DISCUSSION

CHAPTER 4

DISCUSSION

4.1 Isolation of mutants with altered ability to produce citric acid

In previous study, it was shown that *Yarrowia lipolytica* can grow well on 2% NBD palm olein as the main carbon source and produced citric acid (Ajam *et al.*, 1991). However, for industrial purposes the amount of citric acid produced was not cost effective. In attempts to enhance the production of citric acid on NBD palm olein, *Y. lipolytica* ATCC 8661 (Laboratory strains no. M240) (Tan and Gill, 1984) was subjected to mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine (NTG). The choice of this strain was due to its ability to produce lipases and the short lag phase for initiation of growth as shown by its growth in olive oil (Tan and Gill, 1984). These characteristics were desirable because the carbon source chosen for this project was palm olein. Furthermore in commercial process, it is obviously an advantage to have the lag phase as short as possible, for not only long lag phase is time wasting but extra medium is consumed in maintaining the culture viability prior to growth.

Akiyama *et al.* (1972 and 1973) showed that it was possible to obtain mutants with improved production of citric acid by selecting for monofluoroacetate (MFA) sensitive isolates and the inability of these isolates to utilise citrate for growth. MFA inhibits aconitate hydratase which catalyses the reversible isomerisation of citrate to isocitrate. The defect in the functioning of the

interconversion of citrate to isocitrate by this enzyme resulted in accumulation of citric acid (Akiyama *et al.*, 1972 and 1973; Gribble, 1973). Akiyama *et al.* (1972) isolated strain S-22 (IFO no. 1545/ Laboratory strains no. M243) which was sensitive to 0.1 % MFA. Besides that S-22 was also reported not able to grow or could only grow slightly on agar media containing di- or tricarboxylic acids as the sole of carbon source. Relative activities of aconitate hydratase (ACH) in the cells of mutant K-20 (first mutagenesis) and M243 (or S-22, second mutagenesis) were approximately 1/10 and 1/100 respectively compared to the parent strain (ATCC 20111/IFO no. 1437) (Akiyama *et al.*, 1973). These facts supported the statement that the mutant strains (both K-20 and S-22) were extremely sensitive to MFA (Akiyama *et al.*, 1973). Table 25 illustrates the results reported by Akiyama *et al.* (1973).

Table 25: The utilisation of carbon sources by *Yarrowia lipolytica* strains ATCC 20111, K-20 and S-22 (M243) on media containing di- or tricarboxylic acids.

Carbon source	Parent strain (ATCC 20111)	K-20	S-22
Glucose	+	+	+
n-Hexadecane	+	+	+
Acetate	+	+	+
Pyruvate	+	+	-
Lactate	+	±	-
Citrate	+	±	-
Isocitrate	+	-	-
α- ketoglutarate	+	-	-
Succinate	+	±	±
Malate	±	-	-
Fumarate	±	-	-
None	-	-	-

(Source: Akiyama *et al.*, 1973)

\+ Growth

± Slight growth

\- No growth

As shows in Table 25 strain M243 (S-22) was not able to grow on pyruvate, lactate, citrate, isocitrate, α-ketoglutarate, succinate, malate and fumarate. However, this strain grew well on glucose, n-hexadecane and acetate plate. From these findings, it was concluded that the low activity of ACH in the cells caused sensitivity of mutant strains to MFA and resulted in high productivity of citric acid and low productivity of isocitric acid. Due to low activity in ACH, strain M243 produced citric acid and isocitric acid in the ratio of about 97:3 in medium containing n-paraffins (Akiyama *et al.*, 1972 and 1973). However, in this study under the experiment conditions employed, it seems that M243 was able to tolerate up to 1.5 g/l MFA (0.15 %) and grow on citrate and a number of media containing intermediate of TCA as the carbon source, such as pyruvate and malate (Table 18). This implies the effect of mutation in M243 for deficiency in aconite hydratase is not so severe.

Working on similar assumptions that strains sensitive to MFA and unable to utilise citrate would produce high level of citric acid, strains which showed poor growth on citrate and 0.1% of MFA were selected. From 1100 colonies isolated after mutagenesis of M240, only 20 colonies showed poor growth on 0.1% of MFA and unable to grow well on citrate. No colonies showed complete sensitivity to 0.1% of MFA and inability to grow on citrate. Most colonies showed good growth on citrate as well as acetate. Similar observations have also been reported by Wojtatowicz *et al.* (1993), in their attempts to improve strain A-101 of *Y. lipolytica* for citric acid production on n-paraffins, the frequency of mutants of *Y. lipolytica* incapable of growing on citrate were very low and extremely unstable. The inability to isolate MFA-sensitive and low frequency of citrate negative mutants lead them to suggest that *Y. lipolytica* A-101 might not be a haploid strain. In this work similar

results were observed which might suggest similar situation. This was further supported by results from attempts to induce auxotrophic mutants in M240 to accomplish the genetic analysis. Due to inability to mate and sporulate, determination of ploidy in M240 was not possible.

In order to test whether the above characteristics were consistent with the expected results the 20 colonies isolated from mutagenesis of M240 were screened on minimal medium with 0.1% bromocresol. The diameter of the yellow halo around the colony would roughly indicate the amount of acids produced. An isolate, identified as F21 showed 53 % longer diameter compared to it parent strain M240. This was the strain with the larger halo among the 20 isolates selected (Figure 18).

Strain F21 were further tested on agar media containing intermediates of tricarboxylic acid. A number carbon source did not support good growth of F21 compared to its wild type M240 (Table 18). These facts indicated that the mutant strain might have an obstruction concerning the tricarboxylic acid metabolism as suggested by Akiyama *et al.* (1973) for their mutants. In their work, strain K-20 was unable to growth on isocitrate, α-ketoglutarate, malate and fumarate. Strain S-22 also showed the same results as K-20 with additional characteristics, which were the inability to growth on three other intermediates namely pyruvate, lactate, and citrate.

Detailed analysis of citric acid production of strain F21 was carried out by HPLC, however the result was disappointing. It did not indicate significant level of citric acid when compared to its parent strain (M240). Thus, the earlier assumption made was not quite correct. Despite its promising characteristics, strain F21 (6.3 g/l) did not improved its citric acid production on 2% NBD palm olein compared to the parent strain M240 (8.0 g/l) in fact it showed a reduction. The decrease in the

production of citric acid may due to the exposure to NTG. NTG is a very powerful mutagen and usually affected several sites of the DNA during replication. Additional mutations may have affected the production pathway of citric acid. Furthermore, selection for citric acid mutants was made on glucose medium. The change in carbon source to NBD palm olein probably altered the expression of certain genes involved in production and functioning of some of the enzymes involved in TCA cycle.

