

RESULTS

CHAPTER 3

RESULTS

3.1 Isolation of strains with high level of citric acid production

The yeast *Yarrowia lipolytica*, ATCC 8661 (Laboratory strain no. M240) was used in mutagenesis in an attempt to isolate higher citric acid producer strains on NBD palm olein. Mutants were induced by treatment of logarithmic cells with N-methyl-N'-nitro-N-nitrosoguanidine (NTG).

According to Akiyama *et al.* (1972), enhanced citric acid production was shown by strains sensitive to monofluoroacetate (0.1%) and inability to utilise citrate for growth. A mutant strain incapable of growing on citrate as the sole carbon source and sensitive to monofluoroacetate would have low aconitate hydratase activity. With low activity of this enzyme the conversion of citric acid to isocitric acid was inhibited which resulted in the accumulation of citric acid (Akiyama *et al.*, 1972 and 1973). Such colonies were selected, then screened on minimal medium containing 0.1 % bromocresol green to select for high acid producers. Bromocresol green is an indicator which change colour due to the changes in pH, thus high acid producers formed large yellow zone on media incorporated with this indicator.

A mutant strain F21 was selected based on the characteristics mentioned above. Out of 1100 colonies of isolates from M240, 20 colonies showed poor growth on citrate and 0.1% MFA. No strains showed sensitivity to 0.1 % MFA,

even up to 0.15 %. However, at 0.2% concentration of MFA only the wild type strain showed the ability to grow.

The 20 colonies were then screened on 0.1 % bromocresol green and media containing intermediates of the TCA cycle as carbon sources. 12 colonies showed poor growth on pyruvate, citrate, isocitrate, succinate, malate and fumarate. Out of the 12 colonies, 4 isolates showed poor growth on α -ketoglutarate. Strain F21 was one of the 4 colonies, showing significantly larger halo on 0.1% bromocresol green. F21 showed 3.8 cm diameter halo which is 52.6 % longer in diameter compared to its parent strain (M240) (1.8 cm). Unfortunately, the actual citric acid production of this mutant when assessed on HPLC did not increase significantly compared to strain M240 (Figure 17). Strain F21 was further exposed to NTG. This led to the isolation of mutant strain F21A.

Strain F21A was selected based on the same characteristics as strain F21. 1000 colonies were selected after mutagenesis of F21, 32 colonies showed poor growth on citrate and 0.1% of MFA. Table 17 shows the response of all the yeast strains on monofluoroacetate (MFA).

On 0.1% bromocresol green, F21A showed 3.5 cm in diameter of yellow halo which was significant larger when compared to strain M240 (1.8 cm)(at about 56% longer in diameter). Strain M243 (Akiyama *et al.*, 1972 and 1973) an established strain was used as comparison. Strain M243 showed 3.5 cm in diameter, which was comparable with strain F21A. Figure 18 illustrates the acid yielding capacity as shown by halo formed on minimal medium incorporated with 0.1% bromocresol green. After five days growth, strain F21A showed halo size which, was significantly larger then strain M240.

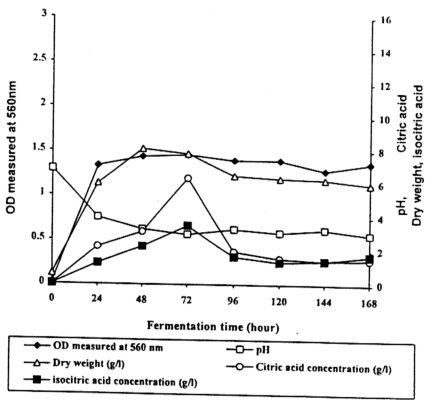


Figure 17 : Growth and citric acid production of *Yarrowia lipolytica* (F21) utilising 2% NBD palm olein as the carbon source.

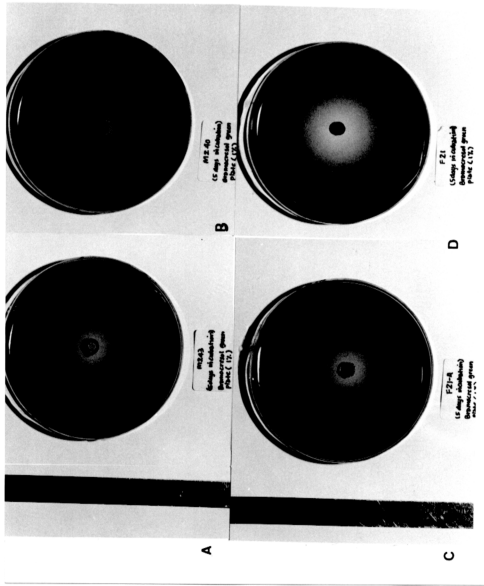


Figure 18: Acid forming capacity as shown by size of halo formed on minimal medium incorporated with 0.1% bromocresol green as pH indicator after 5 days of incubation. A. M243 (IFO no. 1545/S-22) B. Parent strain, ATCC 8661 (Laboratory strain no. M240) C. F21A and D. F21

Table 17: Effects of Sodium monofluoroacetate (MFA) on growth of *Yarrowia lipolytica*.

Strain	MFA concentration (g/l)			
	0.5	1	1.5	2
Parent strain ATCC 8661/M240	++	+	+	+
M243 (IFO no. 1545/ S-22)	++	+	+	-
F21	+	±	±	-
F21A	+	+	+	-

- (++) Good growth
 (+) Growth
 (±) Poor growth/slight growth
 (-) No growth

Growth studies on several carbon sources related to the tricarboxylic acid cycle (TCA) of all strains are shown in Table 18. All strains grew well on acetate. The two mutants, F21 and F21A showed similar growth ability, as M243 except the latter was totally not able to utilise isocitrate. On those intermediates that were utilised, F21 and F21A showed much reduced growth.

The morphology of colonies and cells grown on YEPD plate of all strains are described in Table 19. Strains M240 and M243 have similar, round shape, smooth and wet colonies on YEPD. Strain F21 showed a rough and wrinkled colony. While strain F21A showed fine wrinkle and rather dry colonies (Figure 19). Both strains (F21 and F21A) have the same cell morphology which were elongated with budding. Pseudomycelium was only observed after 4-5 days of incubation on NBD palm olein.

Table 18: Utilisation by different strains of *Yarrowia lipolytica* of carbon sources related to TCA cycle after 24 hours incubation.

Carbon source	Parent strain (ATCC 8661/M240)	M243 (IFO no. 1545/ S-22)	F21	F21A
Acetate	++	++	++	++
Pyruvate	+	±	±	±
Citrate	+	+	±	±
Isocitrate	+	-	±	±
α-ketoglutarate	-	-	-	-
Succinate	+	±	±	±
Malate	±	±	±	±
Fumarate	+	-	±	±

- (++) Good growth
 (+) Growth
 (±) Poor growth
 (-) No growth

Table 19: Morphology of colonies and cells of different strains of *Yarrowia lipolytica* grown on YEPD medium after 48 hours incubation.

Strain	Colony morphology	Cells morphology
Parent strain (ATCC8661/M240)	round shape, smooth and wet	single cells and cells with buds
M243 (IFO no. 1545/ S-22)	round shape, smooth and wet	single cells and cells with buds
F21	rough wrinkled and dried	single cells, elongated cells and cells with buds
F21A	finely wrinkled and dried	single cells, elongated cells and cells with buds

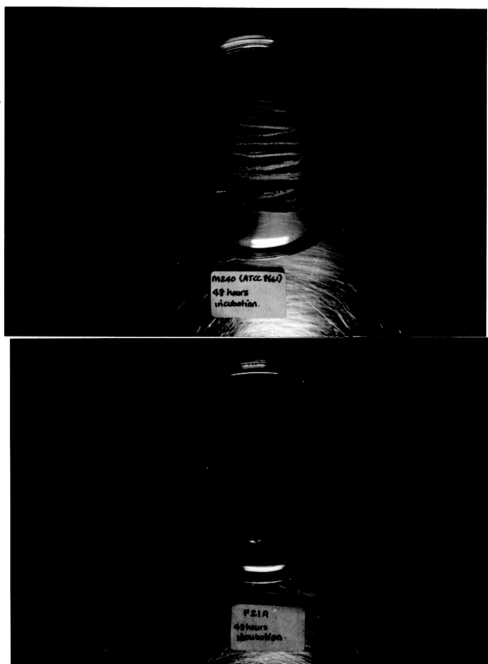


Figure 19: Two different morphological colonies of *Yarrowia lipolytica* grown on YEPD medium after 48 hours incubation. A. Parent strain (ATCC 8661/M240) (Smooth and wet). B. F21A (Wrinkled and dried)

3.2 Growth and production of citric acid on glucose and NBD palm olein

Strain F21A was further tested for its ability to grow and produce citric acid on 2% glucose, 2% and 4% NBD palm olein.

