REGULATION OF VOLATILE FATTY ACID SYNTHESIS IN *MEGASPHAERA ELSDENII* AND HEXANOIC ACID UTILISATION BY *PSEUDOMONAS PUTIDA*

Submitted by

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DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree in any university and that to the best of my knowledge, contains no copy or paraphrase material published or written by any other person, except where due reference is made in the text of this thesis.

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This research thesis is dedicated to my beloved brother-in-law Professor Anwar-us-Salam Shaheed. May Allah (S.W.T) shower His blessings on him and Grant him Jannatul-Firdous (Ameen).

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PUBLICATIONS

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LIST OF SYMBOLS AND ABBREVIATIONS

The following abbreviations have been used throughout this thesis:

A_{600}	absorbance at 600 nm
AcCoA	acetyl coenzyme A
ANGIS	Australian National Genomic Information Services
APS	ammonium per sulphate
ATCC	American Type Culture Collection
BSA	bovine serum albumin
BUTY	*n*-butyric acid
C^{b}_{BUTY}	concentration of *n*-butyric acid in solution (broth) phase (includes both dissociated and non-dissociated forms), (mM)
C^{b}_{HEX}	concentration of *n*-hexanoic acid in solution (broth) phase (includes both dissociated and non-dissociated forms), (mM)
C^{e}_{BUTY}	effective concentration of *n*-butyric acid, (mM)
C^{e}_{HEX}	effective concentration of *n*-hexanoic acid, (mM)
cpm	counts per minute
CSL	Commonwealth serum laboratory
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
dNTPs	equimolar mixture of dATP, dCTP, dGTP, dTTP
DNA	deoxyribonucleic acid
DNS	3'4'-dinitrosalicylic acid
DPN	diphosphopyridine nucleotide
DTT	dithiothreitol

EDTA	ethylenediamine tetraacetic acid
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
HEX	*n*-hexanoic acid
MW	molecular weight markers
HPLC	high performance liquid chromatography
HS-CoA	Coenzyme A (reduced form)
ID	internal diameter
IPTG	isopropyl-β-D thiogalactopyranoside
kDa	kiloDaltons
kPa	kiloPascal
K_m	Michaelis-Menten constant
LacZ	*E. coli* gene for β-galactosidase
LDH	lactate dehydrogenase
ME	2-mercaptoethanol
MIC	minimum inhibitory concentration
m/z	mass/charge ratio
NCBI	National Centre for Biotechnology Information
PAGE	polyacrylamide gel electrophoresis
P_{BUTY}	productivity of *n*-butyric acid, (g/l.h)
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
P_{HEX}	productivity of *n*-hexanoic acid, (g/l.h)
PYG	peptone-yeast-glucose medium
PYL	peptone-yeast-lactate medium
S	glucose consumption, (g/l)
SDS	sodium dodecyl sulphate
T_h	harvest time, (h)

THBAG	Todd Hewitt Blood Agar supplemented with glucose
THBAL	Todd Hewitt Blood Agar supplemented with lactate
TPN	triphosphopyridine nucleotide
Tris	tris(hydroxymethyl)-aminomethane
UV	ultraviolet
V_b	broth volume, (l)
VFA	volatile fatty acids
V_{max}	maximum rate of reaction
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
$Y_{p/s}$	yield of *n*-hexanoic acid from glucose consumed (g *n*-hexanoic acid/g glucose)

Measurements

bp	base pairs
cm	centimetre
Ci	Curie
^oC	degree Celsius
dpm	disintegrations per minute
g	gram
>	greater than
h	hour
kb	kilobase pairs
kg	kilogram
<	less than
l	litre
μg	microgram
μl	microlitre
μm	micrometre
μM	micromole

mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
min	minute
M	molar
MW	molecular weight standards
ng	nanogram
nm	nanometer
N	normality
%	percentage
pmol	picomole
psi	pounds per square inch
rpm	revolutions per minute
s	second
SD	standard deviation
U	unit
V	volt
v/v	volume per volume
w/v	weight per volume
x g	times gravitational force

SUMMARY AND KEY FINDINGS

The research work described in this thesis has two studies on processes that may lead to adipic acid production through hexanoic acid synthesis pathway by the anaerobic bacterium *Megasphaera elsdenii* and its subsequent utilisation by *Pseudomonas* strains. The first part of the research described was concerned with the ability of *Pseudomonas putida* (syn *P. oleovorans*) to convert hexanoic acid to adipic acid. However, despite a sustained effort the research was unable to confirm the formation of adipic acid in these strains and this line of research was not pursued further. The research however, did show for the first time that these *Pseudomonas* strains could produce octanoic acid from *n*-octanol vapours in the presence and absence of *n*-hexanoic acid and has thus led to important new information.