As mentioned earlier F21 showed poor growth on MFA, it may be considered sensitive but not completely negative. It tolerated up to 0.15% of MFA and this is considered more sensitive compared to strain M240 which can tolerate up to 0.2%. Theoretically, F21 showed desirable characteristic in term of MFA-sensitivity. The incomplete sensitivity allowed the continuity of TCA cycles, which would produce sufficient energy, and intermediates, which in turn ensure the flow of acetyl-CoA into the cycle to synthesis citric acid.

Due to the unsatisfactory of citric acid production by strain F21, a second mutagenesis was carried out on F21 leading to the isolation of F21A. Strain F21A was selected based on the same characteristics as strain F21. Strain F21A showed poor growth on 0.1% of MFA and citrate. On minimal medium incorporated with 0.1% bromocresol green, strain F21A showed at about 56% and 37% longer in diameter compared to strain M240 and F21 respectively. This definitely showed that more acids were being produced by strain F21A.

Evaluation of quantity of citric acid production during fermentation on 2% NBD palm olein showed that F21A has increased at about 25% in citric acid production (10.8 g/l) compared to M240 (8.0 g/l). For growth on media containing

tricarboxylic acid intermediates, strain F21A showed similar results as F21. It was not completely sensitive to MFA (0.15%). The colony morphology of F21A was slightly different. It was wrinkled and rather dry as compared to F21. This was probably due to the effects of mutations that occurred in the strain.

4.2 Growth and citric acid production on glucose and NBD palm olein

Palm oil is a cheap renewable resource and its availability in large volume make it an attractive substrate for fermentation process. Since Malaysia represents the largest producer of palm oil in the world, every effort is geared towards exploiting palm oil in fermentative process, one that shows promise is NBD palm olein as the carbon source in microbial fermentation.

The advantage of using NBD palm olein (oil) as compared to carbohydrate has been described by Bader *et al.* (1984). Energetically a typical oil contains about 2.4 time the energy of glucose on a per weight basis.

Several studies carried out recently supported the notion that palm oil is a better carbon source than carbohydrate in supporting cellular growth and metabolites production. Ho *et al.* (1984) established that *Streptomyces peucetius* var *caesius* (H3502) showed better growth and high anthracycline production when grown in palm oil compared to glucose. Palm oil was also found to be superior than glucose for growth and oxytetracycline production by *S. rimosus* (Ho *et al.*, 1994). By replacing lactose with palm oil, high penicillin production by *Penicillium chrysogenum* was obtained (Tan and Ho, 1991). Due to the success of palm oil as the carbon source in the production of valuable metabolites mention above, effort was made in attempts to produce citric acid by *Y. lipolytica* (M240) utilising NBD

palm olein as the carbon source. More available energy will enhance the cell proliferation and may in turn increase the production of citric acid. This can be seen when growth on 2% and 4% NBD palm olein produced more citric acid compared to cells grown in medium containing 2% glucose as the carbon source.

The choice of microorganism was due to the ability of this yeast to produce lipases. *Y. lipolytica* has been shown to produce two types of lipases; lipase I and lipase II (Ota *et al.*, 1973, 1978 and 1982). However, Zvyagintseva (1980) demonstrated that the enzymes were mainly present in the cell wall on the outer surface of the cytoplasmic membrane. Further studies by Ratledge and Tan (1990) reported that there were three forms of extracellular lipase activities: a constitutive cell-associated activity, an inducible cell-associated activity and an inducible cell-free activity as discussed in Section 1.22.

Ajam *et al.* (1991) reported on the conditions for growth of *Y. lipolytica* (M240) and production of citric acid on NBD palm olein as the carbon source. Different concentrations of NBD palm olein ranging from 1% and 10% were experimented. In their report 2% NBD palm olein was found to support optimum growth of the yeast and was able to produce 8.0 g/l of citric acid concomitant with high level of isocitric acid (6.2 g/l) as by product. Palm olein concentration greater than 2% decreased the yield of citric acid and concentration greater than 5% seemed to slow down the growth rate. A large amount of fats remained in the culture broth. These observations were also reported by Koh *et al.* (1983).

In agreement with the results observed by Ajam *et al.* (1991), strain F21A showed good growth in 2% NBD palm olein and able to produce high concentration of citric acid (10.8 g/l). In addition to above, F21A was also able to tolerate higher

concentration of NBD palm olein (4%) where the dry weight was increased but a slight reduction in citric acid production. The reduction of citric acid in 4% NBD palm olein was also shown by the two other strains, M240 and M243 where the dry weights were also reduced. The ability to grow on high concentration of oils was reported by Ikeno *et al.* (1975). A mutant of *Y. lipolytica* was able to tolerate 7% palm oil and produce 102 g/l of citric acid, however they did not report a detailed study of the strain involved. Information on such citric acid improvement would be very valuable in the construction of industrial strains of this yeast. In order to fill the gap, although F21A did not performed as efficient as the mutant reported by Ikeno *et al.* (1975) it was performing significantly much better than the parent strain thus, further characterisation of this mutant was carried out.

Strain F21A was grown in glucose as a comparison to NBD palm olein employed as the main carbon source. F21A showed good growth in glucose. Unlike *Saccharomyces cerevisiae*, growth of *Y. lipolytica* is strictly aerobic where citric acid is the main metabolite produced. Fukui and Tanaka (1981) reported that *Candida* yeasts have different metabolic features from those of *Saccharomyces* yeasts being utilised for the metabolic and physiological studies as typical eukaryotic microorganisms. *S. cerevisiae* produced ethanol when grown in high concentration of glucose. The most important pathway of sugar metabolism in yeast is the Embden-Meyerhof or glycolysis pathway (also known as the hexose diphosphate pathway). The pathway converts glucose to pyruvate, reducing two molecules of NAD^+ coenzyme to NADH and generating two molecules of ATP. The pyruvate formed is the key anabolic precursors and in aerobic conditions, it serves as substrate for oxidation. Pyruvate is further oxidised through oxidative

decarboxylation to form acetyl-CoA by multienzymes complex pyruvate dehydrogenase. Acetyl-CoA is oxidised *via* TCA cycle. TCA cycle thus represents a melting pot where catabolic and anabolic intermediates interconvert. This fact explains the necessity for reactions replenishing the cycle and the existence of reactions converting intermediates of the cycle for biosynthetic purposes (anabolic reactions). Two main anaplerotic routes exist, namely pyruvate and glyoxylate cycles that provides succinate and malate from acetyl- CoA. Theoretically, the TCA cycle is self-perpetuating, but the cycle must also supply intermediates for biosynthetic reactions and at the same time continuously provides energy, therefore synthesis of additional oxaloacetate is necessary to keep the TCA cycle functioning which occurs *via* the carboxylation of pyruvate. As Ratledge (1987) explained, oxaloacetate is also produced from the activity of the TCA cycle, the carboxylation of pyruvate must be regulated so that acetyl-CoA and oxaloacetate are always produced in equal amounts. This is achieved by the pyruvate carboxylase being dependent upon acetyl-CoA as a positive effector, which increases its activity, as more acetyl-CoA was available. As oxaloacetate and acetyl-CoA were removed to form citric acid, the concentration of acetyl-CoA will fall; pyruvate carboxylase will then slow down but, as pyruvate dehydrogenase still operates as before, more acetyl-CoA will be produced. In this way not only will citric acid synthesis always continue, but the two reactions leading to the precursors of citric acid will always be balanced (Ratlegde, 1987).

