA214 were maintained by streaking on LB-agar medium incorporated with the appropriate antibiotic every month.

E. coli HB101 and JM109 were maintained by streaking on LB-agar (section 2.9.1) slant. For the yeast strains, the culture was maintained by growing on YEPD (section 2.8.1) plate at 28°C for 48 hours and kept at room temperature (every week).

2.6 Sterilisation

All plasticware, including microfuge tube, yellow and blue tips, microfuge comfortips, beakers and polypropylene centrifuge tubes were steam-sterilised by autoclaving at 121°C (15 lb) for 20 minutes.

Glassware including beakers, measuring cylinders, conical flasks, pipettes, universal and bijou bottles were sterilised in a dry heat oven at 180°C for 2 $^{1}/_{2}$ hours.

Most of the media and solutions were steam-sterilised at 121°C (15 lb) for 20 minutes in an autoclave. YEPD (section 2.8.1) medium and seed culture fermentation medium were autoclaved at 115°C (10 lb) for 15 minutes.

All antibiotics stock solutions and solutions of chemicals which are not stable to heat were sterilised by filtration through sterile membrane filter (Millipore, type GS, 0.22 μm pore size, 25 mm) using Millipore Swinnex-25 syringe filter holder.

2.7 Storage

All media and solutions were stored at 4°C unless stated.

2.8 Culture media of *Yarrowia lipolytica*

2.8.1 Yeast-extract-peptone-glucose (YEPD) medium

Glucose	20.0 g
Bacto-peptone	20.0 g
Yeast-extract	10.0 g
Distilled water to	1000 ml

One hundred ml of medium were dispensed into 250 ml conical flasks and autoclaved. For solid medium 2% of agar was added before autoclaving.

2.8.2 Yeast-extract-potassium acetate (YEPA) medium

Potassium acetate	20.0 g
Bacto-peptone	20.0 g
Yeast extract	10.0 g
Distilled water to	1000 ml

2.8.3 Minimal solid medium

Glucose	8.0 g
$(NH_4)_2SO_4$	5.0 g
Yeast nitrogen base	1.5 g
Bacto-agar	20.0 g
Distilled water to	1000 ml

Appropriate amino acids was added (Table 9) when ever required after the medium had been autoclaved.

Table 9: Amino acids and nucleic acid bases used in this study.

Compound	Concentration of stock solution (mg/ml)	Stock solution (ml) added per 400 ml of minimal medium	Final concentration (μg/ml) in supplemented MM
L-Leucine	5	4	50
L-Arginine	5	4	50
L-Lysine	5	4	50
L-Methionine	5	4	50
L-Tryptophane	5	4	50
L-Histidine	10	2	50
Adenine	5	4	50
Uracil	2	10	50

2.8.4 Minimal medium with 0.1% Bromocresol green

Minimal medium	90 ml
Bromocresol green	0.1 g

Bromocresol green was disolved in 10 ml of MM medium solution, filter-sterilised and added to autoclaved minimal medium.

2.8.5 Tricarboxylic acid medium (Akiyama *et al.*, 1972)

Defined medium

NH_4Cl	1.0 g
$(NH_4)_2 NO_3$	1.0 g
$KH_2 PO_4$	0.5 g
Thiamine HCl	300 μg

Bacto-agar	20.0 g
Sodium acetate	7.0 g
Distilled water to	1000 ml

Sodium acetate was substituted with other substrates as listed in Table 10 below.

Table 10: Substituted substrates in the medium.

Substrate	Weight (g/l)
Sodium monofluoroacetate	1.0
Sodium succinate	7.0
Sodium citrate (citric acid)	7.0
Malic acid	7.0
Oxalacetic acid	7.0
α-ketoglutaric acid	7.0
Isocitric acid	7.0

The substrate was added after the medium had been autoclaved. Substrates were dissolved with distilled water and filter-sterilised exception only for Sodium acetate, Sodium monofluoracetate and Sodium citrate which can be autoclaved in the media.

2.8.6 Fermentation medium (Tan and Gill, 1984)

$Na_2 HPO_4$	4.3 g
$KH_2 PO_4$	2.6 g
$(NH_4)_2 SO_4$	1.5 g
Yeast-extract	0.1 g
$MgSO_4.7H_2O$	0.02 g
$(NH_4)_2 (FeSO_4)_2$	0.005 g

Thiamine HCl	0.001 g
Surfactant	0.5 g
Distilled water to	1000 ml

Eighteen ml of fermentation liquid medium was dispensed into 250 ml conical flasks. Different concentrations of palm olein were added as required (2% and 4% NBD palm olein (w/v).

The completed fermentation medium was autoclaved and cool at room temperature. Thiamine HCl used was prepared separately by dissolving 20 mg in 20 ml of distilled water. The solution was filter-sterilised and added to an autoclaved medium to a final concentration of 0.002 mg in 20 ml of fermentation medium.

2.8.7 Seed medium

Fermentation medium to	1.0 liter
Glucose	20.0 g
(without surfactant)	

The solution was dispensed into 250 ml conical flasks in 100 ml volume.

2.8.8 Yeast extract-Malt extract medium (YM) (Ogrydziak *et al.*, 1978)

Yeast-extract	1.2 g
Malt-extract	1.2 g
Peptone	2.0 g
Glucose	4.0 g

Bacto-agar	8.0 g
Distilled water to	400 ml

2.8.9 Restrictive growth medium (RG) (Ogrydziak *et al.*, 1978)

Yeast-extract	0.08 g
Peptone	0.08 g
Glucose	0.4 g
Bacto-agar	8.0 g
Distilled water to	400 ml

2.9 Culture media of *Escherichia coli*

2.9.1 Luria-Bertani (LB) medium (Miller, 1972)

Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Distilled water to	1000 ml

For solid medium 2% agar was added before autoclaving.

2.9.2 LB with ampicillin

Ampicillin stock solution of 50 mg/ml was prepared in distilled water, filter-sterilised using sterile membrane filter (type GS, 0.22 μm pore size, Millipore corp.) in Swennex-25 syringe filter holder. The stock solutions were then dispensed in 1 ml aliquot for storage at -20°C. Working stocks were allowed to come to room temperature prior to use. Appropriate

was then added aseptically to autoclaved LB medium to give the final concentration required.

2.9.3 LB with tetracycline

Tetracycline stock solution of 30 mg/ml was prepared and filter sterilised as mentioned in Section 2.9.2. Appropriate volume was then added aseptically to autoclaved LB medium to give the final concentration required.

2.9.4 M9 Minimal medium (Miller, 1972)

$Na_2 HPO_4$	2.4 g
$KH_2 PO_4$	1.2 g
NaCl	0.2 g
$NH_4 Cl$	0.4 g
Bacto-agar	8.0 g
Distilled water to	400 ml

After autoclaving, 4 ml of 0.01 M of solution of $CaCl_2$ (Section 2.10.8) and appropriate amino acids were added to give the final concentration required (L-leucine, 50mM and L-proline, 50 mM).

2.9.5 M9 minimal medium with 0.01% bromocresol green

M9 minimal medium to	400 ml
Bromocresol green	0.04 g

1.5 ml of 1M of NaOH solution (Section 2.10.7) was added after the medium has been autoclaved.

2.10 Solutions

2.10.1 80% (v/v) glycerol stock solution

Glycerol	20 ml
Distilled water to	100 ml

2.10.2 0.1M Phosphate buffer, pH 7.0

Na_2HPO_4	4.3 g
KH_2PO_4	2.6 g
Distilled water to	1000 ml

The chemicals were dissolved in distilled water to a final volume of 1000 ml and kept at room temperature.