The second line of research pursued and which constituted the major research effort was concerned with an investigation on regulation and production of the metabolic intermediates and end-products during glucose and lactate metabolism in *M. elsdenii*. A number of wild type strains were studied as well as mutants isolated as resistant to 3-fluoropyruvate were used to investigate end-product inhibition and the biochemical pathways used for fermentation. Small volume cultures and controlled fermenters were used to investigate the fatty acid end-products during glucose, lactate and mixed substrates and to optimise the effect of pH on growth and metabolism. An important finding was that the mutant ME5 produced relatively more valeric and hexanoic acid compared to its parental strain ATCC 25940 in mixed substrates. While in the presence of glucose, the proportion of valeric acid produced by strain ME5 was higher than seen for its parental strain ATCC 25940 and mutant ME7. Another important finding was that pH control improved the yield of acids compared with uncontrolled fermentation.

The work described in this thesis was also concerned with the recovery of fermentation products to obtain higher productivity and more efficient recovery of the products by adsorption to anion exchange resins. Initially, Amberlite IRA-93 (weak base anion-exchange resin) and Amberlite IRA-400 (strong base anion-exchange resin) resins were tested for their ability to adsorb and desorb butyric and hexanoic acids from water and peptone-yeast-glucose media. It was observed that both resins adsorbed more *n*-hexanoic acid than *n*-butyric acid, which suggested that the VFA chain length might affect the degree of adsorption. Both acids were desorbed more effectively by esterification from Amberlite IRA-93 resin. This indicated that Amberlite IRA-93 might have some attractive features above Amberlite IRA-400 in terms of product recovery in the form of esters. Further studies were carried out on the adsorption and desorption of both acids on the fermentation level in peptone-yeast-glucose media under pH controlled and uncontrolled pH conditions. Results indicated that using the Amberlite IRA-93 had an advantage in that it did not release a counter ion on binding of anions so that it exerts a buffering effect on the fermentation broth, thus reducing the degree of pH control needed and the amount of alkali used to maintain the optimum pH. The outcome of this finding is that better yields and better recovery of products can be achieved.

Another line of research was concerned with the key enzymes involved in the fermentation and the genes coding for them. The research detected pyruvate dehydrogenase (PDH) in strain ATCC 25940 for the first time and showed that the activity of this enzyme was significantly lower in the mutant ME5. However, lactate dehydrogenase (LDH) activity in ME5 was significantly higher compared with ME7 and the parent ATCC 25940. It was also shown that enzyme expression was highest in exponential growth. It was concluded that the relative changes in PDH and LDH in ME5 were responsible for the changes in carbon flow to acid end-products.

The final component of the research was concerned with a preliminary investigation of the molecular biology of the enzymes leading to the formation of hexanoic acid. The sequence of the short-chain acyl-CoA dehydrogenase (SCAD) in ATCC 25940 and mutants ME5 and ME7 were obtained and compared. However, no changes were seen which could explain the altered end-products. Likewise a minor change in the sequence of the electron-transferring flavoprotein was unlikely to affect the metabolic activity. An attempt to design PCR primers from related sequences of lactate dehydrogenase to investigate the LDH genes was unsuccessful.