The glyoxylate cycle has two characteristic enzymes, namely isocitrate lyase and malate synthase which are located in peroxisomes. However, the glyoxylate enzymes were suppressed by the presence of glucose as shown by the enzyme assay

in glucose medium. Both enzymes were only induced when yeast was grown in NBD palm olein.

The first step in the utilisation of lipids before fatty acids are taken into the cells depends on the activity of lipases. Products of lipase activity are free fatty acids (3 moles) with glycerol (1 mole). Glycerol is utilised by the Embden-Meyerhof pathway. The fatty acids transported into the cells were activated to fatty acyl-CoA in peroxisomes (Figure 13B). Fatty acyl-CoA undergoes β-oxidation each turn of the cycle acetyl-CoA is lost and a new fatty acetyl-CoA with two carbon atoms fewer is formed, until the final products are 2 acetyl-CoA, or acetyl-CoA and propionyl-CoA, depending upon whether the original fatty acid has an even or an odd number of carbon atoms (Ratledge, 1987). Since the β-oxsidation system is exclusively localised in peroxisomes of yeast, acetyl-CoA required for the citrate synthesis in TCA cycle must be transported to mitochondria. Carnitine acetyltransferase in peroxisomes and mitochondria might be responsible for this transportation (Fukui and Tanaka, 1979 and 1981). This possible role of mitochondria and peroxisomes can be easily understood as illustrated by Figure 12.

Acetyl-CoA generated enters the TCA cycle. The first reaction is the condensation of acetyl-CoA and oxaloacetate catalyse by citrate synthase resulting in the formation of citric acid. Citric acid is key position in the intermediary metabolism where it has a multifunctional role in regulating both anabolic and catabolic pathway of cells (Scere, 1972). Apart from oxidation of citric acid, the TCA cycle also serves to supply intermediates required for numerous biosynthetic pathways (Evans and Ratledge, 1985). This may explain why citrate synthase activity had the highest activity in all strains compared to two other enzymes of the

TCA cycle, aconitate hydratase, and isocitrate dehydrogenase in medium containing glucose, 2% and 4% NBD palm olein. These observations were consistent with that of Ermakova *et al.* (1986) where *Y. lipolytica* was grown in medium containing glucose and hexadecane.

Obviously in utilisation of palm olein as substrate the production and activity of lipases are the main factor in substrate availability for β-oxidation supplying acetyl-CoA to TCA cycle. Comparison of the lipases produced in the 3 strains, F21A, M240 and M243 indicated this was indeed the case. The high citric acid producers, F21A, which can tolerate high concentration of palm olein concomitantly, exhibited higher extracellular lipase activity. These experiments were conducted twice and for each experiment duplicate samples were examined.

Comparing the activity of citrate synthase in the three strains, M240, M243 and F21A growing in palm olein, positive correlation was indicated between the activity of lipases and this enzyme. The highest activity of lipases in strain F21A was when it was grown in 4% NBD palm olein. Since citrate synthase activity was high in strain F21A growing in the same concentration of NBD palm olein, it was suggested that there is probably relationship between lipase activity with the activity of citrate synthase. This observation was further supported by strain M243 where citrate synthase and lipases activity was rather high in activity on 2% NBD palm olein. The highest activity in citrate synthase and lipases activity in strain F21A in higher concentration of NBD palm olein may explain why this strain was able to tolerate better growth compared to the two other strains which failed to perform. The increased level of lipases stimulates growth by the formation of acetyl-CoA, which undergoes condensation with oxaloacetate to form citric acid. The

availability of more acetyl-CoA would increase the citrate synthase activity not only to satisfy the functioning of **TCA cycle** but also **glyoxylate cycle**. This may also explain why citrate synthase activity was much higher in 2% and 4% NBD palm olein compared to glucose.

The citrate synthase activity in all strains was prominent between 24-48 hours. The activity of this enzyme decreased there after. This suggested that enhanced metabolism occurred resulting in accumulation of dry weight reaching maximum at about after 48 hours incubation.

Isomerisation of citric acid to isocitric acid is the next step of the TCA cycle to supply substrate for the first oxidation and reduction reaction. This is catalysed by aconitate hydratase (Figure 14). Although F21A showed poor growth on monofluoroacetate (MFA), aconitate hydratase (ACH) in this strain was not reduced significantly. As mentioned earlier for F21, F21A also showed desirable characteristics where MFA-sensitivity was not completely negative, this will maintained the supply of isocitrate for continuity of TCA cycles which would produce energy and the flow of intermediates at the desired level. In fact Tani *et al.* (1990) showed that a from MFA-resistant mutant of *Y. lipolytica* strain MA92 was able to produce in citric acid production treble the amount compared to its parent strain *Y. lipolytica sp.* Y-1. In their work no isocitric acid was detected in the culture broth. Tani *et al.*, (1990) explained this observation was probably due modification of fluoroacetate on the gene which resulted in low activity of ACH in strain *Y. lipolytica* MA92.

MFA administered to animals is enzymatically converted to monofluorocitrate (MFC) which causes a competitive inhibition of ACH activity

which catalyses the conversion of citric acid to isocitrate acid and inhibition results in the accumulation of citric acid in the animal tissue (Akiyama *et al.*, 1972 and 1973; Gribble, 1973). The effect of MFA on microorganisms, was reported in a *Vibrio* which excreted citric acid into the medium in the presence of the compound (Akiyama *et al.*, 1972). It was also reported that citric acid accumulation and ACH activity were affected by concentration of ferrous ion in the medium. Ferrous ion is well known to be an essential factor for the activity of ACH and played an important role in determining the ratio of citric and isocitric acid. The value required is extremely low, below 1 p.p.m was sufficient for enzyme activity (Akiyama *et al.*, 1972). However, it was impossible to attain this level in practice. The other choice was to find a mutant with low activity of ACH. Biochemical mechanism of citric acid accumulation by the traditional fermentation of molasses using *Aspergillus niger* was showed to have hardly any activity of ACH. Base on the assumption mutant strains having low ACH would produce more citric acid, Akiyama *et al.*, (1972) isolated strain S-22 that was MFA-sensitive.