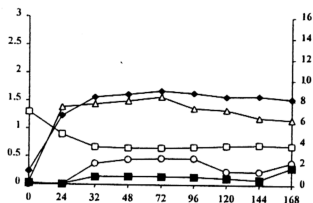
3.2.1 On 2% glucose

Strains M240 and M243 have been reported to be used as strains to produce citric acid utilising n-paraffin as the carbon source (Akiyama *et al.*, 1972 and 1973). In such substrate the yeast has the ability to grow actively and produced high level of citric acid. In this study, all strains were initially grown on glucose and their performance assessed. This provides a reference to compare subsequent results.

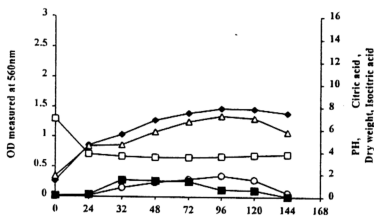
Figure 20 illustrates growth and production of citric acid of all strains on glucose. The pattern of growth of all strains was fairly similar, however the F21A showed the fastest growth rate reaching OD₅₆₀ of 1.68 and also attained much higher dry weight (8.4 g/l) after 48 hours incubation as compared to M240 (7.2 g/l). Strain M243 showed comparable rate of growth and attained the same value of dry weight as shown by strain F21A.

In term of citric acid production, F21A (2.5 g/l) was producing 25% higher citric acid as compared to M240 (1.87 g/l) but 5.3 % lowered as compared to M243 (2.64 g/l). Another favourable observation is that the level isocitric acid of strain F21A was 70% lower compared to its citric acid production at the maximum point of production at 96 hours incubation. The same observation was also shown by strain M243, it produced 63% much lower isocitric acid compared to its citric acid

(A)



(B)



(C)

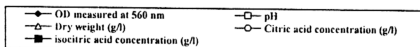
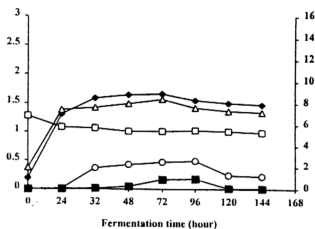


Figure 20 : Growth and citric acid production of different strains *Yarrowia lipolytica* utilising 2% glucose as the carbon source. The cultures were incubated at 32°C on orbital shaker and samples were taken at interval indicated. A. F21A; B. Parent strain (ATCC8661/M240); C. M243 (IFO no. 1545/ S-22).

production at 96 hours incubation. Although both strains showed an increase in citric acid and decrease in isocitric acid production when compared to M240, the values of citric and isocitric acid were still fairly low compared to production during growth in 2% and 4% NBD palm olein (*Appendix IX*).

The excretion of acids into medium resulted in a decline in pH. The pH decreased rapidly reaching steady reading of 3.5-3.8 at 48 hours of incubation.

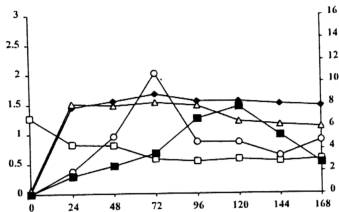
3.2.2 On 2 % NBD palm olein

From previous observation, 2% NBD palm olein seem to be the most suitable concentration for growth and production of citric acid by *Y. lipolytica* (Ajam *et al.*, 1991). Higher or lower concentration retard growth and reduced production of citric acid.

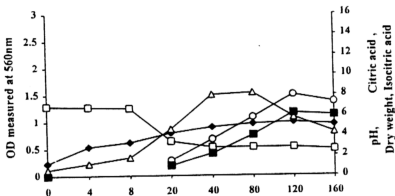
All the strains tested were able to grow and produced citric acid on 2% NBD palm olein. However, the extend of growth and quantity of citric acid production differ. Figure 21 illustrates the growth and production of citric acid of *Y. lipolytica* strain F21A compared to strain M240 and strain M243 on 2% NBD palm olein.

As indicated in Figure 19 the trend of growth is fairly similar. F21A showed the fastest rate of growth compared to M240 and M243. The highest dry weight observed in strain F21A was at about 8.2 g/l at 72 hours incubation. The same value was also observed when it compared to strain M240 (8.2 g/l) in dry weight. Strain M243 (10.5 g/l) showed the highest dry weight exhibiting 22% much higher compared to strain F21A.

(A)



(B)



(C)

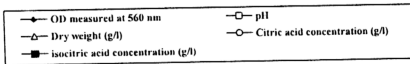
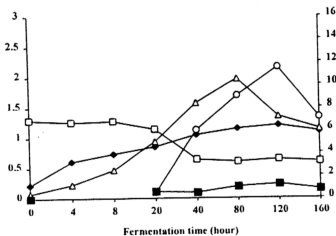


Figure 21 : Growth and citric acid production of different strains *Yarrowia lipolytica* utilising 2% NBD palm olein as the carbon source. The cultures were incubated at 32°C on orbital shaker and samples were taken at interval indicated. A. F21A; B. Parent strain (ATCC8661/M240); C. M243 (IFO no. 1545/ S-22).

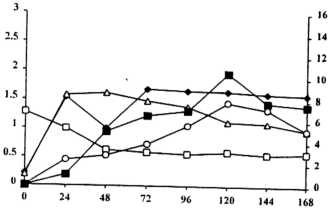
Despite the rather low dry weight compared to strain M243, in term of citric acid production F21A produced 10.8 g/l (135%) of citric acid which showed a 35% increase in the production compared to strain M240 (8 g/l). Another interesting observation, is that the highest level of citric acid in strain F21A was produced at 72 hours incubation which was 48 hours earlier compared to both strains M240 and M243. Isocitric acid of strain F21A reached maximum level at a later phase of incubation after about 120 hours (*Appendix X*).

3.2.3 On 4% NBD palm olein

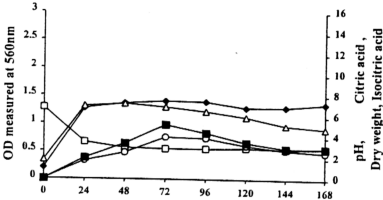
Ikeno *et al.* (1975) reported that *Y. lipolytica* can grow on 7% palm oil and produced 102 g/l of citric acid. This indicated the feasibility of high level of citric acid production using NBD palm olein as the carbon source. Figure 22 illustrates the growth and production of citric acid of strain F21A compared to its wild type and strain M243 on 4% NBD palm olein.

The growth of all strains was comparable. Strain F21A showed faster rate of growth reaching an OD₅₆₀ of 1.62 and attained the highest dry weight at about 8.6 g/l at 48 hours incubation. Comparison with its wild type and M243 indicated that strain F21A was much superior exhibiting 16.3% and 5.0% much higher of dry weight respectively. This indicated that all strains grown on 4% NBD palm olein showed a slight increased in the production of cells compared to on 2% NBD palm olein. However, in term of citric acid production all strains showed reduction in production of this acid including strain F21A.

(A)



(B)



(C)

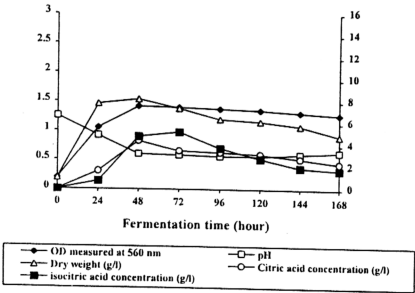


Figure 22 : Growth and citric acid production of different strains *Yarrowia lipolytica* utilising 4% NBD palm olein as the carbon source. The cultures were incubated at 32°C on orbital shaker and samples were taken at interval indicated. A. F21A; B. Parent strain (ATCC8661/M240); C. M243 (IFO no. 1545/ S-22).

Strain F21A produced 7.8 g/l of citric acid while concurrently the isocitric acid reached 10.52 g/l which account to 26% than citric acid. As shown in Figure 20 in strain F21A isocitric acid was produced simultaneously with citric acid reaching maximum on day 5 of incubation. Strains M240 and M243 showed the same pattern of production, however the extend of its acids production differ. Strain M240 showed 23% increase in the production of isocitric acid whereas, only 4% increase in strain M243 compared to citric acid production in 4% NBD palm olein. However when compared to growth in 2% NBD palm olein, isocitric acid production in strain M240 was 16% much lower. Whereas, in strain M243 showed 75% increasing (1.2 g/l to 4.8 g/l) in isocitric acid production in 4% NBD palm olein (*Appendix XI*).