2.10.3 0.05 M Phosphate buffer pH 7.0

0.1 M Phosphate buffer	500 ml
(Section 2.10.2)	
Distilled water to	1000 ml

The solution was stored at room temperature.

2.10.4 20% (v/v) Sodium thiosulphate solution

Sodium thiosulphate	20 g
Distilled water to	100 ml

Storage at room temperature.

2.10.5 N-methyl-N'-nitro-N-nitrosoguanidine (NTG) stock solution

NTG	0.01 g
Distilled water	5 ml

NTG stock was filter-sterilised.

2.10.6 3:2 (v/v) Dioxane : Ethyl acetate solution

Dioxane	300 ml
Ethyl acetate	200 ml

These were mixed in dark bottle and kept at room temperature.

2.10.7 1 M Sodium hydroxide

Sodium hydroxide	40 g
Distilled water to	1000 ml

The solution was autoclaved and stored at room temperature.

2.10.8 10 M Sodium hydroxide

Sodium hydroxide	40 g
Distilled water to	100 ml

The solution was autoclaved and stored at room temperature.

2.10.9 0.1 M Calcium chloride

Calcium chloride	1.147 g
Distilled water to	100 ml

This solution was filter-sterilised.

2.10.10 0.1 M Citric acid

Citric acid	2.1 g
Distilled water to	100 ml

The solution was filter-sterilised and kept at room temperature.

2.10.11 1% (w/v) Citric acid

Citric acid	0.1 g
Distilled water	10 ml

2.10.12 1% (w/v) threo-D_s(+)isocitric acid

threo-D_s(+)isocitric acid	25 mg
Distilled water	2.5 ml

2.10.13 0.5% (w/v) *di*-Ammonium orthophosphate

di-Ammonium orthophosphate	5 g
Distilled water to	1000 ml

di-Ammonium orthophosphate was dissolved in 600 ml of distilled water. The pH was adjusted to 2.8 with concentrated orthophosphoric acid and solution was made up to 1000 ml. This solution was then filtered

through cellulose nitrate membrane filter (Millipore, type HA, 0.45 μm, 47 mm) using a set filter vacuum (Millipore). The filtered solution was degassed by connecting the vacuum tubing at the neck of the bottle. Both filteration and degassing procedure was used Edward Vokes vacuum pump (Cenco Instruments Coporation, Chicargo U.S.A.).

2.11 Lipase assay solutions

2.11.1 2% (v/v) Polyvinyl alcohol (PVA) solution

Polyvinyl alcohol	12 g
Distilled water to	600 ml

Polyvinyl alcohol (PVA) was dissolved in 1 litre beaker containing 400 ml of distilled water which was placed on a hot magnetic stirrer maintaining at 80°C. Distilled water was added after dissolving to a final volume of 600 ml.

2.11.2 Foamy emulsion

Olive oil	25 ml
PVA (Section 2.11.1)	75 ml

The ingredients were blended for 15 minutes until foamy emulsion was formed.

2.11.3 1:2 (v/v) Acetone:Ethanol

Acetone	100 ml
Ethanol	200 ml

The solutions were mixed in dark bottle and kept at room temperature.

2.11.4 0.05 M NaOH

NaOH	2.0 g
Distilled water to	1000 ml

The solution was kept in room temperature.

2.11.5 0.1 M NaOH

NaOH	2.0 g
Distilled water to	1000 ml

The solution was stored at room temperature.

2.11.6 5% (w/v) Phenopthalein

Phenopthalein	5.0 g
Ethanol to	100 ml

The solution was kept at room temperature.

2.12 Protein determination solutions

2.12.1 Bovine serum albumin stock solution (BSA)

BSA	50 mg
Distilled water	1 ml

Solution was stored at -20°C.

2.12.2 Solution A

Na_2CO_3	2.0 g
0.1 M NaOH to	100 ml

2.12.3 1% (w/v) Sodium citrate

Sodium citrate	10.0 g
Distilled water to	1000 ml

2.12.4 Solution B

$CuSO_4.5H_2O$	0.5 g
Sodium citrate (Section 2.12.3)	100 ml

2.12.5 Solution C

Solution A	50 ml
Solution B	1 ml

Solution C was prepared when required.

2.12.6 Solution D

2 N Folin-Ciocalteu's reagent	100 ml
Distilled water	100 ml

The 1:1 gave a final concentration of 1 N.

2.12.7 0.1M Tris-HCl, pH 7.8

Tris-HCl	1.576 g
Sterile distilled water to	100 ml

The pH was adjusted to 7.8 with 10 M NaOH (Section 2.10.7).

2.13 Citrate synthase assay solutions

2.13.1 3M Tris-base, pH 8.0

Tris-base	7.27 g
Sterile distilled water to	20 ml

The pH was adjusted to pH 8.0 with concentrated HCl.

2.13.2 20 mM Tris-HCl

Tris-HCl	0.3152 g
Distilled water to	100 ml

The pH was adjusted to pH 8.0 with10 M NaOH (Section 2.10.7).

2.13.3 0.05 M 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB)

DTNB	0.495 g
Tris-HCl (Section 2.13.2) to	25 ml

2.13.4 0.2 M Oxaloacetate

Oxaloacetate	0.264 g
Distilled water	100 ml

The solution was stored at -20°C.

2.13.5 100 mM Acetyl-CoA

Acetyl-CoA	25 mg
Sterile distilled water	3.08 ml

The solution was stored at -20°C.

2.14 Aconitate hydratase assay salutions

2.14.1 1 M Potassium phosphate

KH_2PO_4	13.61 g
Distilled water	100 ml

The solution was stored at room temperature.

2.14.2 1 M *di*-Potassium phosphate

K_2HPO_4	17.42 g
Distilled water to	100 ml

The solution was stored at room temperature.

2.14.3 0.5 M Phosphate buffer, pH 6.0

1 M KH_2PO_4 (Section 2.14.1)	61.5 ml
1 M K_2HPO_4 (Section 2.14.2)	38.5 ml
Distilled water to	200 ml

2.14.4 1 mM cis-Aconitic acid

Cis-aconitic acid	1.74 g
Sterile distilled water	10 ml

A serial dilution was done to give the final concentration of 0.5 mM.

2.15 Isocitrate lyase assay solutions

2.15.1 0.5 M Phosphate buffer, pH 6.8

1 M KH_2PO_4 (Section 2.14.1)	50.3 ml
1 M K_2HPO_4 (Section 2.14.2)	49.7 ml
Distilled water to	200 ml

2.15.2 15 mM Magnesium chloride

$MgCl_2$ anhydrate	0.305 g
Distilled water	100 ml

2.15.3 10 mM Phenylhydrazine HCl

Phenylhydrazine	0.362 g
Distilled water	25 ml

The solution was kept in -20°C.

2.15.4 6 mM Cysteine HCl

Cysteine HCl	0.094 g
Distilled water	100 ml

2.15.5 0.5 mM Isocitric acid

threo-D_s(+)isocitric acid	50 mg
Sterile distilled water	0.434 ml

The solution was kept at -20°C. A serial dilution was done to give the final concentration required.

2.16 Malate synthase assay solutions

2.16.1 18 mM Tris-HCl, pH 7.1

Tris-HCl	0.284 g
Distilled water to	100 ml

2.16.2 15 mM Magnesium chloride

$MgCl_2$ anhydrate	0.305 g
Distilled water to	100 ml

2.16.3 0.2 µmoles Acetyl-CoA

10 mM of Acetyl-CoA was used as stock solution. A serial dilution was carried out to give the final concentration required.