In report presented by Akiyama *et al.* (1973), in the study of relationship between ACH and citric acid productivity in fluoroacetate-sensitive mutant strain of *Y. lipolytica* in n-paraffin (M243/S-22), activity of ACH was high at early stage of fermentation after 24 hours incubation. The ACH activity decreased there after and the production of citric acid began to increase. This was confirmed by strain M243 /SS-22. Works carried out in the present study indicated the activity of ACH was high at 24 hours of incubation and decreased to a very low level at day 5 of incubation. Low activity of ACH would accumulate high citric acid in the medium. This observation was also shown by strain F21A growing cells in 2% NBD palm

olein where ACH activity was high (0.24 µmol/mg protein/min) at day 1 of incubation. The decrease in activity of ACH at day 3 to 0.015 µmol/mg protein/min coincides with the highest in citric acid production. The same situation was also observed in strain M240 (0.19 µmol/mg protein/min)(day 2) where the activity of this enzyme was high. The data seemed to suggest that high activity of ACH at early stage of the growth is to fulfil high demand in the functioning of **TCA cycle** and **glyoxylate cycle** when cells were growing **actively in oil**. The two enzymes involved in glyoxylate cycle, which are localised in peroxisomes; isocitrate lyase and malate synthase were high in activity at 24 to 48 hours of incubation. This may indicate that the peroxisomes were well developed in this yeast. However, no electron microcsopy examination was conducted in this study.

Isocitrate lyase converted isocitrate to glyoxylate where isocitrate was supplied from TCA cycle in mitochondria. Condensed the glyoxylate with acetyl-CoA to form malate, malate dehydrogenase catalysed the oxidation of malate to oxaloacetate (Figure 12). The overall reaction of the glyoxylate cycle is the formation of oxaloacetate from 2 molecules acetyl-CoA (2 acetyl-CoA + $2NAD^+$ + FAD $\rightarrow$ oxaloacetate + 2CoASH + 2NADH + $FADH_2$ + $2H^+$). The formation of oxaloacetate is important during active growth to supply TCA cycles so that energy and intermediates can be continuously produced.

In 4% NBD palm olein the activity of ACH in strain F21A was highest on day 3 of incubation (0.19 µmol/mg protein/min) and remained high until day 5 of incubation (0.165 µmol/mg protein/min). The increase in activity of ACH in 4% NBD palm olein probably stimulated by the increase in activity of citrate synthase, which supplies citric acid, at the same time fulfilling the demand of the glyoxylate

cycle. This probably explains why excessive isocitric acid was produced when cells were growing in higher concentration palm olein. Maximum value of ACH attained in 4% NBD palm olein where the activity of this enzyme, was high until at day 5 of incubation, which explained the high production of isocitric achieved at this time. Besides the high ACH on day 5, the low NAD^+: ICDH also reached a low level (0.036 µmol/mg protein/min). At the same time isocitrate lyase in strain F21A decreased at day 5 of incubation to a very low level of 0.050 µmol/mg protein/min. The changes in the activities of the two latter enzymes further enhanced the accumulation of isocitric acid. The decrease in isocitrate lyase activity may be caused by depletion of growth requirements. Accumulation of isocitrate due to reduction in activity of isocitrate lyase has been observed by Matsuoka *et al.* (1980) where isocitrate lyase-deficient strains of *Y. lipolytica* grown in n-alkane accumulate more isocitrate then citric acid. Ermakova *et al.* (1986) also reported that isocitrate producer stains has high aconitate hydratase, low NAD^+ and NADP isocitrate dehydrogenase and very low isocitrate lyase activity. Marchal *et al.*, (1977) reported the accumulation of isocitric acid can also be attributed by the localisation of this acid in different compartments. Isocitric acid occurs in mitochondria, cytosol and peroxisomes. Whereas, citric acid is strictly in the mitochondria. Since ACH is absent cytosol, isocitric acid can be accumulated here in high concentration, which is then excreted into the medium. From the observation, it can be concluded that in strain F21A, the high availability of fatty acids due to high extracellular lipase activity when the cells were growing in 4% NBD palm olein increased growth and elevated TCA cycle. Furthermore, in the same time *Y. lipolytica* peroxisomes were formed when it was grown in oil (Fukui and Tanaka, 1981; Ermakova *et al.*, 1986),

this elevated the functioning of the glyoxylate cycle concomitant with TCA cycle, inducing high activity of isocitrate lyase and malate synthase activities. However, due to certain limitation isocitrate lyase and NAD^+: ICDH were reduced while ACH activity was still remained high on day 5 of incubation, which led to the accumulation of isocitric acid.

In both cases when all strains were grown on 2% and 4% NBD palm olein, malate synthase activity seems to be higher compared to isocitrate lyase. This was probably due to the excess of acetyl-CoA generated by ß-oxidation of fatty acids in the yeast cells which can enter the glyoxylate cycle directly through reaction with this enzyme in peroxisomes. In the study of isocitric and citric acid production in *Y. lipolytica* utilising n-alkane by Aiba (1986) it was shown, that enhancement of malate synthase activity could be accounted by an accumulation of intracellular inducer of acetyl-CoA. This is consistent with the data from this study where lipases activity was high when strains were grown in palm olein especially shown by strain F21A which has elevated level of lipases activity in 4% NBD palm olein.

4.3 Production of citric acid by *Yarrowia lipolytica* at fermenter level

In batch or shake flask fermentation of *Y. lipolytica*, a small population of yeast is inoculated into a suitable medium and growth of the cells starts immediately under non-limiting conditions. Once growth commences, an exponential increase in population density occurs; which is indicated by the increase in optical density and dry weight. However these increases reached a limit due to depletion of certain growth requirements or changes in pH . The cells entered stationary phase followed by lysis if conditions did not improve.

The energy and intermediates for biosynthesis during growth are largely supplied by the TCA cycle. As long as carbon substrates are available the cycle continue to fulfil the cell requirement.