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CHAPTER 1

1 LITERATURE REVIEW

1.1 Introduction

The development of biological processes that can produce vital industrial chemicals from renewable resources is of considerable long-term importance as many of these chemicals are currently made only from fossil fuels. The earth's reserves of fossil fuels are strictly finite and are being utilised at a rate well in excess of the earth's capacity to replace them. It is currently estimated that the earth's total economically recoverable oil reserves amount to approximately 1,700 billion barrels of oil. The earth's reserves of coal are six to eight times larger than the supplies of oil (The New Encyclopaedia Britannica, 1991), however, these reserves are also finite and the continued large-scale use of coal and other hydrocarbons raises a number of issues related to pollution, the environment, and the greenhouse effect. In order to ensure that the impact of the approaching depletion of fossil fuels reserves is kept to a minimum, the development of alternative renewable resources for the production of useful chemicals would be intelligent. Fossil fuels are burnt for energy and used in the production of drugs, petrochemicals, plastics, synthetic fibres, dyestuffs, lubricants and a wide range of other useful materials and industrial chemicals (McTigue *et al.*, 1986). Adipic acid (a six carbon di-carboxylic acid) is an example of a vital product from petrochemical sources that could be hypothetically produced from renewable resources. It is used in the manufacture of Nylon 6,6 a useful synthetic polyamide used in fibres and nylon-based plastics and is currently produced only from fossil fuels.

One of the most common bio-molecules available in the world is starch. Starch is a plant storage molecule that consists mostly of glucose in α(1,4)-linked D-glucose, with a smaller amount being in α(1,6)-linked D-glucose (Lehninger, 1986). Starch is the major component of many agricultural crops and these include most cereals, rice, tuber crops (such as potatoes and sago) and many others. The potential for using starch as a feedstock for the production of industrially useful chemicals is immense, but only if economic ways can be found to convert glucose in starch into industrially useful chemicals. Many bacteria are able to convert glucose (and other simple sugars) into a range of useful chemicals as by-products and end-products of their metabolism. The primary aim of this project was to demonstrate the conversion of glucose to adipic acid, a chemical feedstock of commercial importance used in the production of nylon. The production of adipic acid from starch is theoretically possible, and can be done in two principal stages. The first stage has three phases:

(i) the conversion of starch into glucose, probably using the enzyme amylase;

(ii) the microbial conversion of glucose into hexanoic acid in a fermentation system; and

(iii) the recovery and purification of hexanoic acid from the fermentation medium.

Some aspects of the conversion of glucose into hexanoic acid are described in Part B of this thesis.

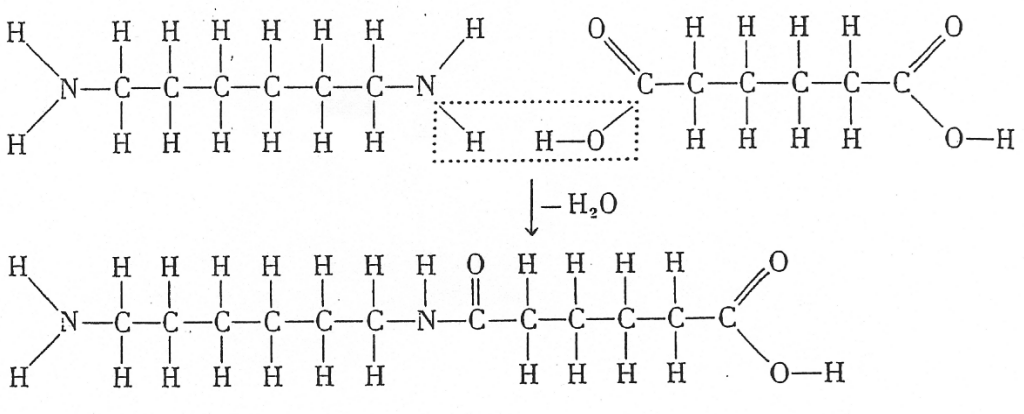
The second stage involves the microbial conversion of hexanoic acid into adipic acid, and/or the bioconversion of alkanes into corresponding alkanoic acids in the presence and absence of hexanoic acid, *via* a second bacterial fermentation system, using pseudomonads in particular. The potential conversion of hexanoic acid into adipic acid by *Pseudomonas* species is the subject of Part A of this thesis. The overall flow diagram for the major intermediates is shown below:

Starch $\rightarrow$ Glucose $\rightarrow$ Hexanoic Acid $\rightarrow$ Adipic Acid

Adipic acid can theoretically be produced from hexanoic acid, which is a product of anaerobic glucose metabolism by *Megasphaera elsdenii*. This bacterium converts glucose or maltose into acetic, butyric and hexanoic acids by serial condensation of C_2 units produced *via* their metabolism through the common intermediate, pyruvate. Yields of hexanoic acid are 5-8 g/l from 40 g/l glucose in stirred-batch fermentation with manual pH control (Britz and Wilkinson, 1984): there appears to be scope to increase these yields by product removal and fermentation optimisation (Roddick and Britz, 1986).