2.16.4 1 mM Glyoxylate

Glyoxylic acid	0.0114 g
(Sodium salt)	
Distilled water to	100 ml

2.17 Isocitrate dehydrogenase assay solutions

2.17.1 0.5 M Phosphate buffer

1 M K_2HPO_4 (Section 2.14.1)	49.7 ml
1 M KH_2PO_4 (Section 2.14.2)	50.3 ml
Distilled water to	200 ml

2.17.2 0.1 M Magnesium chloride

$MgCl_2$ anhydrate	2.035 g
Distilled water to	100 ml

2.17.3 25 mM Diphosphopyridine nucleotide (DPN)

DPN	25 mg
Sterile distilled water	1.51 ml

The solution was stored at -20°C.

2.17.4 5 mM Isocitrate

This was obtained from stock solution as mentioned in Section 2.15.5.

2.18 Electrophoresis gel and solutions

2.18.1 0.7% Agarose gel

Agarose	0.35 g
1X TBE solution	50 ml

The mixture was dissolved in a microwave (2 min and 50 seconds, power 70%).

2.18.2 10X strength TBE buffer

Tris-base	108 g
Boric acid	55 g
Na_2. ETDA.$_2H_2O$	9.3 g
Distilled water to	1000 ml

For routine electrophoresis, 10X strength stock solution was diluted to IX strength. The pH of the 1X solution should be 8.3. For electroelution the TBE buffer was sterilised by autoclaving. The solution was kept at room temperature.

2.18.3 6X strength bromophenol blue (BPB) loading dye

(Sambrook *et al.*, 1989)

1% (w/v) BPB	0.9 ml
Ficoll (type 400)	0.45 g
Glycerol	2.0 ml
Sterile distilled water to	5 ml

The final concentration of BPB in the loading dye was 0.18% (w/v), ficoll (type 400) 9% (w/v) and glycerol 40% (w/v), filter-sterilised and kept at room temperature.

2.18.4 Ethidium Bromide solutions

Ethidium bromide	1 g
Distilled water	100 g

The mixture stirred on a magnetic stirrer for several hours to ensure that the dye completely dissolved. It was stored in dark bottle at room temperature.

2.19 Solutions for the isolation of the chromosomal DNA of *Yarrowia lipolytica*

2.19.1 0.89% (w/v) Saline solution

Sodium chloride	8.9 g
Distilled water	1000 ml

The solution was autoclaved.

2.19.2 Solution A (0.2M Tris-base, pH 10.3)

Tris-base	2.422 g
Distilled water	100 ml

The pH was not adjusted.

2.19.3 Solution B

Lyticase	0.0084 g
Phosphate buffer (pH 7.0) (Section 2.10.2)	1 ml

2.19.4 0.8 M Sodium chloride

Sodium chloride	4.675 g
Distilled water to	100 ml

This was autoclaved and kept at room temperature.

2.19.5 0.4 M Ethylenediaminetetra-acetic acid, disodium salt (EDTA), pH 8.0

EDTA	14.88 g
Distilled water to	100 ml

The disodium EDTA was stirred vigorously in 70 ml of distilled water. The pH was adjusted to 8.0 with 10 M NaOH (2.10.7) and the final volume was adjusted to 100 ml. This was autoclaved and kept at room temperature.

2.19.6 20% (w/v) Sodium dodecyl sulphate (SDS)

SDS	2 g
Distilled water to	10 ml

The SDS was dissolved in distilled water. The resulting solutions was filter-sterilised and stored in at room temperature.

2.19.7 0.2 M Tris-HCl, pH 8.5

Tris-HCl	3.152 g
Distilled water to	100 ml

The Tris-HCl was dissolved in 70 ml of distilled water. The pH was adjusted to 8.5 with 10 M NaOH (Section 2.10.7) and the solution was made up to 100 ml. This solution was autoclaved and kept at room temperature.

2.19.8 Solution C

NaCl (Section 2.19.4)	1 volume
EDTA, pH 8.0 (Section 2.19.5)	1 volume
SDS (Section 2.19.6)	1 volume
Tris-HCl pH 8.5 (Section 2.19.7)	1 volume

2.19.9 1 M Tris-HCl , pH 8.8

Tris-HCl	15.8 g
Distilled water to	100 ml

The pH was adjusted to 8.0 or 8.8 with 10 M NaOH (Section 2.10.7).

2.19.10 Neutral phenol

Neutral phenol buffer was prepared as the following the method of Sambrook *et al* (1989). Phenol was equilibrated in a brown bottle with 0.5X volume 1M tris-HCl, pH 8.8 (Section 2.19.9), overnight by stirring with a magnetic stirrer. The aqueous phase was removed and the phenol was then

similarly equilibrated in 1 M Tris-HCl, pH 8.0 (Section 2.18.9) until pH > 7.6.

2.19.11 Isoamyl alcohol-chloroform (1:24, v/v)

Isoamyl alcohol-chloroform was prepared by mixing 24 volumes of chloroform and 1 volume of isoamyl alcohol and stored at room temperature.

2.19.12 Buffered phenol: chloroform (1:1, v/v)

Neutral phenol (Section 2.19.10)	1 volume
Isoamy alcohol-chloroform (Section 2.19.11)	1 volume

The mixture was stored in the dark bottle at 4°C.

2.19.13 20 mM Tris-HCl, pH 7.5

Tris-HCl	0.316 g
Distilled water to	100 ml

The pH was adjusted to 7.5 with 10 M NaOH (Section 2.10.7).

2.19.14 30 mM Sodium Chloride

Sodium chloride	0.176 g
Distilled water to	100 ml

2.19.15 RNase A (10 mg/ml)

A stock solution of RNase A (10 mg/ml) was prepared in 10 mM Tris-HCl, pH 7.5 (1 volume) (Section 2.19.13) and 15 mM NaCl (1

volume)(Section 2.19.14) (Maniatis *et al.*, 1982). It was then boiled at 100°C for 15 min to denature DNase, cold down to room temperature and distributed into small volumes (1 ml/microfuge tube) and stored at -20°C.

2.19.16 0.02 M Tris-HCl, pH 8.0

Tris-HCl	0.315 g
Distilled water to	100 ml

The pH was adjusted with10 M NaOH (Section 2.10.7). This was autoclaved and kept at room temperature.

2.19.17 0.002 M EDTA disodium salt

EDTA	0.0744 g
Distilled water	100 ml

2.19.18 Tris-HCl-EDTA (TE) Buffer, pH 8.0

Tris-HCl, pH 8.0 (Section 2.19.15)	1 volume
EDTA disodium salt (Section 2.19.16)	1 volume

The mixed of tris-HCl and EDTA will give 0.01 M and 0.001 M of final concentration appropriately.

2.19.19 3 M Sodium acetate

Sodium acetate	2.461 g
Distilled water	10 ml

2.20 Solutions for plasmid DNA isolation from *Escherichia coli* (Sambrook *et al.*, 1989)

2.20.1 1 M D-Glucose

D-Glucose	3.602 g
Distilled water to	20 ml

The mixture was filter-sterilised.

2.20.2 1 M Tris-HCl, pH 8.0

Tris-HCl, pH 8.0	12.11 g
Distilled water to	100 ml

Tris-HCl was dissolved in 70 ml of distilled water. The pH was adjusted to 8.0 with concentrated 10 M NaOH (Section 2.10.7). The solution was made up to 100 ml. This was autoclaved and kept at room temperature.