Citric acid is the first intermediate of the cycle. This primary metabolite is produced during active metabolic activities. Ideally, for high citric acid production, the yeast should be producing excessive citric acid more that the requirement for production of energy and intermediates. This surplus then excreted into the medium, which can then be harvested. Working towards this goal, F21A has been indicated to have the potential to be employed as an industrial strain. The next step is to develop a system for large scale production. The work must now shift from batch culture to fermenter, so initially 1 liter fermenter was employed. Using 1 liter fermenter the nutrients are constantly supplied and equal fraction of the reaction mixture including cells and medium in which the medium is fed in and from which effluent is removed at the same rate, the steady state will be achieved. The formation of new biomass by the culture is balanced by the lost of cells from the fermenter. Therefore the yeast is always growing actively and citric acid as the first intermediate of the TCA cycle is produced continuously.

However, the change in conditions from batch culture to fermenter system altered the performance of strain F21A. In 2% palm olein, no production was detected using HPLC. Since growth occurred, all the citric acid must have been utilised by the yeast. This is also reflected by the dry weight and optical density, which was high, compared to shake flask. Only in 4% palm olein, the citric acid was detected. This was a good sign, because the yeast was not inhibited by high concentration of palm olein. Trilli (1986) suggested in wider sense, scale-up is the

study and exploitation of the effects of fermentation scale on the various fermentation parameters both physical and biology. In his report the scaling-up fermentation affects a number of physical and biological parameters, altering the capabilities of yeast to produce citric acid.

In the study of the importance of citric acid in the metabolism of oleaginous yeasts, the intracellular and extracellular concentrations of this metabolite have been extensively monitored in batch, transition and continuous. By growing *Candida curvata* in batch fermentation (Boulton and Ratledge, 1983a), it was found that citric acid was rapidly transported out of the cells into the culture medium soon after exhaustion of nitrogen in the medium. Citric acid accumulated intracellularly for first 40 hours growth, then decreased steadily as the cellular lipid content increased. Similar fluctuations in citric acid concentration were also seen in chemostate transition experiments using *Lipomyces starkeyi* it was shown as the culture become progressively nitrogen-limited, citrate was excreted from the cells accompanied by marked increase in the intracellular citric acid content (Boulton and Ratlegde, 1983b). Under the steady-state condition of continuos culture citrate was detected only at the lower dilution rates. No citrate could be detected in cell or medium when the growth rate was increased above $0.1h^{-1}$ (Boulton and Ratledge, 1984).

The above observations were interpreted by the authors as the differences in metabolic activity in the relevant cellular compartments. The observation was during the early growth stages where an unimpaired tricarboxylic cycle ensures that all mitochondrial citrate is rapidly oxidised. However, the onset of nitrogen limitation appears to provide the metabolic signal necessary to promote the accumulation intracellular citrate. The citrate must then leave the mitochondria and

accumulated in the cytosol prior to its excretion from the cell. Further experiment by Evans, Scragg and Ratledge (1983) showed that cytosolic citrate metabolism, at the time of excretion from the cell, was considerably lower than the rate of citrate efflux from the mitochondria It was found that after 40 hours growth, only small amount of citrate was expelled from the cells and was accompanied by steady decrease in the intracellular citrate pool.

The excretion of citric acid is always related to nitrogen limitation. The decreased in nitrogen concentration would also affect the AMP level thus inhibits the NAD^+:ICDH. This would lead to the accumulation of citric acid. Evans and Ratledge (1986) reported ATP inhibited NAD^+:ICDH activity, especially in oleaginous yeasts, which emphasised that the ATP: AMP ratio is probably the major regulatory parameter controlling the oxidation of citric acid in the TCA cycle. Since the study in this project was carried out using the continuous system nitrogen limitation did not occur therefore the citric acid did not accumulate as indicated by Boulton and Ratledge (1984) where the citric acid was only detected at low dilution rate.

Another important factor is the pattern of aeration and agitation, thus in turn affected the characteristics of the cells, their efficiency and productivity and their response to the conditions in the fermenter. Trilli (1986) reported in stirred fermenter cultures, the agitation system stirs and mixes the medium and enables the uptake oxygen, mainly by diffusion from the medium. In shake flask system, on the other hand, the cells seem to obtain oxygen largely by direct absorption from the air, as the culture swirls around the walls of the flask in a thin layer, enabling good growth to take place. Thus, shake flask fermentation provides much better growth

with high citric acid production. Calam (1986) reported the differences in agitation and aeration tend to create different metabolic pattern in shaken and stirred cultures, which in turn can cause the two systems to yield different amounts of the desired product. Attempts were made to increase the degree of mixing in the fermenter system, however this led to excessive foaming. Further experimental works should be carried out to overcome this problem and maximise the conditions in the fermenter system in order to attain the high yield of citric acid characteristic of this strain.

4.4 Mating type determination and genetic analysis

In most yeasts, mating occurs only between cells carrying different mating types. In *Y. lipolytica* the mating types are designated as A and B. This mating reaction is initiated by cell agglutination involving complementary glycoproteins located on cell surfaces (Crandall and Brock, 1968). The principal function of sexual mating is to increase the ploidy level (Wickerham and Burton, 1962). Once mating of haploid cells is successful the yeast become diploid and has heterologous at the mating type locus. These changes enable the yeast to undergo sporulation, which is regulated by environmental conditions. Sporulation involves the meiotic process which give rises to haploid in the form of ascuspores. Meiotic segregation allows the study of genetic recombination and chromosome segregation in the progeny. These could give information on the location of the mutant gene in respective chromosome.

In order to study the mutated gene (s) in F21A the strain has to be prepared for mating and sporulation depending out its ploidy. To facilitate mating,

auxotrophic mutants were required. These were obtained by mutagenesis and screening on selective plates. The auxotrophic requirements were useful markers for checking the mating process. However, under the experimental conditions employed in this study mating type of F21A and M240 could not be determined and genetic analysis was not possible.

Mating of the tester strains, ATCC 32338 A *ade* 1 and ATCC 323339 B *trp* 1 was successful, and exposure to conditions where sporulation was induced, showed low level of ascus formation. Herman (1971) and Bassel and Mortimer (1973) reported that *Y. lipolytica* exhibited low mating frequencies and low spore viability. Ogrydziak *et al.* (1978) reported low mating frequency is a major obstacle to the rapid development of *Y. lipolytica* genetics. All crosses must be forced using complementary genetic markers. Therefore, complementation analysis, scoring of mating type and scoring involving complementation is quite laborious. However, in his work an extensive program of inbreeding has led to greatly increased spore viability from 15% to 85% as well as an increased number of four-spored asci.