Adipic acid is currently produced mainly from petrochemicals. Nylon is formed *via* the conversion of adipic acid into adiponitrile then its polymerization of adipic acid and 1,6-hexadiamine in the presence of a small amount of heat to give a polyamide polymer as shown in Figure 1.1 (Sienko and Plane, 1961).

Figure 1.1: The formation of Nylon; the first step involves the condensation of adipic acid and 1, 6-hexadiamine to form a nylon dimer, followed by a series of reaction at either end of the molecule to give a giant nylon polymer.



It would be of considerable long-term economic benefit if the reagents for nylon manufacture could be produced from biological sources. This would ensure a continuation of the supply of nylon even when fossil fuels finally become depleted or too expensive as a substrate for this purpose. Few microorganisms are capable of producing adipic acid directly, and those that can do are usually inefficient and/or require the use of non-renewable substrates such as aliphatic hydrocarbons or

aromatic compounds, such as toluene (Hsish, 1984). Adipic acid production by a *Nocardia* strain uses aliphatic amines or diamines (e.g. dodecamethylenediamine) for the production of adipic acid at concentrations of 60 mg/l *via* an unspecified route (JP 58149687 [assignee Nissan Chemicals Industries Ltd]). The substrate is inappropriate for our purposes, the yields are low and the biochemistry/genetics of the system are not well advanced. The production of dicarboxylic acids by fermentation of yeast *Pichia carboniferus* converts straight-chain monocarboxylic acids, such as myristic acid, into dicarboxylic acids including adipic acid, which is produced at a yield of 200 mg/l from 2 g/l myristic acid (JP 82129694 [assignee Dainippon Ink and Chemicals, Inc.]).

In view of the need to develop an efficient process that uses renewable feedstock, the use of a two-stage process that involves the efficient production of each intermediate explains the necessity for the two-stage fermentation based on starch. The surge of recent biotechnical advances has increased interest in the potential application of bioconversion systems for the production of high volume chemicals such as adipic acid and other carboxylic acids and commercially established commodities. An overview of the various alternative ways in which adipic acid could be produced from renewable resources is covered in "Some Possibilities Concerning the Production of Adipic Acid from Renewable Sources" (Britz *et al.,* 1986). The remainder of the introduction provides a literature review relevant to the two parts of the proposed process studied in this thesis.

1.2 Part A

1.2.1 The conversion of hexanoic acid to adipic acid

1.2.1.1 Omega-oxidation (ω-oxidation)

The conversion of *n*-hexanoic acid to adipic acid can theoretically occur through a biochemical process called *omega*-oxidation and was first discovered in the urine samples by Verkade *et al*. (1932). A minor pathway for the oxidation of fatty acids involves oxidation of the terminal methyl, called the ω-carbon, or the adjacent methylene carbon of fatty acids by NADPH and molecular oxygen. ω-Oxidation is important for the metabolism of fatty acids with 6 to 10 carbons.

$$^{-}OOC\text{-}(CH_2)_8\text{-}CH_3 \xrightarrow{\omega\text{-oxidation}} {}^{-}OOC\text{-}(CH_2)_8\text{-}COO^{-}$$

The reaction occurs in three steps. The first is the hydroxylation of the ω-terminal carbon of a fatty acid carried out by cytochrome P-450 (Walter and Klaus, 1985). Oxidation of the hydroxyl group to an aldehyde, and then to a carboxyl group, follows and this completes the conversion of the monocarboxylic to the dicarboxylic acid in a mammalian system (Mitz and Heinrikson, 1961; Bjorkhem and Hamberg, 1971; Bjorkhem, 1972).