2.20.3 0.25 M EDTA disodium salt, pH 8.0

EDTA disodium salt	18.61 g
Distilled water to	100 ml

The EDTA disodium salt was dissolved in 70 ml of distilled water. The pH was adjusted with 10 M NaOH (Section 2.10.7) and made up to 100 ml. This solution was autoclaved and kept at room temperature.

2.20.4 Solution 1

	Final concentration
D-Glucose (Section 2.20.1)	50 mM
Tris-HCl, pH 8.0 (Section 2.20.2)	25 mM
EDTA, pH 8.0 (Section 2.20.3)	10 mM

The solution 1 was mixed with 5 ml of 1 M D-glucose, 2.5 ml of Tris-HCl, pH 8.0 and 4.0 ml of 0.25 M EDTA, pH 8.0. The mixture then was top up to 100 ml with sterile distilled water.

2.20.5 0.4 M Sodium hydroxide

Sodium hydroxide	1.6 g
Distilled water to	100 ml

This was autoclaved and kept at room temperature.

2.20.6 2% (w/v) Sodium dodecyl sulphate

SDS	2 g
Distilled water	100 ml

This was filter-sterilised.

2.20.7 Solution II

NaOH (Section 2.20.5)	1 volume
SDS (Section 2.20.6)	1 volume

This alkaline SDS solution was usually mixed just before being used.

2.20.8 5 M Potassium acetate

Potassium acetate	49.07 g
Distilled water to	100 ml

This was autoclaved and kept at room temperature.

2.20.9 Solution III

Potassium acetate (Section 2.20.8)	60 ml
Acetic acid	11.5 ml
Sterile distilled water to	100 ml

2.21 Solutions for the transformations of *Yarrowia lipolytica*

2.21.1 0.1 M Citric acid

Citric acid	2.10 g
Distilled water to	100 ml

This solution was autoclaved and kept at room temperature.

2.21.2 0.1 M Lithium acetate, pH 6.0

Lithium acetate	1.02 g
Distilled water to	100 ml

The pH of the Lithium acetate was adjusted to pH 6.0 with acetic acid.

2.21.3 Carrier DNA for transformation of *Yarrowia lipolytica*

Salmon sperm DNA	5 mg/ml

This was sonicated to an average size of 500 bp.

2.21.4 40% PEG 4000 in 0.1 M Lithium acetate

PEG	40 g
Lithium acetate (Section 2.21.2)	100 ml

2.22 Citric acid fermentation

2.22.1 Seed culture preparation

An active colony of *Y. lipolytica* (M240) was obtained by growing the yeast on YEPD plate for 48 hours at 28°C. Single colony was streaked on another YEPD plate and incubated at the same temperature for 48 hours. This colony was inoculated into seed medium (Section 2.8.7). The cells were grown for 16-18 hours to reach logarithmic phase at 32°C at 220 rpm orbital shaking. At this stage approximately 10^7 cells/ml can be obtained.

The cells were transferred into Nalgene bottles (250 ml) and harvested by centrifugation at 8,000 rpm (Rotor GSA, Sorvall Intruments RC5C) for 10 minutes at 4°C. Pellet was washed twice with 10 ml of 0.1 M phosphate buffer, (Section 2.10.2) and centrifuged at 6,000 rpm. The cells were resuspended in 100 ml of the same buffer and ready for inoculation.

2.22.2 Fermentation

Two ml of the seed culture (Section 2.22.1) was dispensed into fermentation medium (Section 2.8.6). Duplicated shake flask cultures were incubated at 32°C on orbital shaker (220 rpm). Optical density, pH, dry weight of the cells and citric acid level were determined at various intervals throughout the fermentation.

2.22.3 Determination of optical density

Optical density (OD) was determined according to the method of Tan and Gill (1984). One ml culture was added to 2 ml of dioxane:ethyl acetate (Section 2.10.6) and OD was taken at 560 nm.

2.22.4 Measuring of the pH

The pH value of every samples were taken using the pH meter (Model pH M64).

2.22.5 Dry weight

Diethyl ether method was used to determine the cell dry weight (Tan and Gill, 1984). Ten ml of fermentation culture was washed and put in separating funnel. Ten ml of diethyl ether with two drops of 1 N H_2SO_4 was added to the separating funnel. The mixture was shaken and allowed to settle. The lower portion was filtered and weighed on dried filter paper (Whatman No. 1). The cells were dried for 24 hours at 80°C to a constant dry weight.

2.23 Analysis of citric acid

2.23.1 Samples preparation

Five ml of the fermentation culture (Section 2.22.2) was filtered on Whatman no. 1 filter paper in the funnel. Supernatant was refiltered with sterile swenex (Millipore, type GS 0.22 μm pore size, 25 mm). Two ml of the filtered sample was kept in HPLC tube at -20°C.

2.23.2 Analysis of citric acid by High Performance Liquid Chromatography (HPLC)

Samples from Section 2.23.1 were thawed and analysed by HPLC. Commercially obtained pure citric acid (Section 2.10.11) and isocitric acid (Section 2.10.12) were each prepared at concentration 10 mg/ml in water. Equal volume of each was mixed to give a standard mixture, in which the concentration of each is known, to be used as standard for HPLC runs. This mixture is prepared fresh for each run.

A reverse phase chromatography column, an analytical Inertsil ODS-2 column (5μm, 4.6 X 150 mm) (GL, Sciences Inc.) was used for the separation of citric acid. The samples were run at a flow rate 1.2 ml/minute and at isocratic with mobile phase of 0.5 % $(NH_4)_2HPO_4$ (Section 2.10.13). Detection of peaks was at absorbance 214 nm with 0.5 auf sensitivity.

Washing of the column was done before and after each run. Prior to the injection of samples into the column, a mixture of methanol (Gradient grade for chromatography): water (70:30 by volume) is allowed to run through for least 15 minutes, after which is followed by actual solvent system or mobile phase. The

stability of the column is shown by consistency of the values usually 0.000, displayed on the digital detector on the System Controller. After each run, the column is again washed but with 100% methanol for several minutes before switching over to the methanol;water (70:30 v/v) mixture, where further washing is carried out before the column is stored.

A specific volume of standard mixture of known concentration (e.g. 20 µl of standard mixture consisting of 100 µg of each acid) was injected to obtain the retention time of the peaks (Figure 15). Then following the protocol given in the data Module Instruction Manual calibration, the known value of each citric acid and isocitric acid (in this case, 10 µg/µl) is keyed in. The Data Module would then quantify each peak corresponding to the retention time.

2.24 Induction of the mutants with high citric acid production

2.24.1 Culture preparation for mutagenesis

An active colony of *Y. lipolytica* as mentioned in Section 2.22.1 was inoculated into YEPA liquid medium. The cells were incubated for 18-24 hours at 32°C at 220 rpm orbital shaking. Cells were harvested, and treated as indicated in Section 2.22.1.

2.24.2 Mutagenesis of *Yarrowia lipolytica* M 240 (ATCC 8661) with N-methyl- N'-nitro-N-nitrosoguanidine.

The induction of mutants was based on Akiyama's method (1972). Cell pellet (Section 2.24.1) was resuspended in 7.5 ml 0.1 M phosphate buffer pH 7.0 (Section 2.8.2). NTG solution from Section 2.10.5 was added to a final

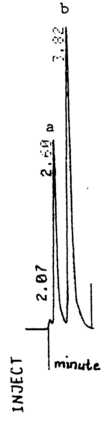

Figure 15 : HPLC of standard mixture used for calibration of the chromatography system. a. Isocitric acid b. Citric acid. Each component in the mixture has a concentration 10 μg/μl.

concentration of 250 μg/ml. The NTG treated-cells suspension was transferred into 125 ml conical flask and incubated in shaking waterbath at 30°C for 30 minutes. The treated cells were placed into sterile screw cap culture tube and spun down at 6,000 rpm at room temperature. Supernatant was discarded in 20% (w/v) of sodium thiosulphate solution (Section 2.10.4). The pellet was washed twice with 10 ml of 0.1 M phosphate buffer (pH 7.0) and resuspended with same volume of buffer. 0.1 ml of the cell suspension was plated on YEPD solid medium after 10^{-3} and 10^{-4} dilution.