3.3 Lipase production

There are several factors that may contribute in the production of citric acid in strain F21A. Supply of carbon source is vital to ensure the proper functioning of the TCA cycle where citric acid is being produced. The first step in the utilisation of palm olein as the carbon source is the breakdown of lipids to fatty acids before absorption into the cells. This depends on the production of lipases. In this study extracellular lipase activity was assayed on 2% glucose, 2% and 4% NBD palm olein.

3.3.1 2% glucose

As expected, glucose suppressed the production of lipases. None of the strain showed any positive result when grown in glucose.

3.3.2. 2% NBD palm olein

Figure 23 illustrate the growth and extracellular lipases activity of strain F21A compared to its wild type strain and M243. The lipase activity of strain F21A increased rapidly after 8 hours incubation, similar to activity exhibited by strain M243. However, the value achieved between these strains differ. Strain F21A reached its maximum production at about 13.42 $\mu\text{mol/ml}$ at 16 hours incubation. Whereas, for strain M243 at about 18.87 $\mu\text{mol/ml}$ at 20 hours incubation. The lipase activity of both strains decreased drastically after reaching its maximum. For strain M240 the lipase activity increased rapidly after 4 hours incubation reaching 13.0 $\mu\text{mol/ml}$ at 12 hours incubation and decreased there after.

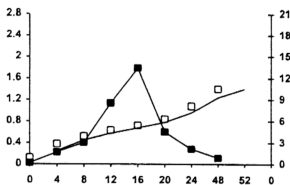
3.3.3 On 4% NBD palm olein

On 4% NBD palm olein, as shown before strain F21A grew much better compared to strain M240 and M243 (Figure 24). Concomitant with the good growth, F21A had increased lipase activity at about 17.40 $\mu\text{mol/ml}$. For strain M240 and M243 at maximum activity only had 12.03 $\mu\text{mol/ml}$ and 12.90 $\mu\text{mol/ml}$ respectively.

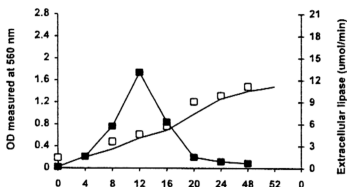
3.4 Activity of key enzymes in TCA cycle

Citric acid, the acid of interest in this study, is formed via the condensation of oxaloacetate and the incoming acetyl-Coenzyme A catalysed by citrate synthase.

(A)



(B)



(C)

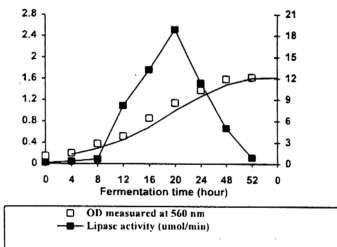
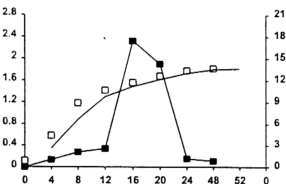
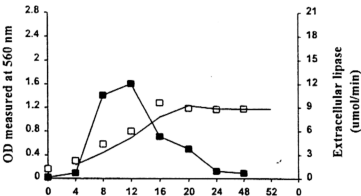


Figure 23 : Growth and extracellular lipases activity of different strains *Yarrowia lipolytica* utilising 2% NBD palm olein as the carbon source. The cultures were incubated at 32°C on orbital shaker and samples were taken at interval indicated. A. F21A; B. Parent strain (ATCC8661/M240); C. M243 (IFO no. 1545/ S-22).

(A)



(B)



(C)

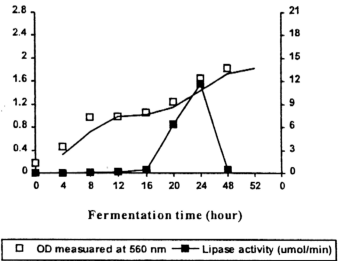


Figure 24 : Growth and extracellular lipases activity of different strains *Yarrowia lipolytica* utilising 4% NBD palm olein as the carbon source. The cultures were incubated at 32°C on orbital shaker and samples were taken at interval indicated. A. F21A; B. Parent strain (ATCC8661/M240); C. M243 (IFO no. 1545/ S-22).

The activity of this enzyme was examined to see whether the level of activities differ in different concentration of substrate at the same time whether this would affect the production of citric acid. Aconitate hydratase then catalyses the reversible isomerisation of citrate to isocitrate thus this enzyme determines the balance of the two substrates in the cell. Isocitrate dehydrogenase oxidised isocitrate to the β -keto acid intermediate oxalosuccinate with the coupled reduction of NAD^+ to NADH. The studies of aconitate hydratase and isocitrate dehydrogenase were conducted since these enzymes have been reported by Tabuchi and Hara (1975) as a key of enzymes responsible for the accumulation of citric acid. The defects in aconitate hydratase by mutation or exposure to MFA will lead to the accumulation of citric acid (Akiyama *et al*, 1972). In this study, these three enzymes were assayed in *Y. lipolytica* strain F21A, M240 and M243 grown on 2% glucose, 2% and 4% NBD palm olein.

3.4.1 On 2% glucose

As shown in Figure 25, the activity of all enzymes in all strains increased up to day 2 of fermentation before declining gradually. Strain F21A (1.5 $\mu\text{mol/mg}$ protein/min) showed 20% more citrate synthase activity compared to strain M240 (1.20 $\mu\text{mol/mg}$ protein/min) and 2.7% (1.46 $\mu\text{mol/mg}$ protein/min) higher than strain M243. The trend was similar for aconitate hydratase activity where strain F21A showed much higher activity compared to strain M240 and M243 at about 9.7% and 34.0% respectively. The activity of isocitrate dehydrogenase differ slightly, strain F21A still showed much higher activity (0.045 $\mu\text{mol/mg}$ protein/min) compared to strain M240 (0.042 $\mu\text{mol/mg}$ protein/min) on day 1 incubation.

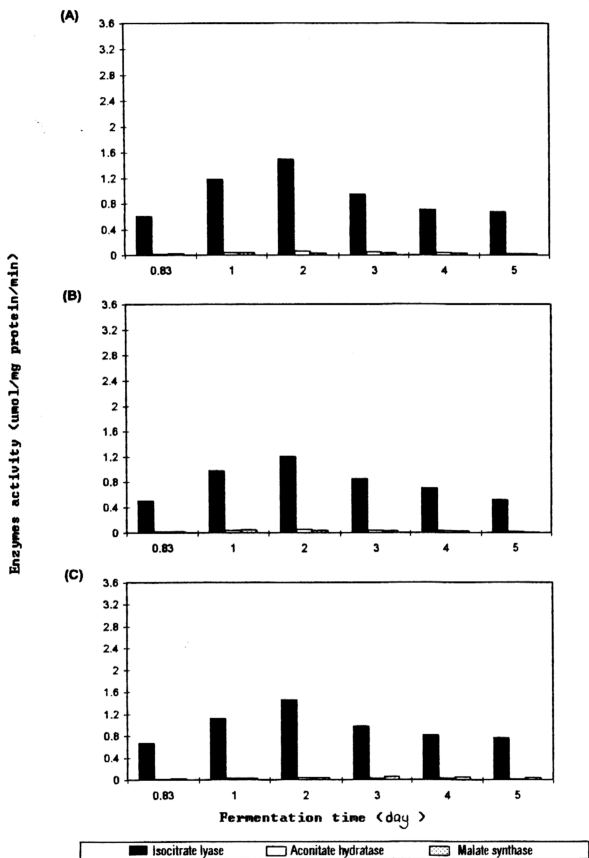


Figure 25: The TCA cycle enzymes activity of different strains *Yarrowia lipolytica* utilising 2% glucose as the carbon source. (A) Strain F21A; (B) Parent Strain M240 (ATCC 8061); (C) Strain M243(S-22/FO no. 1545).

Whereas, for strain M243 the activity was increased up to day 3 of fermentation and remain at more or less at the same level there after (0.056-0.046 $\mu\text{mol/mg protein/min}$).

3.4.2 On 2% NBD palm olein

Figure 26 illustrates the activity of citrate synthase, aconitate hydratase and isocitrate dehydrogenase when cells were grown on 2% NBD palm olein. Citrate synthase activity in all strains attained it maximum at day 2 of incubation where strain F21A showed 7.7 % much higher in activity compared to strain M240 and 44.0% much lower in activity compared to strain M243. The aconitate hydratase and isocitrate dehydrogenase activities of all strains increased when compared to growth on 2% glucose. The increase in aconitate hydratase activity reaching to about 71% and the activity of isocitrate dehydrogenase touched to nearly 65% in 2% NBD palm oil. Strain F21A showed the highest activity of aconitate hydratase among the strains which attained 0.24 ($\mu\text{mol/mg protein/min}$) at day 1 fermentation. This shows that F21A has 20.8% much higher in activity of aconitate hydratase compared to strain M240 (0.19 $\mu\text{mol/mg protein/min}$) and 57.5% much higher in activity compared to strain M243 (0.102 ($\mu\text{mol/mg protein/min}$)).