As planned if F21A was haploid and mating between the auxotrophic derivatives of F21A and known tester strains was successful the diploid obtained would carry the mutated gene(s) in heterozygous condition. Tetrad analysis would give an expected 3:1 ratio of wild type and the mutant phenotype. Genetically three types of asci were produce (AA Aa aa). The relative frequencies of these three types of asci are dependent upon the location of the two genes with respect to each other or with respect to their centromeres if they are located on different chromosomes. The usual criterion of linkage is PD (Parental ditype) / NPD (Non-parental ditype) > 1.

The statistical significance of this test has been discussed by Perkins (1953, cited by Beckerich *et al.*, 1984). If the two genes are located sufficiently close on the same chromosome, their distance D in centimorgans can be expressed, according to the equation established by Perkins (1949, cited by Beckerich *et al.*, 1984), D = 100 (T + 6 NPD)/ 2 (PD + NPD + T). When the two genes are located on different chromosomes, the ratios PD/NPD is close to unity, because the two centromeres segregate randomly at the first meiotic division. The proportion of tetratypes (T) is a function of the distance of each gene to its own centromere. If **x** and **y** represent the probability of segregation of each locus at the seconds meiotic division, the proportion of tetratypes will be f (T) = **x** + **y** - $^{3xy}/_{2}$. Probabilities **x** and **y** are estimates of distance of these genes to their centromeres.

If hybrids were obtained from the cross of a known haploid and diploid cells, the survival of the ascuspores were expected to be only $^{1}/_{3}$ of the total number of ascuspores. This was due to unstable duplex state (A/A/a) where $^{2}/_{3}$ of the population was unable to grow. A tetraploid duplex state of A/A/a/a segregates 4:0, 3:1 and 2:2 for phenotypic characteristic determined by the dominant allele and the recessive allele respectively.

4.5 Improvement of citric acid production in *Yarrowia lipolytica* using recombinant DNA techniques.

Research on yeast has been concentrated on *S. cerevisiae*. There is an extensive knowledge on almost every aspects of this yeast. It is thus not surprising that, this yeast was again the chosen eukaryote host when the DNA recombinant technology developed not too long ago. The significant understanding of its genetic

and molecular biology fulfilled the requirement for high-level expression of foreign proteins as well as the development of systems for regulated gene expression and the efficient secretion of foreign proteins. *Y. lipolytica* came into the picture when it was realised that this yeast has several other advantages compared to *S. cerevisiae*. *Y. lipolytica* is an industrially important yeast which can be grown on a number of substrates such as on hydrocarbons (Rohr *et al.*, 1983) palm oil (Ikeno *et al.*, 1975; Ajam *et al.*, 1991) and carbohydrate raw materials such as cane molasses, beet molasses and starch (Rohr *et al.*, 1983). Patents have been issued to protect its use for the production of various metabolites, such as 2-ketoglutaric acid, erythritol, mannitol or isopropylmalic acid (Gaillardin and Heslot, 1988). *Y. lipolytica* is also a natural producer of exocellular proteins and secretes an alkaline protease in gram per liter amounts. Yamada and Ogrydziak (1983) reported different strains of *Y. lipolytica* would produce various combinations of extracellular proteases. This includes the extracellular alkaline, acid and neutral proteases. Alkaline protease was widely used in detergents in 0.5% (w/w) which contribute 3% of an active enzyme in detergent (Best, 1985). Whereas, neutral protease is important in the hydrolysis of protein in brewing substrates. Acid protease has been reported by Best (1985) will cause the coagulation of milk and gives palatable cheese flavour. However, further hydrolysis of casein produces an unpalatable cheese.

The ability of *Y. lipolytica* to secrete extracellular proteases is in sharp contrast to *S. cerevisiae*, which does not secrete large proteins at high levels in its growth medium (Gaillardin and Heslot, 1985). Moreover, genetically engineered strains of the baker's yeast have proved by and large to be disappointing for the secretion of heterologous proteins, mostly because the secretion apparatus of this

yeast tends to be rapidly overloaded, when a secretable protein is overproduce (Gaillardin and Heslot, 1985). It therefore seems reasonable to investigate the potential of other yeast as an expression system.

An integrative transformation system was developed by Gaillardin *et al.* (1985) where standard procedure for transformation system based on the lithium acetate was adapted. Using this technique two genes have been cloned as selectable markers in *Y. lipolytica* (auxotrophic yeast). The two genes are *LYS5* which encoded saccharophin dehydrogenase (Xuan *et al.*, 1988) and the *LEU2* gene, which encoded isopropyl malate dehydrogenase (Gaillardin and Ribet, 1987). There are three other genes which were explored employing the DNA recombinant techniques, *XPR2* gene which resulted in reduced ability to produce extracellular protease (Davidow *et al.*, 1987), lipase gene (Ng, 1989) and isocitrate lyase gene (*ICL1*) (Barth and Scheuber, 1993).

Since it has been shown that it was possible to develop and employ the transformation system in this yeast it would be appropriate to attempts this technique in improving strains of *Y. lipolytica* for industrial production of citric acid. There is very little work that has been done in this area. To date, strain improvement employing this technique in *Y. lipolytica* has not been reported. Apart from providing a technique to improve commercial strains, this recombinant system would also provide a system where any genes of interest for example gene coding for useful novel protein to be inserted and express in the yeast. Fermentation would then be carried out using palm olein as the carbon source, giving variation to oil palm utilisation and at the same time producing useful products at a reduced cost.

As a working model, the mutation in F21A where enhanced citric acid was produced, was targeted as the gene to be cloned. The strategy for developing the transformation system was divided into three phases. **First** the expression of the cloned gene, has to be ensured. Since the gene whose mutation resulted in higher production of citric acid was derived from wild type strain of *Y. lipolytica* (M240), theoretically when reintroduced in appropriate recipient strain derivative of M240 should not pose any problem. **The second** strategy was to obtain an appropriate recipient strain which for the initial phase M240 will be utilised. Strain M240 was a prototroph, appropriate stable auxotrophs have to be selected. Selection based on antibiotic resistance could no be used in *Y. lipolytica* because most strains are naturally resistant to antibiotics commonly used such as chloramphenicol or G418 (Jamenez and Davies, 1980; Cohen *et al.*, 1980). Davis and Jamenez (1980) demonstrated that G418, an aminoglycoside antibiotic inhibited the growth of a wide range of prokaryotic and eukaryotic organisms. In fact, examination of all auxotrophs derivative of M240 on LB medium incorporated with ampicillin (50μg/ml) or tetracycline (30 μg/ml) showed good growth, which indicated that this strain of Y. *lipolytica* is resistant to these two antibiotics. **Thirdly**, we have to identify the gene from the donor (F21A) and deliver it to the recipient (M240).