2.24.3 Selection of the mutants

After 2 days growth colonies were transferred using tooth pick onto YEPD medium to be kept as master plate. High acid producers were screened on Tricarboxylic acid medium mentioned in Section 2.8.5. The plates were incubated at 28°C for 48 hours. Tentative mutant strains were isolated by their colony-forming abilities on acetate, poor growth on citrate and sensitive to MFA (Akiyama *et al.*, 1972).

2.24.4 Screening for citric acid over production

The putative mutants obtained (Section 2.24.3) were further tested on 0.1% Bromocresol medium (Section 2.8.4). After 5 days growth at 28°C, the diameter of the yellow halo of the putative mutant strains was compared with strain M240 and citric acid over production strain M243. Selected over producer strains were then

grown in shake flasks. Supernatant was tested quantitatively for citric acid using HPLC.

2.25 Lipase activity

Lipase activity experiments were conducted on cells grown on glucose, 2% and 4% NBD palm olein. Samples were taken at interval throughout the fermentation.

2.25.1 Fermentation

The fermentation was carried out as in Section 2.22.2.

2.25.2 Samples preparation for lipase activity

Ten ml of fermented culture broth from Section 2.25.1 were spun down at 12,00 rpm (Rotor SS34, Sorvall Instruments RC5C) for 10 minutes at 4°C. The supernatant was transferred into 2 labelled test tubes (One for control and one for lipase activity) and covered with parafilm. For control, the samples prepared were boiled in hot water for 10 minutes, whereas for lipase, samples were placed on ice.

2.25.3 Lipase activity

Five ml of the foamy emulsion were added to 4 ml 0.05 M phosphate buffer (Section 2.10.3) in 125 ml conical flask. The experiment was done in duplicate. The conical flasks were pre-incubated in an incubator shaker (New Brunswick Scientific Environment Incubator Shaker, Edison, N.J., USA) at 45°C and 500 rpm for 10 minutes (to allow the content of the flasks to reach 45°C). One ml of the

sample prepared in Section 2.25.2 was added to each of the preincubated flasks. These were then reincubated for a further half an hour at the same temperature and speed. The reaction was then asserted using 20 ml of 1:2 (v/v) acetone-ethanol (Section 2.11.3) and titration was done with 0.05 M NaOH (Section 2.11.4) using 5% phenopthalein (Section 2.11.6) as indicator. Lipase activity was calculated as shown in *Appendix I.*

2.26 Tricarboxylic acid cycle enzymes activity

2.26.1 Fermentation

The fermentation was carried out as in Section 2.22.2.

2.26.2 Sample preparation for enzymes activity

Ten ml culture were spun down at 10,000 rpm (Rotor SS34, Sorvall Instruments RC5C), 4°C for 20 minutes. Pellet was washed twice with 0.1 M phosphate buffer (Section 2.10.2) and resuspended in 2 ml of the same buffer. Cells suspension was homogenised (Cells homogeniser, B. Braun) for $1^1/_2$ minutes to break the cells. The cell lysate was then divided into two equal volume and each samples was diluted with 1 ml of 0.05 M phosphate buffer (Section 2.10.3). One was used for protein determination and the other one for enzyme assay. These were spun down at 17,000 rpm for 20 minutes at 4°C. Supernatant was discarded into Eppendorf tubes and placed on ice for enzyme assay.

2.26.3 Protein preparation for standard curve

The 50 mg per ml of BSA (Section 2.12.1) was used to prepare different concentrations of protein (0.01 mg, 0.05 mg, 0.1 mg, .15 mg, 0.20 mg, 0.25 mg, 0.30 mg, 0.35 mg, 0.40 mg, 0.45 mg and 0.5 mg).

2.26.4 Protein determination

A total volume of 1 ml protein samples were placed in appropriate labelled test tubes. For example 0.8 ml of BSA solution (10^{-2}) will give 0.4 mg of protein. This was added with 0.2 ml of distilled water to a total volume of 1 ml solution. 5 ml of solution C (Section 2.12.5) was added and vortexed immediately, to the BSA solution and the various samples (Section 2.26.2). A tube containing no BSA (0.1 M Tris-HCl, pH 7.8)) (Section 2.12.7) was included as a blank. All the test tubes were then incubated for 10 minutes at room temperature.

0.5 ml Folin-Ciocalteu's (Section 2.12.6) reagent was then added to the samples and vortexed immediately. The test tubes were further incubated for 30 minutes at room temperature. OD was read using spectrophotometer at 750 nm (Copeland, 1994). A curve of OD_{750} versus concentration (mg) of the BSA was used as the standard curve to determine the protein concentration of the samples *(Appendix II)*.

2.27 Citrate synthase activity

Citrate synthase activity was assayed by the method of Scere (1963). The components of 1 ml of the reaction mixture are shown in Table 11.

Table 11: Components in 1 ml reaction mixture of citrate synthase activities.

Components	Concentration of stock solution	Solution added per ml (µl)	Final concentration
Tris-HCl, pH 8.0 (Section 2.13.1)	3.0 M	666.67	100 mM
DTNB (Section 2.13.3)	0.05 M	125	0.25 mM
Oxaloacetate (Section 2.13.4)	0.2 M	10	200 µM
Acetyl-CoA (Section 2.13.5)	10 mM	30.8	100 µM
Enzyme extract (Section 2.20.2)	-	40.0	-
Distilled water	-	127.3	-

The assay was performed at room temperature using a blank with all components except the cell extract. Reading was started by adding oxaloacetate and the appearance of the mecaptide ion formed from 5.5,-dithio-bis(2-nitrobenzoic acid)(DTNB) and acetyl CoA. The absorbance was at 412 nm in a spectrophotometer 20. Specific activity of citrate synthase activities was calculated as show in *Appendix III.*

2.28 Aconitate hydratase activity

Aconitate hydratase was carried out by the method of Anfinsen (1962). The components of 1 ml of the reaction mixture are shown in Table 12.

The assay was performed at room temperature. Reading was started by adding 0.1 ml of cis-aconitate solution and OD was measured using a spectrophotometer at 240 nm. One unit of enzyme is defined as the amount needed to cause an initial rate of decrease in optical density (- ΔE_{240}) of 0.001 per minute

under the above conditions. Specific activity of aconitate hydratase was calculated as shown in *Appendix IV*.

Table 12: Components in 1 ml reaction mixture of aconitate hydratase activities.

Components	Concentration of stock solution	Solution added per ml (µl)	Final concentration
Phosphate buffer pH 6.0 (Section 2.14.3)	0.5 M	100	0.5 mM
Cis-aconitic (Section 2.14.4)	0.5 mM	100	0.05 mM
Enzyme extract (Section 2.20.2)	-	100	-
Distilled water	-	700	-

2.29 Isocitrate lyase activity

Isocitrate lyase acitivity was performed by the method of Dixon and Kornberg (1959). The components in 1 ml reaction mixture is shown in Table 13.

Table 13: Components in 1 ml reaction mixture of isocitrate lyase activities.