Activity of isocitrate dehydrogenase in strain F21A exceeded strain M240 and M243 by 23.2% and 60% respectively. The highest TCA cycle enzymes activities of all strains on 2% NBD palm olein were summarised in Table 20 (*Appendix XIV and XV*).

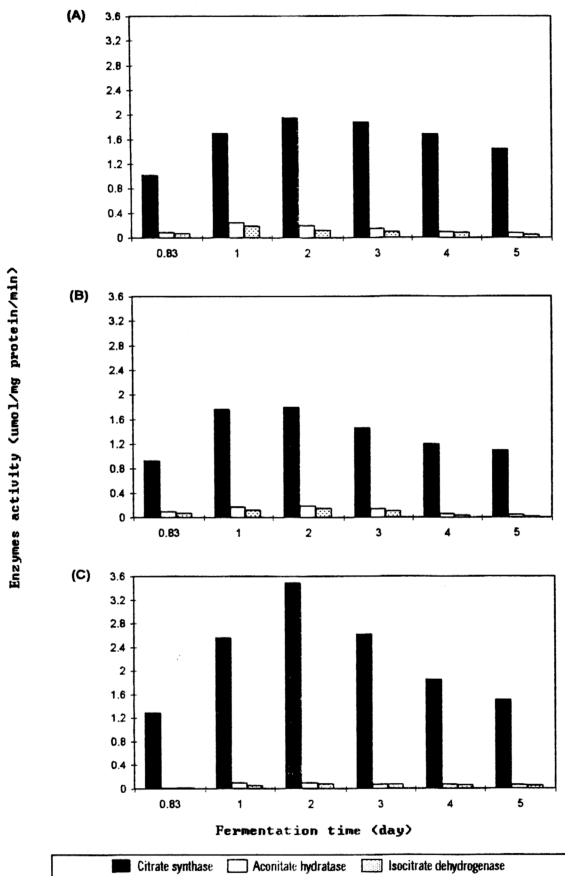


Figure 26: The TCA cycle enzymes activity of different strains *Yarrowia lipolytica* utilising 2% NBD palm olein as the carbon source. (A) Strain F21A; (B) Parent Strain M240 (ATCC 8661); (C) Strain M243(S-22/IFO no. 1545).

3.4.3 On 4% NBD palm olein

Figure 27 showed the enzymes activities in strain F21A, strain M240 and M243 when grown on 4% NBD palm olein. Citrate synthase was the most prominent enzyme where strain F21A showing higher activity compared to strain M240 and M243 by 41% and 35.4% respectively.

Aconitate hydratase activity in strain F21A achieved its maximum activity at day 2 of incubation ($0.19 \mu\text{mol/mg protein/min}$) and the activity remained until the last day of the experiment. Strain M240 showed the highest activity of aconitate hydratase which was achieved on day 1 of incubation reaching $0.23 \mu\text{mol/mg protein/min}$, at about 17.4 % much higher compared to F21A, the activity was decreased thereafter (day 2) to $0.145 \mu\text{mol/mg protein/min}$. Strain M243 showed the lowest activity except on day 1 of fermentation which was comparable to F21A.

The isocitrate dehydrogenase activity was the highest in strain M240. Strain F21A showed lower activity, only reaching $0.1 \mu\text{mol/mg protein/min}$ on day 2 incubation. Table 20 summarised the maximum activities of all enzymes on 2% glucose, 2% and 4% NBD palm olein (*Appendix XVI and XVII*).

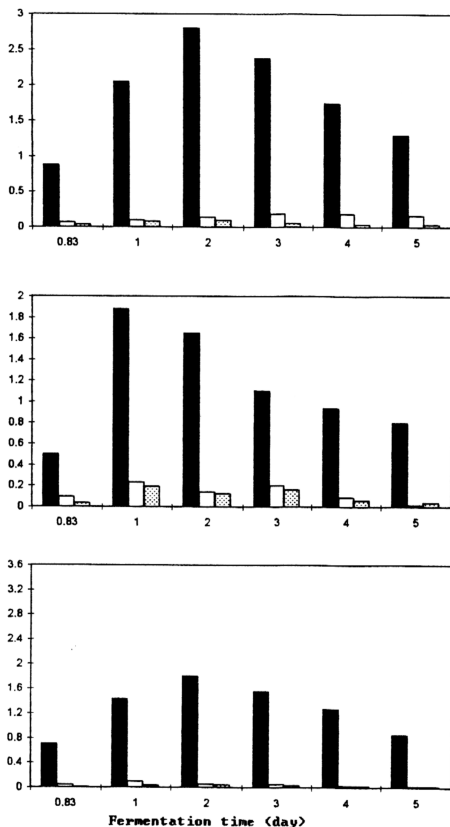
Enzymes activity ($\mu\text{mol}/\text{mg protein}/\text{min}$)

Figure 27: The TCA cycle enzymes activity of different strains *Yarrowia lipolytica* utilising 4% NBD palm olein as the carbon source. (A) Strain F21A; (B) Parent Strain M240 (ATCC 8661); (C) Strain M243(S-22/IFO no. 1545).

Table 20: The maximum activity of TCA cycle enzymes assayed during growth of different strains *Yarrowia lipolytica* on glucose, 2% and 4% NBD palm olein as the carbon source.

Enzymes	2% glucose			2% NBD palm olein			4% NBD palm olein		
	Strain F21A	Parent strain (ATCC 8661/M240)	M243 (IFO no. 1545/ S-22)	Strain F21A	Parent strain (ATCC 8661/M240)	M243 (IFO no. 1545/ S-22)	Strain F21A	Parent strain (ATCC 8661/M240)	M243 (IFO no. 1545/ S-22)
Citrate synthase ($\mu\text{mol}/\text{mg}$ protein/min)	1.50 (48 hrs)	1.20 (48 hrs)	1.46 (48 hrs)	1.95 (48 hrs)	1.80 (48 hrs)	3.49 (48 hrs)	2.80 (48 hrs)	1.88 (24 hrs)	1.81 (48 hrs)
Aconitase hydratase ($\mu\text{mol}/\text{mg}$ protein/min)	0.062 (48 hrs)	0.056 (48 hrs)	0.041 (48 hrs)	0.24 (24 hrs)	0.19 (48 hrs)	0.102 (24 hrs)	0.19 (72 hrs)	0.23 (24 hrs)	0.092 (24 hrs)
Isocitrate dehydrogenase ($\mu\text{mol}/\text{mg}$ protein/min)	0.042 (48 hrs)	0.048 (24 hrs)	0.056 (72 hrs)	0.19 (24 hrs)	0.15 (48 hrs)	0.080 (48 hrs)	0.10 (48 hrs)	0.19 (24 hrs)	0.042 (48 hrs)

3.5 Involvement of glyoxylate cycle

Growth on substrates such as glycerol, acetate and n-alkane induce another pathway, the glyoxylate cycle to become operative to supplement the TCA cycle. Two key enzymes in this cycle are malate synthase and isocitrate lyase. In order to determine whether the glyoxylate cycle is operative during growth of *Y. lipolytica* strain F21A, M240 and M243 on 2% and 4% NBD palm olein, these two enzymes were assayed. All strains were also grown on 2% of glucose as comparison where the glyoxylate enzymes were suppressed by the present of glucose.

3.5.1 On 2% glucose

As shown in Figure 28, glucose suppressed the production of both enzymes malate synthase and isocitrate lyase in all strains. The enzymes were only induced when the yeast was grown on 2% and 4% NBD palm olein.

3.5.2 On 2% NBD palm olein

Generally the activity of malate synthase was much higher compared to isocitrate lyase. As indicated in Figure 29, strain F21A showed maximum level of activity at day 2 of incubation at about 1.88 $\mu\text{mol/mg protein/min}$. This is 36% increased in activity compared to M240 (1.2 $\mu\text{mol/mg protein/min}$) on the same day of incubation. Strain M240 attained its maximum activity at day 1 of incubation at about 1.48 $\mu\text{mol/mg protein/min}$. Comparing with M243, strain F21A showed 46.3% increased in activity of malate synthase at day 2 of incubation. The maximum activity for strain M243 was at day 1 incubation which showed the highest activity compared to strain F21A and M240 (*Appendix XIV and XV*).