A brief schematic of the method employed for cloning segments of foreign DNA into *Y. lipolytica* is shown in Figure 41. Before the experiments were conducted the vectors which will carry the foreign DNA as a passenger have to be identified. In this work, plasmid pINA62 was chosen (Figure 7). The 9.6 kb of plasmid pINA62 derived from pBR322, which has been manipulated to facilitate the cloning process in *Y. lipolytica*. It is an integrative plasmid, which carries 5.25 kb of

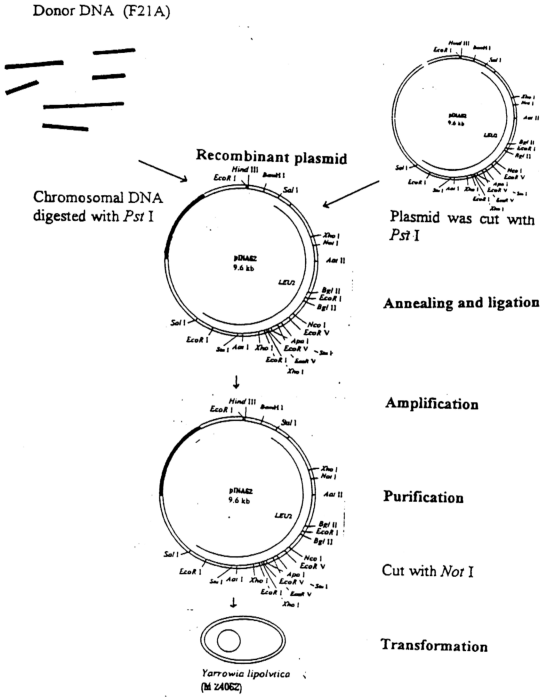

Figure 41: A method employed for cloning segments of foreign DNA into *Yarrowia lipolytica*.

Y. lipolytica LEU2 gene at the *Sal*I site of pBR322. By introducing the *LEU2* marker at *Sal*I site, tetracycline resistance has been deleted but the ampicillin resistance remains. The present of *LEU2* maker in the cloning vector and its expression in the *leu*$^-$ recipient auxotrophic mutant permits the identification of transformed cells. Since M240 was decided as the recipient cell, it has to have this complementary auxotrophic mutation. Mutagenesis using NTG was carried out on M240. Auxotrophic mutants requiring leucine were isolated. After several screening a non-reversible leucine requiring isolate was found that can be complemented by the *LEU2* gene in pINA62. Plasmid pINA62 was also chosen as a vector because it has been shown to have a high transformation rate and stable once incorporated into the host organism (Gaillardin and Ribet, 1987).

To achieve high volume of plasmid extract, amplification of pINA62 was done in *E. coli* JM109 instead *E. coli* HB101. The plasmid DNA from *E. coli* HB101 was highly contaminated with chromosomal DNA and the yield was fairly low (3-5 μg/μl) compared to same volume of *E. coli* JM109 (1.28-1.35 X 10^8 cells/ml)(10-15 μg/μl). Therefore in subsequent experiment, *E. coli* JM109 was used to amplified pINA62. The incorporation of the plasmid into *E. coli* was also confirmed on agarose gel. To purified the plasmid the agarose gel was placed on UV transluminator (Model TM-36 UV products Inc.) and the excised plasmids DNA band were transferred into 1.5 ml of microfuge tubes (Section 2.36.1). Purification was carried out with GENE CLEAN II (Bio. 101 Inc.) which resulted in a distinct plasmid band on agarose gel (Figure 35). The purified plasmid was then used in attempts to transform *Y. lipolytica* (M24062) derivative of M240 with the leucine requirement. In order to enhance the transformation rate the plasmid has to be

linearised. This was done by a cleavage at *Not*I site. This cut did not disturb the *LEU*2 gene (Gaillardin and Ribet, 1987). Although the yield of transformants was a bit low the results was very promising. Transformants obtained grew very well on minimal medium without leucine. Transference of transformants to new medium and further prolonged growth indicated that the transformants were very stable. This indicated that the plasmid has been integrated into the chromosomal DNA of the recipient strain and the *LEU*2 gene seemed to be well expressed.

The third step was to attempt to build a library containing the gene whose mutation resulted in higher of production of citric acid in F21A. The procedure was divided into three parts. First, the vector of pINA62 must be cleavaged at the chosen site to insert DNA fragments from F21A. The *Pst* I site was chosen for this purpose. The insertion of the foreign DNA would result in the ampicillin resistance factor to be inactivated. The lost of ampicillin would provide an indicating for selecting colonies with successful insertion of DNA fragments. However, the construction of gene library at *Pst* I could not progress because for some unknown reason of pINA62 was not amenable to cleavage despite several modification. A possible explanation was that the plasmid pINA62 has mutated, changing the sequence at the *Pst* I site which make the *Pst* I endonuclease unable to recognise the site.

Instead of this plasmid, two other plasmids, pINA214 and pINA230 carrying a shorter version of *LEU*2 marker were assessed for their suitability as a cloning vector. pINA214 and pINA230 were both 6.7 kb long lacking the *Not* I site but allowing the selection of *E. coli* transfomants by both ampicillin and tetracycline resistance. Therefore, the identification of recombinant plasmids expressed in *E. coli* will be lost of either marker. Cleavage at *Bam* HI site at tetracycline resistance

gene in pINA230 and inserting of a compatible DNA fragment would inactivate the tetracycline resistance. *E. coli* transformed with such a plasmid can still be detected because the ampicillin marker would form ampicillin resistant colonies, which can be selected on plates containing ampicillin. Those colonies that have plasmids with DNA inserts can be detected by testing for sensitivity to tetracycline. The transformants would lose the tetracycline resistance because the tetracycline gene would be inactivated by the insertion. Since *Bam* HI site was chosen for the purpose in cloning work, both plasmids supplied were examined to confirm that both of them have not mutated. They were cleavage with *Bam* HI at tetracycline gene site. The digestion of the plasmids were then confirmed on agarose gel which was shown as a distinct band.

Plasmid pINA230 was finally chosen in this work due to its unique and more workable site of *Bam* HI. *Bam* HI restriction endonuclease cleaved both strands of double-stranded DNA to generate new 5' phosphate and 3' hydroxy ends. The *Bam* HI-digested plasmid would result in a staggered double stranded DNA with an end containing 5' phosphate termini (Figure 42). Therefore, the donor chromosomal of *Y. lipolytica* must be digested completely with *Sau* 3AI to ensure that they have compatible ends. *Sau* 3AI recognises the same site as *Bam* HI (isoschizomers). These experiments were conducted according to the method of Ng (1989) (total digestion) which successfully isolated recombinant plasmid containing lipases gene.