Component	Concentration of the stock solution	Solution added per ml (µl)	Final concentration
Potassium phosphate pH 6.80 (Section 2.15.1)	0.5 M	80	200 µM
Magnesium chloride (Section 2.15.2)	15 mM	100	15 µM
Phenylhydrazine HCl (Section 2.15.3)	10 mM	25	10 µM
Cysteine HCl (Section 2.15.4)	6 mM	100	6 µM
threo-D_s(+)isocitric acid (Section 2.15.5)	0.5 mM	100	5 µM
Enzyme extract (Section 2.21.2)	-	50	
Sterile distilled water	-	465	

The reaction is started by the addition of 5 μM of threo-D_s(+)isocitric acid. After a lag of approximately 1 min., E_{324} is linear for 4-5 minutes. Reading was measured by a spectrophotometer at 324 nm. E_{324} for glyoxylic acid phenylhydrozone = 1.7 x 10^4. Specific activity of isocitrate lyase was calculated as shown in *Appendix V.*

2.30 Malate synthase activity

Malate synthase activity was performed by the method by Dixon and Kornberg (1959). The components of 0.5 ml reaction mixture is shown in Table 14.

The OD reading was taken at 232 nm using a spectronic 20. Sodium glyoxylate was added after detecting the presence of acetyl coenzyme-A deacylase from the extract. The initial rate of the rapid decrease in optical density is proportional to the amount of malate synthase present. E_{232} for the cleavage of the thio-ester bond of Acetyl CoA is 4.5 x 10^3. Specific activity of malate synthase activities was calculated as shown in *Appendix VI.*

Table 14: Components in 1 ml reaction mixture of Malate synthase activities.

Component	Concentration of stock solution	Solution added per ml (μl)	Final concentration
Tris-buffer, pH7.1 (Section 2.16.1)	18 mM	1000	18 μM
$MgCl_2$ (Section 2.16.2)	15 mM	14.18	2 μM
Acety-CoA (Section 2.16.3)	0.2 μM/100 μl	12.5	0.025 μM
Glyoxylate (Section 2.16.4)	1 mM	50	1 μM
Enzyme extract (Section 2.21.2)	-	40.0	
Distilled water	-	283.32	

2.31 Isocitrate dehydrogenase activity

Isocitrate dehydrogenase was assayed by method of Kornberg (1955). The components of 1 ml reaction mixture are shown in Table 15.

Table 15: Components in 1 ml reaction mixture of isocitrate dehydrogenase activities.

Component	Concentration of stock solution	Solution added per ml (µl)	Final concentration (µM)
Potassium phosphate buffer (Section 2.17.1)	0.5 M	200	200
$MgCl_2$ (Section 2.17.2)	0.1 M	100	100
DPN (Section 2.17.3)	0.025 M	20	373
Isocitrate (Section 2.17.5)	0.005 M	100	500
Enzyme extract (Section 2.21.2)	-	50	-
Distilled water	-	340	-

The OD reading was measured at 340 nm (UV light). The molecular extinction coefficient of 6.22×10^6 cm^2 mol^{-1} is used. Specific activity of isocitrate dehydrogenase activity was calculated as shown in *Appendix VII.*

2.32 Genetic analysis

Y. lipolytica strain M240 and F21A are prototrophs. In order to carry out the genetics analysis, auxotrophs were isolated by mutagenesis using the NTG treatment.

2.32.1 Mutagenesis

Mutagenesis was carried out as mentioned in Section 2.24.1 except YEPD liquid medium was used instead of YEPA.

2.32.2 Screening of the auxotrophic colonies

Treated cultures were plated on YEPD plates. Surviving colonies were picked up with sterile tooth-picks and plated on YEPD plate. The culture was incubated at 28°C for 3 days. The colonies were then transferred onto minimal medium and minimal medium supplemented with the appropriate amino acid and incubated at 28°C for 3 days. Auxotrophic mutants were detected by their inability to grow on minimal medium and ability to grow on minimal medium supplemented with the relevant amino acids. These putative auxotrophic mutants were further tested on 9 different classification plates as given in Table 16.

2.32.3 Characterisation of auxotrophic mutants

Nine different classification plates containing the various amino acids used are shown in Table 16.

Cells of putative auxotrophic mutants were rescreened on each of the 9 classification plates to confirm their requirement.

Table 16: Minimal medium supplemented with appropriate amino acids and nucleic acid bases.

Minimal medium supplemented with amino acids and nucleic acid bases	Amino acid or nucleic acid base which was not supplied
MM + trp + arg + met + his + lys +ade + ura	leu
MM+ leu + arg + met + his + lys +ade + ura	trp
MM+ leu + trp + met + his + lys +ade + ura	arg
MM+ leu +trp + arg + his + lys +ade + ura	met
MM+ leu +trp + arg + met + lys +ade + ura	his
MM+ leu +trp + arg + met + his + ade + ura	lys
MM+ leu +trp + arg + met + his + lys + ura	ade
MM+ leu +trp + arg + met + his + lys +ade	ura
MM+ leu +trp + arg + met + his + lys +ade + ura	0

2.32.4 Genetic crossing by mass mating

Mass mating was carried out on RG medium (Ogrydziak *et al.*,1978) (Section 2.8.9) in parallel streaks. After 24 hours incubation at 25°C, grown colonies were thoroughly mixed on the same plates and were further incubated for another 2 days. The colonies grown were then transferred onto MM. These MM plates were incubated for 3 to 4 days at 30°C.

2.32.5 The induction of sporulation in *Yarrowia lipolytica*

Sporulation was induced on YM medium (Ogrydziak *et al.*, 1978) for 3-7 days at 28°C.

2.33 Isolation of high molecular weight DNA of *Yarrowia lipolytica* (Ng, 1989)

2.33.1 Culture preparation

An active colony from a plate culture was grown in YEPD liquid medium at 28°C for 18 hours. The cells were placed in 250 Nalgene contina and harvested by centrifugation at 6,000 rpm for 10 minutes at 20°C. The supernatant was discarded and cells were resuspended in 2 ml of solution A (Section 2.19.2) 0.5 ml of cells suspension was transferred into microfuge tube.

2.33.2 Isolation of chromosomal DNA

Twenty five µl of β-mercaptoethanol was added into the cell suspension prepared in Section 2.33.1. Each tube was left to stand at room temperature for at least 30 minutes. Cells were centrifuged at 12,000 rpm for 5 minutes and supernatant was discarded. The cells were resuspended in 0.5 ml of solution B (Section 2.19.3). The tubes were incubated for 1 hour at 30°C. The cells were then spun down at 12,000 rpm for 5 minutes. The supernatant was discarded. The cells were resuspended in 0.4 ml of solution C (Section 2.19.8), 5 µl of proteinase K (1 mg/ml in sterile water) was added immediately to each tube. The tubes were incubated at 50°C for 30 minutes until the cell debris disappeared, 0.5 ml of phenol:chloroform (Section 2.19.10) was added. Protein was extracted by inverting the tubes at least 30 times. The tubes were centrifuged at 12,000 rpm for 5 minutes.