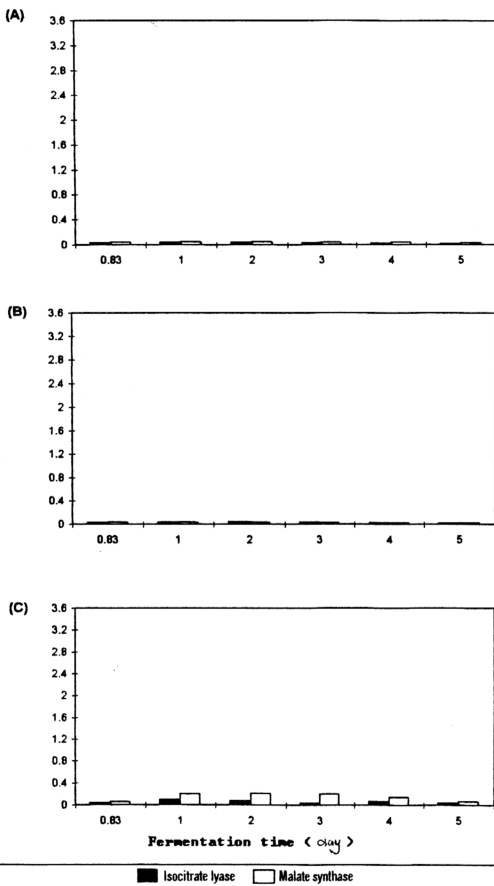
Enzymes activity ($\mu\text{mol}/\text{mg protein}/\text{min}$)

Figure 28: The glyoxylate enzymes activity of different strains *Yarrowia lipolytica* utilising 2% glucose as the carbon source. (A) Strain F21A; (B) Parent Strain M240 (ATCC 8661); (C) Strain M243(S-22/IFO no. 1545).

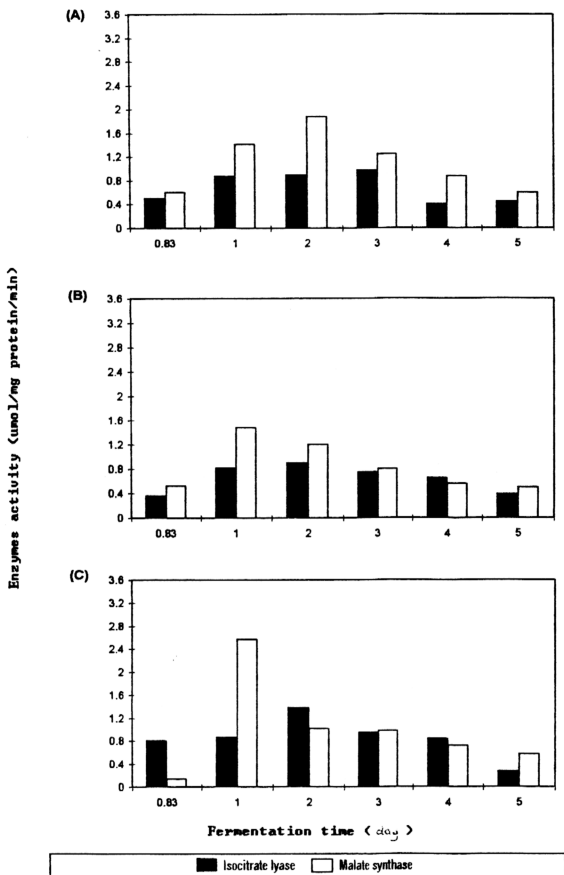


Figure 29: The glyoxylate enzymes activity of different strains *Yarrowia lipolytica* utilising 2% NBD palm olein as the carbon source. (A) Strain F21A; (B) Parent Strain M240(ATCC 8661); (C) Strain M243(S-22/IFO no. 1545).

Isocitrate lyase in strain F21A showed the highest activity on day 3 of incubation at about 0.98 $\mu\text{mol}/\text{mg}$ protein/min. Comparing the activity of this enzyme at day 3 incubation with strain M240 and M243, F21A showed 23.5% and 3.1% much higher in activity respectively. Both strains, M240 and M243 achieved the highest activity on day 2 of incubation. Table 21 summarises the highest activity of the glyoxylate cycle enzymes activities in all strains grown on glucose, 2% and 4% NBD palm olein.

3.5.3 On 4% NBD palm olein

Malate synthase in strain F21A was produced and active from day 1 up to the end of fermentation. As show in Figure 30, at the maximum activity of this enzyme, F21A produced 2.38 $\mu\text{mol}/\text{mg}$ protein/min which is 47% and 66% increased in activity compared to M240 and M243 respectively on the same day of incubation (day 1).

Isocitrate lyase in strain F21A showed the highest activity among the strains at about 1.50 $\mu\text{mol}/\text{mg}$ protein/min on day 2 of incubation. Strain M240 reached it maximum activity at about 1.25 $\mu\text{mol}/\text{mg}$ protein/min on day 1 and 1.17 $\mu\text{mol}/\text{mg}$ protein/min on day 3 for strain M243. Table 21 summarised the highest activity of the glyoxylate enzymes of all strain grown on glucose and 2% and 4% NBD palm olein (*Appendix XXVI and XVII*).

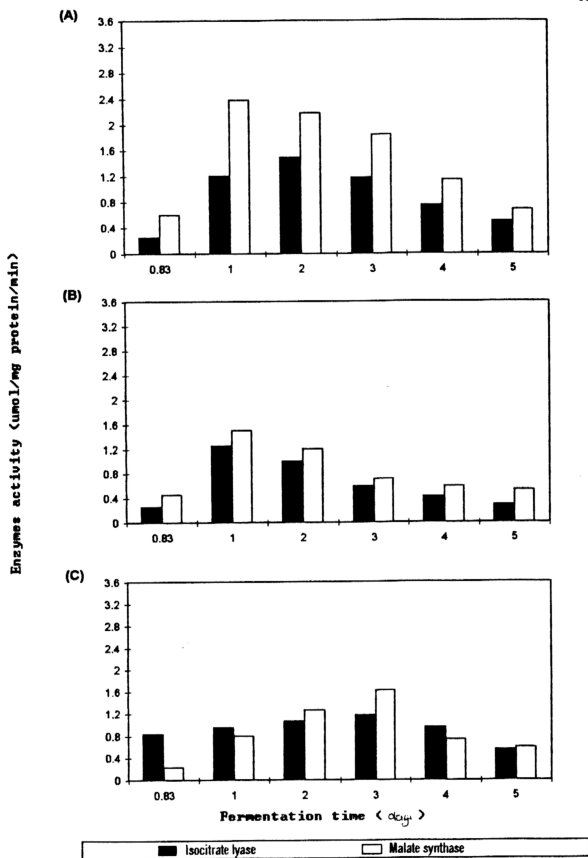


Figure 30: The glyoxylate enzymes activity of different strains *Yarrowia lipolytica* utilising 4% NBD palm olein as the carbon source. (A) Strain F21A; (B) Parent Strain M240 (ATCC 8001); (C) Strain M243(S-22)/FO no. 1545).

Table 21: The maximum activity attained by enzymes of the glyoxylate cycle during growth of different strains *Yarrowia lipolytica* on glucose, 2% and 4% NBD palm olein as the carbon source.

Enzymes	2% glucose			2% NBD palm olein			4% NBD palm olein		
	Strain F21A	Parent strain (ATCC 8661/M240)	M243 (IFO no. 1545/ S-22)	Strain F21A	Parent strain (ATCC 8661/M240)	M243 (IFO no. 1545/ S-22)	Strain F21A	Parent strain (ATCC 8661/M240)	M243 (IFO no. 1545/ S-22)
Malate synthase ($\mu\text{mol}/\text{mg}$ protein/min)	0.051 (48 hrs)	0.039 (48 hrs)	0.061 (24 hrs)	1.88 (48 hrs)	1.48 (24 hrs)	2.57 (24 hrs)	2.38 (24 hrs)	1.50 (24 hrs)	1.63 (72 hrs)
Isocitrate lyase ($\mu\text{mol}/\text{mg}$ protein/min)	0.041 (24 hrs)	0.041 (24 hrs)	0.095 (24 hrs)	0.98 (72 hrs)	0.90 (48 hrs)	1.38 (48 hrs)	1.50 (48 hrs)	1.25 (24 hrs)	1.17 (72 hrs)

3.6 Production of citric acid by *Yarrowia lipolytica* F21A at fermenter

level

As an initial stage to commercialise this strain, fermentation process was carried out in fermenter as a means of scaling up the production of citric acid. Fermenter used in this study was the 2 liter bench-top glass fermenter (Bioflo, New Brunswick Scientific C. O. Inc., U. S. A.). The working volume employed for this study was 1 liter where 100 ml of seed culture was inoculated into the fermenter. The culture/medium was agitated at 300 rpm. The air flow rate was fixed at 1 liter/minute and internal temperature was set at 32°C. The flow rate of medium fed in, was fixed at 1ml/min.