It was observed that the digestion of chromosomal DNA of *Y. lipolytica* was successfully digested with *Sau* 3AI when the concentration of chromosomal DNA

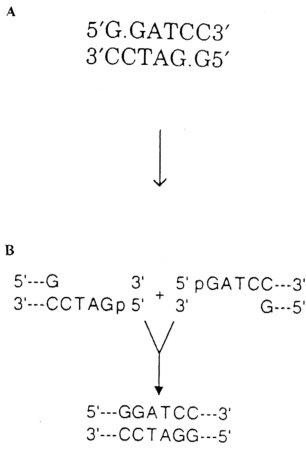

Figure 42: A. A cleavage of two strands of DNA results by restriction endonuclease *Bam* HI.
B. Ligation of identical cohesive *Bam* HI ends with T_4 DNA ligase. (Source: Davis *et al.*, 1994).

was not more than 0.20 μg/μl. Excessive amount would result in incomplete digestion. Under the UV transluminator the ethidium bromide-stained agarose gel of the digest showed as Figure 40. Lane 1 illustrated where the DNA was excessive. Prolonging the incubation period did not change the result.

For future work, it is advisable to do partial digestion of chromosomal DNA of *Y. lipolytica* with *Sau* 3AI to generate random segments of insert DNA. This is because the length of its target sequence determines the average size of DNA fragments generated by the action of a restriction enzyme. The frequency of any particular nucleotide sequence in a polynucleotide of random base composition is 1 in 4^n, where n is the number of bases in the sequence (Murray, 1987). Thus, a given tetranucleotide sequence would occur once in 256 base pairs example *Sau* 3AI, and specific hexanucleotide once in 4096 base pairs such as *Pst* I). Therefore, the chances to get more fragments and isolate a required gene were high using *Sau* 3AI target sequence with different digestion time (call partial digestion) compared to total digested of chromosomal DNA using *Pst* I.

Before reintroduction into *E. coli* cells, the final step in construction of a recombinant DNA molecule is the joining together of the vector and DNA fragment to be cloned. This process is referred to as ligation and conducted *in vitro* using T_4 DNA ligase. The recombinant plasmids resulted from the ligation were then used to transform *E. coli* (HB101). The screening for pINA230 recombinants was performed in the following way. After transformation the cells were plated on to ampicillin medium and incubated until colonies appear. All colonies will be transfomants, untransformed cells were ampicillin sensitive therefore would not grow, only a few of the transformants would contain recombinant pINA230 molecules, most would

contain the normal plasmid. To distinguish recombinants, the colonies were replica on to agar medium plate, which contained tetracycline where the recombinant was unable to grow due to the destruction of the tetracycline gene by the insert.

Ligation of complementary sticky ends such as *Bam* HI and *Sau* 3AI is much more efficient compared to two blunt ended fragments being joined together (Brown, 1990). This is because compatible sticky ends can base pair with one another by hydrogen bonding forming a relatively stable structure for the enzyme to work on. The phosphodiester bonds must be synthesised quickly, to prevent the sticky end circularisation. To increase ligation rate between the fragments and vector the proportion of used was two is to one respectively. However, different trend was obtained. Selection of transformants (Table 24) on LB incorporate with tetracycline did not yield any sensitive colony. This indicated that *Bam* HI digested plasmid pINA230 has a compatible termini which self-ligate and to form circular DNA without incorporation of any insert.

Davis *et al.* (1994) described during the ligation reaction of foreign DNA and plasmid, the plasmid has capacity to circularise and to form tandem oligomers. The failure to clone fragments from *Sau* 3AI-digested fragments, probably due to 5' phosphate termini of pINA230 that tend to ligate. It would have been an advantage to treat the digested vector with calf intestinal phosphatase, which remove the 5' phophates, inhibiting intramolecular circularisation and ligation (Figure 43). Phosphate treatment has been reported to be most efficient for 5' protruding termini, useful but less efficient on blunt termini and relatively inefficient on 3' protruding termini (Davis *et al.*, 1994). This treatment could not be carried out because of time

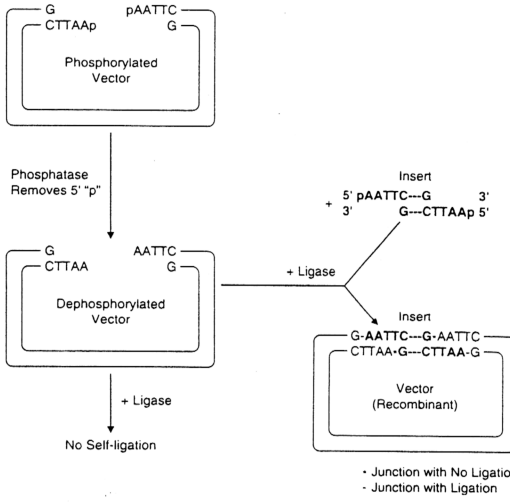

Figure 43: Ligation of fragment with compatible cohesive ends into plasmid vector treated with calf intestinal alkaline phosphatase. Phosphate-treated of the vector inhibits self-ligation and formation of the circles that do not contain an insert fragment. □ = covalent linkage; * = ends juxtaposed but not covalently linked.
(Source: Davis *et al.*, 1994).

constraint in this study. It was not possible to get the calf-intestinal phosphatase in time.

Although recombinant plasmids were unsuccessful to be isolated, the subsequent step was to identify the mutated gene in F21A. It was proposed that if the gene is expressed in *E. coli* selection on calcium carbonate plate (McKay *et. al.*, 1990) or bromocresol green should be sufficient to indicate the presence of the suitable fragment. This was based on formation of clear zone or yellow halo around excessive acid-producing colonies. However, initial screening indicated that *E. coli* (HB101) or JM109 showed clear zone on calcium carbonate plate and yellow halo on bromocresol green plate after 10 days incubation. These indicated that the *E. coli* itself produced acids during glucose fermentation. This was further confirmed by report of Barry *et al.*, (1970), *E. coli* produced a high level of lactic, succinic, acetic and formic acids when grown on media in containing glucose. All the acids would resulted on clear zone on calcium carbonate plate and yellow halo on bromocresol green plate.

The expression of the gene also resulted in higher ability to produce lipases. This feature can be utilised to identify the required fragment by zone clearing on medium containing tributyrin. Ng (1989) had successful shown that the lipase gene of *Y. lipolytica* was expressed in *E. coli.* A halo will be formed around the colony where the tributyrin is hydrolysed.