At the end of the centrifugation, the upper aqueous layer of each tube was carefully transferred to a fresh microfuge tube. The extraction procedure was repeated once. To each aqueous solution, 1 ml of cold ethanol and the 50 µl of 3 M

sodium acetate (Section 2.19.19) were added. The tubes were left to stand at -70°C for 15 minutes and the contents were then pelleted at 12,000 rpm for 5 minutes at room temperature. The supernatant was discarded, 1 ml of cold 70% ethanol was added to rinse the DNA pellet. The tubes were centrifuged at 12,000 rpm for 1 minute and the supernatant removed completely. The DNA obtained was dried in a desicator with aspirator, 300 µl of TE buffer (Section 2.19.18) was added to each tube to dissolve the DNA pellets. Three µl of RNase A (Section 2.19.15) was added to each tube. The tubes were incubated at 37°C for 30 minutes. Protein was extracted as mentioned earlier by adding 300 µl of phenol:chloroform. One ml of cold ethanol was added to each tube and the tubes were inverted 10 times. DNA was precipitated from each tube as described above. The DNA pellets were dissolved in 50 µl TE buffer (Section 2.19.18) and stored at 4°C.

2.34 Transformation of *Escherichia coli*

2.34.1 Preparation of competent cells

An overnight culture of *E. coli* HB101 or JM109 grown at 37°C in 1 ml LB broth was transferred aseptically into 50 ml of LB broth in a 250 ml flask. The flask was shaked at 220 rpm for 3 hours at 37°C. The culture was then chilled at 0°C for 10 minutes and centrifuged at 6,000 rpm at 0°C for 5 min in a Sorvall SS34 rotor. The supernatant was discarded and the pellet was resuspended in 20 ml chilled 0.1 M calcium chloride (Section 2.10.8) and incubated at 0°C for 30 minutes. The cell suspension was again centrifuged at 6,000 rpm at 0°C for 5 minutes. Finally, the cell pellet was resuspended in 0.5 ml of chilled 0.1 M calcium chloride

and kept on ice until use. The competence of the cells were found to be at its maximum when kept overnight.

2.34.2 Transformation experiments

Plasmid DNA (pINA62, pINA230 and pINA214), 0.1-0.5 μg in TE buffer (Section 2.19.18) was added to 100 μl of competent cells (Section 2.34.1) and the mixture was incubated at 0°C for 40 min. It was then given heat shocked at 42°C 1-1 $^{1}/_{2}$ min. One ml of LB broth was added aseptically and the culture was incubated for one hour at 37°C with shaking at 200 rpm to allow phenotypic expression of genetic determinants on the transforming plasmid DNA. The cells were then pelleted, resuspended in a suitable volume of LB broth and spread on the appropriate selective and non-selective plates.

2.35 Small scale plasmid isolations

The method used was modified from that of Birnboim and Doly (1979). An overnight culture of *E. coli* grown at 37°C in 5 ml of LB supplemented with 50 μg/ml of ampicillin was centrifuged at 10,000 rpm for 5 minutes in microfuge tube (sterile). Supernatant was discarded and pellet was resuspended thoroughly in 100 μl of solution I (Section 2.20.4). The suspension was transferred to a 1.5 ml microfuge tube and placed on ice for 5 minutes. 200 μl of solution II (Section 2.20.7) was added. The tube was inverted gently a few times. The lysate was placed on ice for 5 minutes and 150 μl of cold solution III (Section 2.20.9) was added. The tube content was gently mixed by inversion and left on ice for 5 minutes. The tubes were spun down for 10 minutes at 10,000 rpm to remove all

unwanted material, then the supernatant transferred to a new microfuge tube. 250 µl of phenol-chloroform (Section 2.19.12) was added and the contents were mixed. The mixture was centrifuged for 5 minutes at 10,000 rpm at room temperature. The upper aqueous phase containing the plasmid DNA was carefully transferred to a new microfuge tube. 2 volumes of ice cold absolute ethanol were added to the aqueous phase and the mixture incubated at -70°C for 30 minutes. DNA precipitate was pelleted by centrifugation for 5 minutes. The supernatant was decanted and the pellet was washed with 70% ethanol by the centrifugation for 5 minutes at 10,000 rpm at room temperature. The pellet was briefly dried in vacuum at room temperature. The pellet was dissolved in 20 µl of RNase A solution (Section 2.19.15). The DNA solution was left for 1 hour at 37°C before it was used for electrophoresis or restriction endonuclease digestion.

2.36 Large scale preparation of plasmid DNA

The method used was modified from that of Sambrook *et. al.* (1989). An overnight culture of *E. coli* grown at 37°C in 200 ml of LB supplemented with 50 µg/ml of ampicillin was centrifuged at 6,000 rpm for 10 minutes in 250 ml Nalgene contina (sterile). The supernatant was discarded and the pellet was resuspended in 5 ml of ice-cold solution I (Section 2.20.4). Lysozyme was not used. The suspension was transferred into 50 ml of Nalgene tubes; an aliquot of 10 ml freshly prepared solution II (Section 2.20.7) was added to the cell suspension and thoroughly mixed. The tubes were then kept on ice for 10 minutes and 7.5 ml of solution III (Section 2.20.9) was added to the bacterial lysate, mixed well and kept on ice for another 10 minutes. The contents of the tubes were centrifuged at 15,000 rpm for 30 minutes at

4°C in SS34 Sorvall rotor. The supernatant was transferred to Nalgene tubes (50 ml) and 0.6 volume of isopropanol was added and mixed gently. The suspension was left at room temperature for 20 minutes before it was centrifuged at 15,000 rpm for 15 minutes. The supernatant was discarded; the pellet was dried at 37°C, 0.5 ml of sterile TE buffer (Section 2.19.18) was added to dissolve the plasmid. The mixture was transferred into appendorf tubes; 50 µl of RNase A solution (Section 2.19.15) was added and incubated at 37°C for 30-45 minutes. Equal volume of buffered phenol: chloroform was added, mixed and spun down at 10,000 rpm for 5 minutes. The aqueous phase was carefully transferred into new microfuge tubes; 2 volumes of ice cold absolute ethanol were added followed by $^{1}/_{10}$ volume of 3 M sodium acetate (Section 2.19.19). The mixture was incubated at -70°C for 30 minutes and centrifuged at 12,000 rpm for 5 minutes. The pellet was dissolved in 100-200 µl of TE buffer (Section 2.19.18) and stored at -20°C.

2.36.1 Elution and purification of plasmid from the agarose gel using the GENECLEAN II

The plasmids to be used for transformation were first excised from the agarose gel using a sharp razor blade. Individual slices of gel were transferred into separate sterile 1.5 ml microfuge tubes. The plasmids were purified according to technique indicated on the GENECLEAN II manual.

2.37 Large-scale preparation of pure covalently closed circular (ccc) DNA from *Escherichia coli*

This method was used to prepare pure plasmid of pINA230 for cloning experiments.

For plasmid preparation, an overnight 200 ml *E. coli* culture was harvested. Covalently closed circular (ccc) DNA was extracted by the alkaline lysis method of Birnboim and Doly (1979). Lysozyme was not used. The ccc DNA was then purified by Cesium chloride-ethidium bromide density gradient equilibrium centrifugation (Sambrook *et al.*, 1989) and dialysed against TE buffer (Section 2.19.18).

2.38 Electrophoresis of DNA (Sambrook *et al.*, 1989)

DNA were resolved by electrophoresis in submerged horizontal agarose slab gels (0.7 w/v) in 1X TBE buffer pH 8.3 (Section 2.18.2)

DNA samples were mixed with a one-fifth volume of 6X BPB loading dye (Section 2.18.3). The mixture was then loaded into the sample well and electrophoresis on an agarose gel (with or without ethidium bromide), performed at room temperature from cathode (-) to anode (+) at constant voltage (50 to 100 v) until the tracking dye reached the anode end of the gel.

An agarose gel without ethidium bromide was stained by soaking the gel in ethidium bromide solution (0.5 μg/ml) for 15 minutes and viewed on a 302 nm uv transilluminator (model TM-36, UV products, Inc.). The gel was photographed with a polaroid MP-4 land camera filled with a yellow filter and polaroid land 665 black and while films. The exposure time varied from 20 seconds to 25 seconds.