The initial work (McKenna and Coon, 1970) established that the ω-oxidation system of *P. oleovorans* enabled the organism to grow on C_6 to C_{14} *n*-alkanes as a sole source

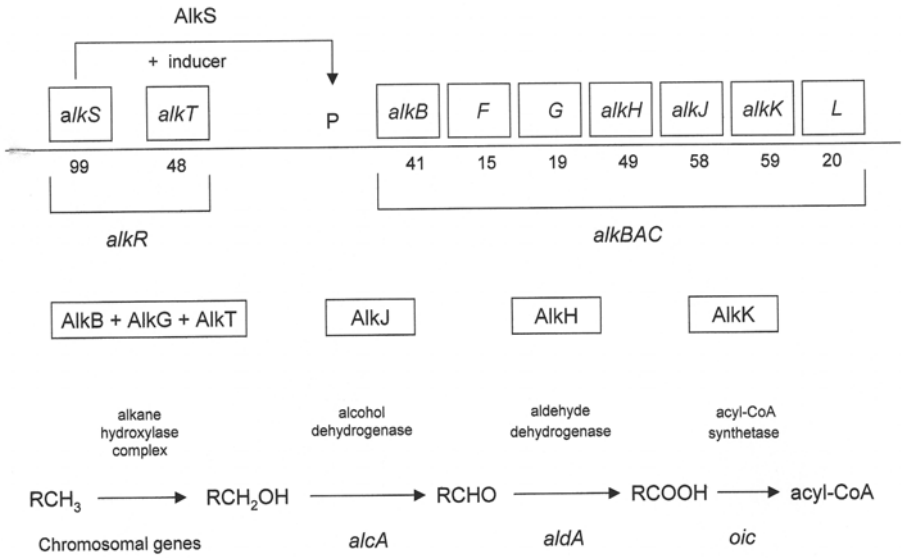
of energy. Any C_6-C_{14} substrate, alkane or alkanol, is suitable for induction of the *alk*-regulon, though octane is the strongest inducer (Mckenna and Coon, 1970; Grund *et al*., 1975). Aliphatic carboxylic acids are not inducers of the alkane oxidation system and alkanals induce only weakly. Compounds with sub-terminal hydroxyl groups act as strong inhibitors of induction (Grund *et al*., 1975). The entire ω-oxidation pathway is obligatory aerobic (May *et al*., 1973; May and Katopodis, 1990; Peterson, 1969; McKenna and Kallio, 1965), and directs the organisms to carboxylate the terminal methyl group of C_6-C_{12} mono-carboxy acids, giving rise to the corresponding dicarboxylic acids (McKenna and Coon, 1970). Unfortunately, these products are utilised as carbon and energy source by *P. oleovorans* through β-oxidation due to failure in blocking the β-oxidation pathway.

Whole cells of various *n*-alkane-degrading pseudomonads were found to ω-oxidize hexanoate and its corresponding ω-hydroxy- and aldehyde (6-oxohexanoate [6OxoHA]) intermediates into adipic acid (Kunz and Weimer, 1983). These findings agreed with a preliminary report (Thijsse, 1964) showing that alkane-induced cells of *Pseudomonas* species strain 473 produced traces of adipate from hexanoate when acrylate was present to inhibit β-oxidation (high energy yielding pathway that produces one $FADH_2$ and one ($NADH + H^+$) for every acetyl-CoA group removed from the fatty acid). The conversion of hexanoate, 6-hydroxyhexanoate (6OHHA) and 6OxoHA to adipic acid by ω-oxidation was also reported by a group working for du Pont (Kunz and Weimer, 1983), which agrees with previous results showing that cell extracts of *P. oleovorans* strain PpG6 catalyzed the conversion of octanoate, 8-hydroxy octanoate, decanoate, and laurate into the corresponding dicarboxylic acids (Kusunose *et al*., 1964). Hexane-induced cells of *P. oleovorans* strain PpG6 were reported to accumulate small amounts of adipic acid when incubated with hexanoic acid or 1,6-hexanediol (1,6-HAD) *via* ω-oxidation (Kunz and Weimer, 1983), suggesting that *n*-hexane induction of the primary alcohol dehydrogenase encoded on the OCT (Octane degrading) plasmid was at least involved in the initial step of this oxidation (Kunz and Weimer, 1983). High levels of adipic acid were not detected presumably due to its subsequent degradation by β-oxidation, evidence that suggests that ω-oxidation plays a physiological role in these bacteria (Kunz and Weimer, 1983).

1.2.1.2 The *alk*-regulon

The *alk*-regulon consists of two distinct regions, the *alkBFGHJKL* (earlier known as *alkBAC*) operon, which codes for structural components, and the *trans*-acting regulatory locus *alkST* (also called *alkR*) region, which encodes the positive regulatory functions required for the expression of *alkBFGHJKL* (Eggink *et al*., 1987a; Kok *et al*., 1989b; Eggink *et al*., 1988; 1990) as shown in Figure 1.2.