From the results obtained in the shake flask system, it was anticipated that similar pattern of production of citric acid can be attained. Figure 31 and 32 indicate the results of citric acid production by strain F21A grown in fermenter on 2% and 4% NBD palm olein. From the previous data in shake flask system, the production of citric acid on 2% palm olein, showed a mark increase up to 10.8 g/l. However, this did not seem to apply to fermenter system. Analysis of supernatant on HPLC chromatograph did not show production even after 9 days incubation.

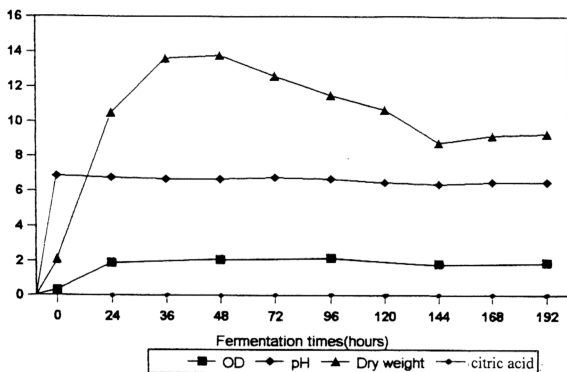


Figure 31: Growth and production of citric acid by *Yarrowia lipolytica*(F21A) on 2% NBD palm olein at fermenter level

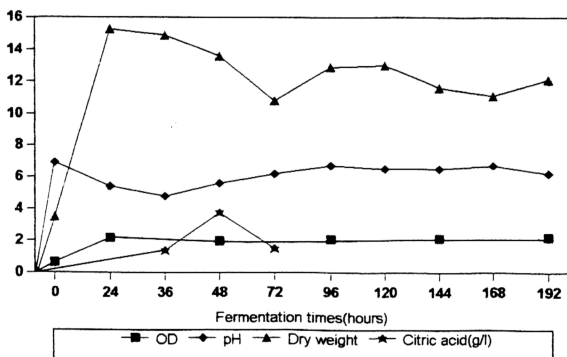


Figure 32: Growth and production of citric acid by *Yarrowia lipolytica*(F21A) on 4% NBD palm olein at fermenter level.

On 4% NBD palm olein citric acid was detected on day 2, 3 and 4 of incubation. however production was reduced to undetectable there after. Repeated experiment on 4% NBD palm olein showed similar results. Another interesting observation was fermentation in both 2% and 4% NBD palm olein did not yield appreciable amount of isocitric acid.

3.7 Genetic analysis of strain F21A

Mutation can affect any gene in an organism. The observed characteristics in the mutant can be due to several form of alterations in the genetic make-up of the organism. Strain F21A exhibited an altered genotype compared to M240. It was highly likely that the alteration was brought about by the mutation, a detailed genetic analysis was planned.

The first step was to find out the mating type of strain M240 and F21A. This was done by inducing the strains to undergo sporulation. If they were heterozygous diploid they should produce asci and the ascospores could be dissected and analysed. The results can be compared with the expected ratio such genotype should produced. If they are haploids, mating with the appropriate tester strains should produced heterozygous diploid, which could then be induced to undergo sporulation. Spore dissection and spore analysis can then indicate the nature of the mutation. Mating of homozygous diploid would create triploid, which will only show $\frac{1}{3}$ survival after spore dissection.

3.7.1 Screening for auxotrophic mutants

To facilitate the above procedure auxotrophic mutants were first isolated. Survivors from NTG treated logarithmic cultures were picked up and inoculated on YEPD, a total of 500 colonies for strain M240 and 480 colonies for strain F21A. Auxotrophic mutants were detected by their inability to grow on Minimal medium (Section 2.32.2). These colonies were further tested on 9 classification plates as mentioned in Section 2.32.3. Table 22 listed the mutants that were induced from strain M240 and F21A.

Table 22: Auxotrophic mutants of *Yarrowia lipolytica* derived from strain M240 (A) and its mutant F21A (B).

A.

Auxotrophic mutants derived from M240	Requirement
M24002	Leu
M24062	Leu
M24024	Leu his tyr
M24025	Leu tyr met
M24117	Leu his tyr ade
M24135	Leu tyr ura
M24165	Leu lys
M24360	Leu met

B.

Auxotrophic mutants derived from F21A	Requirement
FA213	Arg
FA281	Arg his

3.7.2 Induction of sporulation in auxotrophic derivatives of strain M240

and F21A

Sporulation was induced on YM medium for 3 days at 28°C (Ogrydziak *et al.*, 1978). Negative results were shown by all strains derived from M240 and F21A. The incubation was prolonged to 10 days unfortunately both strains did not yield any ascus. Repeated experiment still showed negative results. Potassium acetate medium which enhances sporulation in *S. cerevisiae* (Miller, 1989) was also experimented but no asci were formed.

3.7.3 Mating of auxotrophic derivative of M240 and F21A

Y. lipolytica auxotrophic mutants derived from strain M240 (ATCC 8661) and F21A were crossed two standard strains, under conditions described in section 2.32.4. Repeated crosses yielded negative results. Mating of ATCC 32338 A *ade1* and ATCC 32339 B *trp1* however showed growth on minimal medium which indicate the formation of diploids where the adenine and tryptophan requirements were complemented by the respective haploid strains. Exposure of this mating product to conditions where sporulation was induced, showed low level of ascus formation. This indicates the medium and protocol for mating were suitable. Due to the failure of strain F21A to sporulate and mate, genetic analysis by spore dissection was not feasible.

3.8 Improved citric acid production in *Yarrowia lipolytica* via DNA recombinant techniques

In *Saccharomyces cerevisiae*, recombinant techniques for cloning foreign genes into this yeast is very advanced (Morris and Roth, 1985). Considering fairly similar biological background of *S. cerevisiae* and *Y. lipolytica*, similar techniques for *S. cerevisiae* would be able to be adapted to *Y. lipolytica*. In fact a number of successful attempts have been reported (Gaillardin *et al.*, 1985; Davidow *et al.*, 1985). However, improved production of citric acid either in yeast or other fungi via DNA recombinant techniques has not been receiving much attention. In this study an attempt was made to develop a cloning system where any foreign genes can be introduced in *Y. lipolytica*. The ultimate aim is to clone genes involved in elevated citric acid production to improve commercial strains of *Y. lipolytica*.

3.8.1 Assessment of vectors to be used as cloning vehicles

There are a number of criteria that have to be considered in choosing the vector for the transformation. The plasmids in the pINA group seemed to be most suitable. They were kindly donated by Dr. B. Y. Treton, Institute de la Recherche Agronomique, Thiverval-Grignon, France. These plasmids were derivative from plasmid pBR322 and carries the *LEU2* gene of *Y. lipolytica*.

The *LEU2* gene, which codes for an enzyme (β -isopropylmalate dehydrogenase) in the leucine biosynthetic pathway in *Y. lipolytica*, can complement the *leuB* mutation in *E. coli*. These allow shuttling between the two microorganisms. Transformed *E. coli* can be easily selected on M9 plate (M9 plate acts as minimal medium of *E. coli*). The three available, plasmids pINA62,

pINA214 and pINA230 have also been shown to have high transformation rate and is stable once incorporated into the host genome (Gaillardin and Ribet, 1987; Treton, personal communication).

3.8.1.1 pINA62

Figure 7 shows pINA62, the first plasmid assessed. The 9.6 kb plasmid was derived from pBR322 which has been manipulated to facilitate the cloning process in yeast. It is an integrative plasmid which carry 5.25 kb of *Y. lipolytica* *LEU2* gene located at the *Sal*I site of pBR322. By introducing the *LEU2* marker at *Sal*I site, tetracycline resistance was deleted but the ampicillin resistance remains. Insertion of foreign gene was planned at the *Pst*I because this is the only site with the highest probability of insertion of foreign DNA into pINA62. The insertion of foreign DNA will also resulted in the ampicillin resistance gene to be inactivated. Selection in *E. coli* would be depending on the expression of the *LEU2* gene which means those colonies have plasmids with DNA inserts were able to grow on M9 plate (without leucine requirement) and sensitive to ampicillin.