2.39 Determination of DNA concentrations

Plasmid DNA sample was diluted 10X with sterile deionised water and transferred into a 1 ml quartz cuvette (Sigma chemical Co., U. S. A.). Its optical density reading at wave lengths 260 mm (OD_{260}) and 280 nm (OD_{280}) were measured using a Beckman DU 7500 spectrophotometer. As a solution with OD_{260} of 1 contains approximately 50 µg/ml of double stranded DNA (Maniatis *et al.*, 1982) the following formula was used to calculate the DNA concentration:

$$DNA\ (\mu g/ml) = OD_{260} \times 50 \times 10$$

The ratio of absorbance at OD_{260} to OD_{280} should be 1.8 or greater (Maniatis *et al.*, 1982). A lower ratio indicates a significant amount of protein still remain in the preparation.

2.40 Lithium acetate method for the transformation of *Yarrowia lipolytica*

2.40.1 Preparation of recipient cells

Recipient strain *Y. lipolytica* M24062 was grown on YEPD plate at 28°C for 24 hours. A single colony was inoculated into YEPD liquid medium (Section 2.8.1) at pH 4 incubation at 28°C, 220 rpm to obtain logarithmic phase (OD=1). The cells were harvested and washed twice with 10 ml of TE buffer (Section 2.19.18) by centrifugation at 6,000 rpm for 7 minute. The cells were resuspended at a density 10^7 cells/ ml in 0.1 M LiAC pH 6.0 (Section 2.21.2). The cell suspension was transferred to a sterile 250 ml conical flask and incubated at 28°C for one hour with gentle shaking (140 rpm). After incubation the cells were spun down at 6,000 rpm

for 7 minutes and resuspended in 0.1M LiAC pH 6.0 at a cell density of 10^7 cells/ml (100 ml solution).

2.40.2 Transformation of the yeast cells

In each 1.5 ml of sterile microfuge tube, 5 µl of carrier DNA (Section 2.21.3) and 1 µl of transforming DNA (1 µg DNA linearised) were added and mixed with 100 µl competent cells (2.0-2.3 X 10^7/ml)(Section 2.40.1).

The tube was incubated in a water bath at 28°C for 15 minutes. 0.7 ml 40% PEG 400 in 0.1M LiAC pH 6.0 (Section 2.21.4) was added to the tube. The tube was incubated for one hour at 28°C with shaking at 220 rpm. The cells were given a heat shock at 39°C for 10 minutes. 0.7 ml of TE buffer pH 8.0 was added to the tube. 100 µl aliquot was very gently spread onto minimal medium plates. Recipient cells, which had not undergone transformation were also plated at the same cell density onto MM plates. These plates served as control for the transformation experiments. The MM plates were incubated at 28°C. Colonies appeared in two days.

2.41 Digestion of high molecular weight DNA of *Yarrowia lipolytica* by *Sau* 3A1 and gel electrophoresis

High molecular weight DNA of *Y. lipolytica* isolated in Section 2.33.2 was digested by *Sau* 3A1.

A typical reaction mixture contains; 10 µg of high molecular weight *Y. lipolytica* DNA, 10 units of *Sau* 3A1, 3 µl 10X *Sau* 3A1 buffer and distilled water added to make up to a volume of 30 µl. The reaction mixture was incubated at 37°C

for 5 hours. The reaction mixture was put on ice and left at 65°C for 10 minutes before being loaded into wells of an agarose gel. Gel electrophoresis was conducted in a 0.7% (w/v) agarose as in Section 2.38.

2.42 Restriction endonuclease digestion of plasmid DNA

Digestion was carried out in sterile 1.5 ml microfuge tubes to a final volumes of 20 to 40 µl, containing 0.5 to 5 µg substrate DNA and 1 unit of restriction endonucleases. The reaction mixture was incubated for 3 to 5 hours. At the end of incubation the tube was put at 65°C for 10 minutes, before being loaded into wells of an agarose gel (Section 2.38).

2.43 Ligation of fragments DNA with compatible ends

Sau 3AI fragments from Section 2.14 were ligated with *Bam* HI-digested pINA230. The components of reaction mixture contain; 1 µg of *Sau* 3AI-generated DNA fragments of *Y. lipolytica*, 0.5 µg of *Bam* HI-digested pINA230, 1 units of T4 DNA ligase in 1X ligase buffer and distilled water added to make up to a volume of 10 µl. A *Sau* 3AI-generated DNA fragments of *Y. lipolytica* with compatible ends for ligation was first heated at 65°C for 10 minutes, then cooled to room temperature for 20 minutes and placed on ice. The reaction mixture was incubated overnight at 12°C (Ng,1989) and 40 µl of sterile water was added after that. The ligation mixture was further incubated overnight at 12°C, then 5-10 µl was used to transform competent cells. Transformants were selected when growth occurred on plates with appropriate antibiotic resistance.

2.44 Production of citric acid in continuous culture

Fermenter used in this study was the 2.0 litre bench-top glass fermenter (Bioflo, New Brunswick Scientific C. O. Inc., Edison, U. S. A.). Figure 16 indicates the anatomy of fermenter used. The working volume used was at 1 litre. Upon cooling 100 ml of seed culture (Section 2.22.1) at volume of 100 ml (which made up 10 % of the total volume of fermentation medium (Section 2.8.6) with 0.05 % surfactant (sucrose palmitic), was inoculated into the fermenter. The culture was agitated at 300 rpm, the air-flow rate was fixed at 1 litre/minute and internal temperature was set at 32°C. The flow rate of medium fed into the fermenter was a fixed volume of 1 ml/min. Thirty ml samples were collected at 0, 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. Samples collected then underwent similar steps as explained in Sections 2.22.3, 2.21.4, 2.22.4, 2.22.5, 2.23.1, and 2.23.2.

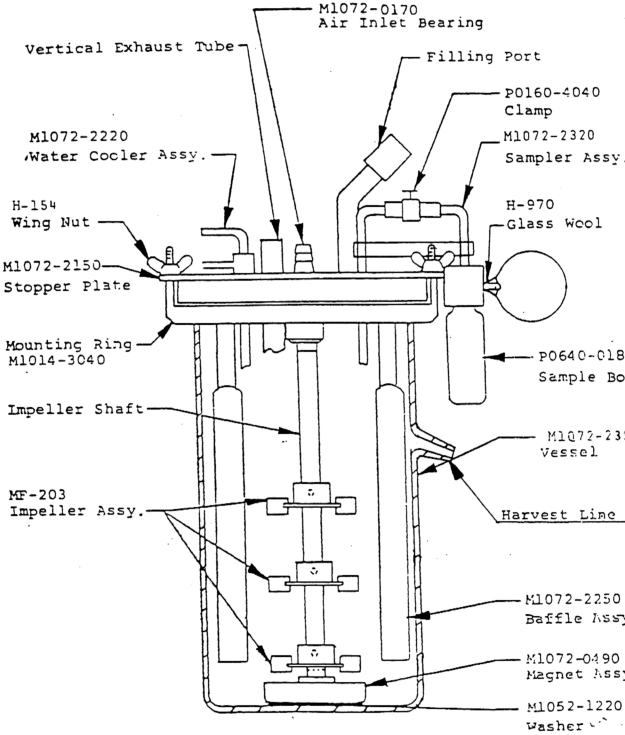

Figure 16 : The anatomy of fermenter (Bioflo, New Brunswick Scientific C. O. Inc., Edison U. S. A.)