Amplification was initially done in *E. coli* HB101. Selection in *E. coli* having this plasmid depends on resistance to ampicillin and showed good growth on M9 plate medium (Figure 33). It also showed a formation of yellow zone around the colony when grown on M9 containing 0.01% bromocresol green (BCG). Control plate showed no growth if medium were not supplemented with leucine. To confirm the integration of the plasmid into *E. coli*, small scale isolation of plasmid DNA was carried out as described in Section 2.35, and electrophoresed on an

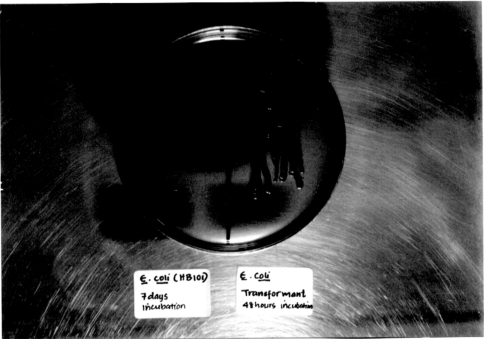


Figure 33: Growth of *Escherichia coli* transformant on M9 medium without leucine.

minigel. The plasmid DNA from *E. coli* strain HB101 was highly contaminated with chromosomal DNA (Figure 34) and the yield was fairly low. In order to obtain a better yield of plasmids, *E. coli* strain JM109 was used instead of HB101. As shown in the same Figure, the same amount of *E. coli* cells ($1.28-1.35 \times 10^8/\text{ml}$), strain JM109 harboring the plasmid pINA62 showed 3-5 times higher yield (10-15 $\mu\text{g}/\mu\text{l}$) compared to *E. coli* strain HB101 (3-5 $\mu\text{g}/\mu\text{l}$). Purification with GENECLEAN II (Bio101, Inc.) gave a distinct plasmid band on agarose gel as shown in Figure 35.

The purified plasmid was used in attempts to transform *leu*- mutant of *Y. lipolytica* strain derived from M240. In order to enhance the transformation rate the plasmid has to be linearised. This was done by cleavage at the *Nor1* site. This cut did not disturb the *LEU2* gene (Gallardin and Ribet, 1987). Among the *leu*-auxotrophs derived from M240, strain M24062, a non-reversible *leu*-derivative showed positive complementation with the *LEU2* gene in pINA62. Transformants grew very well on minimal medium. At the moment the yield of transformants were still fairly low at about 10 transformants per μg of plasmid (Table 23). Modification should be carried out to enhance the transformation rate.

3.8.1.2 Digestion of pINA62 with *Pst*I

In order to create an insertion point, pINA62 was digested with *Pst*I under conditions described in Section 2.42, unfortunately the plasmid was resistant to

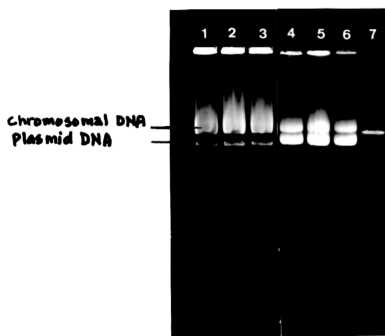


Figure 34.: Agarose (0.7%) gel electrophoresis of plasmid pINA62 extracted from *Escherichia coli* transformant.

Lane 1 to 3 : *E. coli* HB101

4 to 6 : *E. coli* JM109

7 : λ DNA *Hind* III/ *EcoR* I markers

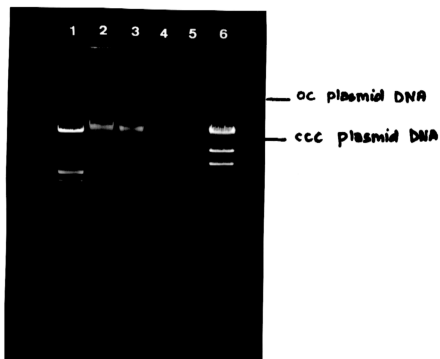


Figure 35: Agarose (0.7%) gel electrophoresis of chromosomal DNA and plasmid pINA62 extracted from *Escherichia coli* JM109 transformant.

- Lane 1 : λ DNA *Hind* III/ *EcoR* I markers
 2: Chromosomal DNA of *Yarrowia lipolytica*
 3: Chromosomal DNA of *Y. lipolytica* digested with *Pst* I
 4: Plasmid pINA62
 5: Plasmid pINA62 digested with *Not* I
 6 : λ DNA *Hind* III/ *EcoR* I markers

Table 23: Transformants obtained after transformation of *Yarrowia lipolytica* with pINA62. The cells were spread on minimal medium and incubated at 28°C for 48 hours.

Cell suspension	Dilution	Volume transferred (ml)	Average colony count per plate	CFU/ml
Before transformation (MM + leu)	10^{-4}	0.1	139	1.39×10^7
	10^{-4}	0.1	125	1.25×10^7
After transformation (MM)	10^0	0.1	1	10
	10^0	0.1	0	0

digestion by this enzyme. Increasing restriction endonuclease used and prolonging the time of incubation did not yield successful result. Other sites were not suitable such as insertion point at *Bam* HI because this site is the remainder of tetracycline marker where *LEU2* gene of *Y. lipolytica* was inserted. Although cut by *Bam* HI will not affect the expression of the *LEU* genes, detection of foreign DNA insertion into the plasmid would be impossible in *E. coli* because the characteristic of the plasmid would remain the same, unless hybridisation was carried out (which is time consuming). Insertion at *Pst* I would inactivated the ampicillin resistant genes. Transformed *E. coli* carrying an insert would lost resistance to ampicillin. Selection in *E. coli* would then be depended on the expression of the *LEU2* gene, which complement *leuB* mutation.

3.8.1.3 pINA214 and pINA230

Due to the failure to yield a suitable insertion point in pINA62, two other plasmids were screened as vectors. Figure 36 and 37 show the genetic map of pINA214 and pINA230. These vectors are both 6.7 kb long which carrying a shorter version of the *LEU2* marker and lacking the *NotI* site but allowing the selection in *E. coli* transformant by both ampicillin and tetracycline resistance. Both antibiotic resistance gene possess unique cloning sites for insertion of DNA fragments. For example pINA214 contains single site recognised by the restriction enzymes *Bam* HI, *Sph* I, *Sal* I, *Nru* I (at tetracycline gene) and *Sca* I, *Pvu* I and *Pst* I (at ampicillin gene). However, *Bam* HI could not be used because there were two other sites in this plasmid. Plasmid pINA230, contains similar single site recognised by the same restriction enzymes as in pINA214 but no extra *Bam* HI site. These two vectors (pINA214 and pINA230) are also low molecular weight vectors compared to pINA62, which is an advantage during plasmid preparation, because damages by shearing forces is reduced (Boffey, 1987). In addition, low molecular weight is usually accompanied by a high copy number (Gingold, 1993).

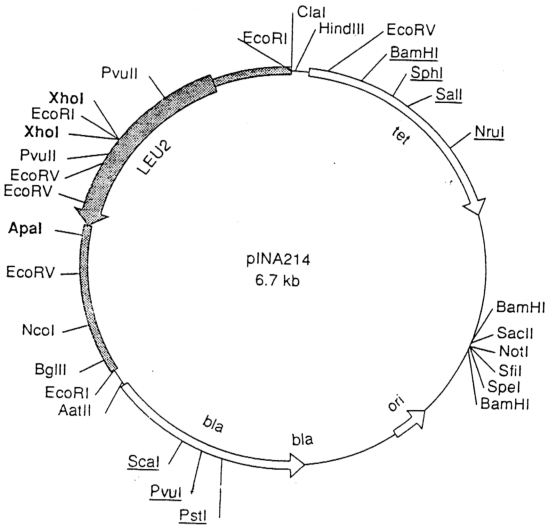


Figure 36 : Genetic map of plasmid pINA214.
(Source: Treton, personal communication)

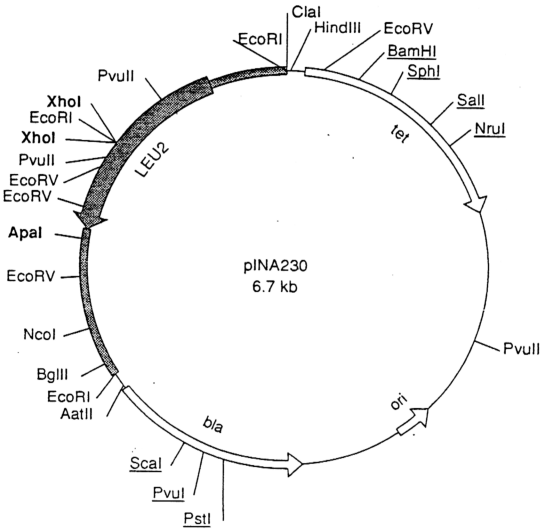


Figure 37 : Genetic map of plasmid pINA230.
(Source: Treton, personal communication)

Cleavage at the plasmid and inserting a compatible DNA fragment would alter the characteristics of the plasmid. For example, insertion of fragment into *Bam* HI site of pINA230 inactivates tetracycline resistance, but bacteria transformed with such a plasmid can still be detected because they are resistant to ampicillin. Therefore, those colonies that have plasmids with DNA inserts can be detected by testing for sensitivity to tetracycline. If insertion was carried out at *Pst* I site of pINA214 and pINA230, this inactivate the ampicillin resistance and allows selection of *E. coli* transformants by tetracycline resistance. The sensitivity to ampicillin indicates that DNA was inserted in the plasmid. Thus, after transformation of the *E. coli* with the ligation mixture three types of cells can be differentiated on the appropriate media, cells carrying the (religated) vector exhibited tetracycline resistance as well as ampicillin resistance; cells carrying the hybrid plasmid exhibits only ampicillin resistance because the tetracycline resistance is inactivated by the integration of foreign DNA; and cells carrying no plasmid that are sensitive to both antibiotics and grown on medium without ampicillin and tetracycline.

The identification of yeast transformants by both plasmids were selected on minimal medium. Those colonies that have plasmids can be easily selected on this medium. Since *LEU2* gene of pINA62 complemented the non-reversible *leu* derivative of M240, M24062 (Figure 38), it is therefore should be of no problem for pINA214 and pINA230 to complement this derivative, *leu* M24062 because these two plasmids were derived from pINA62.

To ensure that the plasmids pINA214 and pINA230 supplied do not have similar problem as pINA62 they were cut with *Bam* HI at the tetracycline site before amplification. Agarose gel electrophoresis showed a distinct band.

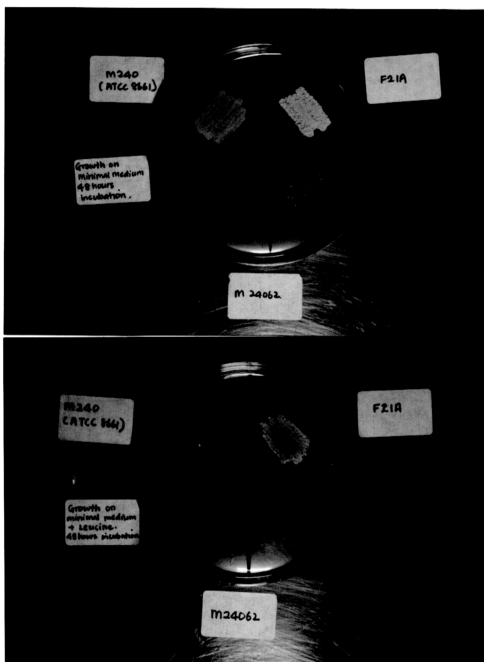


Figure 38: Growth of different strains *Yarrowia lipolytica*, strain M240, F21A and non-reversible *leu* strain M24062 on minimal medium after 48 hours incubation. A. Without leucine; B. With leucine.

Amplification of these plasmids was done both in *E. coli* strains HB101 and JM109. Plasmid isolation from *E. coli* HB101 was highly contaminated with chromosomal DNA and proteins and the yield was fairly low when compared to JM109. Subsequent amplification were carried out in JM109 to reduce contamination with chromosomal DNA, high molecular weight RNA and protein. Purification was also carried out using cesium chloride-ethidium bromide density gradient after alkaline lysis described in Section 3.36.

The most suitable site of insertion in the two plasmids is the *Bam* HI site. Insertion of foreign gene was planned at the *Bam* HI. *Bam* HI is a four base cutter (Boffey, 1987; Davis *et al.*, 1994). These restriction endonucleases cleave both strands of double-stranded DNA to generate a staggered end or new 5'-phosphate over hang (Davis *et al.*, 1994). Ideally, both vector and insert DNA should be cut with the same restriction enzyme however, some pairs of restriction enzymes that have different recognition sequences generate identical staggered ends that are compatible for ligation to each other. For example a *Bam* HI end can be ligated to *Mbo* I end; or *Bam* HI can be ligated to *Sau* 3AI end to generate a compatible sequences (Davis *et al.*, 1994). In fact *Mbo* I and *Sau* 3AI recognise the same site but both of these enzymes were obtained from different source of microorganisms (isoschizomers).

In this study, *Bam* HI of pINA230 was found most suitable to be ligated with *Sau* 3AI because pINA214 has 3 sites of cleavage for *Bam* HI. Other site in this plasmid was not favourable because complementation fragments to be inserted would be too large and too few for example *Pst* I at ampicillin site or *Nru* I at

tetracycline site. Although there was only a one site of cleavage on the plasmid but they were six base cutters comparing to *Bam* HI which was a four base cutter (Boffey, 1987).

3.8.1.4 Digestion of pINA230 with *Bam* HI

Purification of pINA230 was as described in Section 3.37. It was completely digested with *Bam* HI under conditions described in Section 2.42. To confirm that the reaction of the enzyme has gone to completion a sample (5 μ l) was electrophoresed on an agarose minigel (Figure 39). The digestion reaction was extracted with one volume of TE-saturated phenol-chloroform (Section 2.18.12) followed by another extraction with equal volume of chloroform-isomyl alcohol (Section 2.19.11). The upper aqueous phase was transferred to a fresh tube and was precipitated at -70°C for 15 minutes with two volumes of ethanol and one tenth volume of 3 M sodium acetate (Section 2.19.19). The DNA pellet obtained after centrifugation at 12,000 rpm at 0°C for 15 minutes was rinsed with 70% ethanol and dried in a vacuum desiccator. The DNA pellet was resuspended in 20 μ l of TE-buffer (Section 2.19.18) and the OD reading was measured as described in Section 2.39.

As given in Figure 39. Two bands were observed for undigested pINA230 (Lane 2 and 3). The upper bands showed open circular (OC) DNA and at the bottom were covalently closed circular (CCC) DNA. Whereas, only one band was observed for complete digestion of pINA230 with *Bam* HI (Lane 4 and 5).

3.8.2 Digestion of chromosomal DNA of *Yarrowia lipolytica* with

Sau 3AI

A digestion of chromosomal DNA of *Y. lipolytica* was performed according to the technique shown in Section 2.41. To confirm that the reaction has gone to completion a sample (5 µl) was electrophoresed on an agarose minigel. Figure 40 illustrates the results obtained from digestion. The digestion reaction was treated as described for pINA230 in Section 3.8.1.4 above before the cloning reaction was carried out.

3.8.3 Cloning of *Sau* 3AI-generated DNA fragments of *Yarrowia lipolytica* using *Bam* HI- digested pINA230 as vector

Sau 3AI-generated DNA fragments were ligated to *Bam* HI-digested pINA230 (Section 2.43). After transformation of the ligated mixture into competent *E. coli* (Section 2.34.1), ampicillin resistant transformants were obtained after 1 and 2 days of incubation at 37°C on LB plate incorporated with ampicillin (Table 24). The transformants obtained were further tested on LB plate incorporated with tetracycline, however all of them showed good growth on tetracycline which indicated that no fragment has been inserted. The procedure should be repeated in order to obtain the suitable recombinants. However, due to the time constraints further experiment was not able to be carried out.

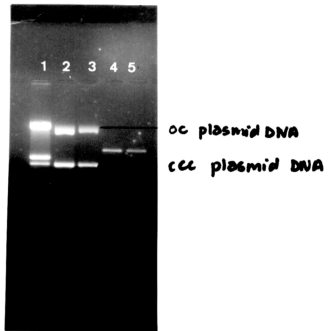


Figure 39: Agarose (0.7%) gel electrophoresis of plasmid pINA230 extracted from *Escherichia coli* JM109 transformant.

Lane 1 : λ DNA *Hind* III/ *Eco*R I markers

2 to 3 : pINA 230 undigested

4 to 5 : pINA230 digested with *Bam* HI

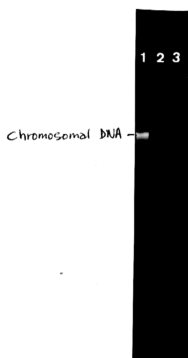


Figure 40: Agarose (0.7%) gel electrophoresis of chromosomal DNA *Yarrowia lipolytica* with *Sau* 3AI.

Lane 1 : 1 μ g of chromosomal DNA of *Y. lipolytica*.

2: 0.2 μ g of chromosomal DNA of *Y. lipolytica*.

3: 0.2 μ g of chromosomal DNA of *Y. lipolytica*.

Table 24: Transformants of *Escherichia coli* (HB101) after transformation with pINA230 (*Bam* HI-digested) and ligated with *Sau* 3AI-generated of *Y. lipolytica*.

Cell suspension	Dilution	Volume transferred (ml)	Average colony count per plate	CFU/ml
Before transformation (LB medium)	10^{-6}	0.1	6	6.0×10^8
	10^{-6}	0.1	8	8.0×10^8
After transformation with pINA230 (<i>Bam</i> HI-digested) ligated with <i>Sau</i> 3AI-generated fragments of <i>Y. lipolytica</i> (LB + ampicillin)	10^0	0.1	25	2.5×10
	10^0	0.1	19	1.9×10
	10^{-2}	0.1	0	0
	10^{-2}	0.1	0	0