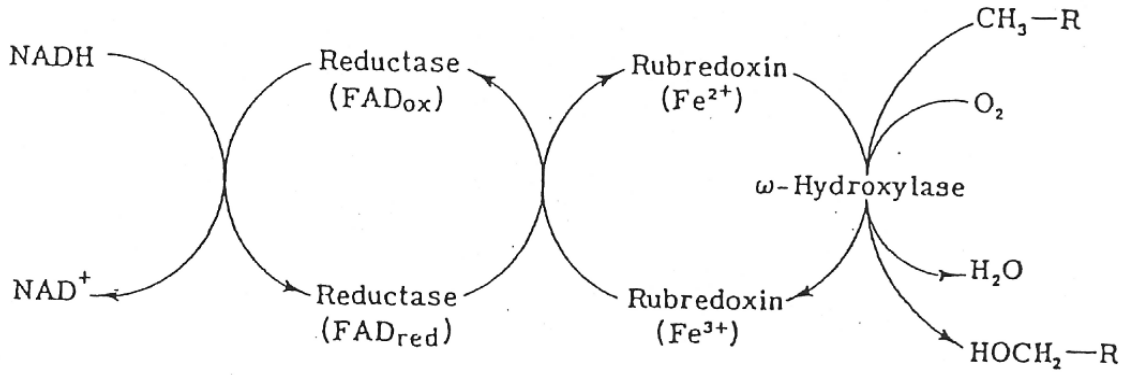
Figure 1.2: The genetic organization of the *alk*-regulon in *P. oleovorans* (Eggink *et al*., 1987a, 1990).



alkB =	membrane-bound monooxygenase	*alkF* =	rubredoxin 1
alkG =	rubredoxin 2	*alkH* =	aldehyde dehydrogenase
alkJ =	alcohol dehydrogenase	*alkT* =	rubredoxin reductase
alkS =	positive regulator of the *alk* system	*alkK* =	encodes acyl-CoA synthase
alkL =	function remains unknown	*alcA* =	codes for growth on alcohol
aldA =	codes for growth on aldehydes	*oic* =	codes for growth on straight chain fatty acids

The *alkBFGHJKL* genes are organized in a single 7.3-kb operon and encode seven polypeptides (Witholt *et al*., 1990; Eggink *et al*., 1987b; Kok *et al*., 1989a) *alkB-L,* that represent for most of the enzymes necessary to convert alkanes to fatty acids. The *alkST* locus consists of two cistrons of which the *alkS* (99 kDa protein) is responsible for inducer recognition and positively regulates the expression of *alkB-L* (Fennewald and Shapiro, 1977, 1979; Eggink *et al*., 1987a; Eggink *et al*., 1988). AlkT encodes rubredoxin reductase that is part of the alkane hydroxylase complex (Eggink *et al*., 1990; Ueda *et al*., 1972), which transfers electrons from NADH to rubredoxin (AlkG). Rubredoxin (AlkG, 19 KDa protein) (Kok *et al*., 1989a, 1989b; Peterson *et al*., 1966; Benson *et al*., 1977) is localized in the cytoplasm and is an iron-sulfur electron transfer protein. AlkB (41 KDa protein) (Kok *et al*., 1989a; Van Beilen *et al*., 1992a) is an integral cytoplasmic membrane protein usually referred to as alkane hydroxylase (Benson *et al*., 1977; Fish *et al*., 1983) or as ω-hydroxylase monooxygenase (McKenna *et al*., 1970; Ruettinger *et al*., 1977) that catalyses the oxidation of alkanes with one oxygen atom derived from molecular oxygen. The second oxygen atom is reduced with the electron transferred from NADH *via* rubredoxin reductase to rubredoxin (AlkG) (Lovenberg and Walker, 1978) (Figure 1.3).

Figure 1.3: Schematic representation of omega-hydroxylation reaction in *P. oleovorans* (Lovenberg and Walker, 1978).



The AlkH (49 KDa protein) gene encodes an NAD-dependent aldehyde dehydrogenase (Kok *et al*., 1989b), and AlkF (15 KDa protein) is a non-functional rubredoxin (Kok *et al*., 1989b). The distal part of the operon, earlier known as *alk*C (Owen *et al*., 1984), encodes three polypeptides (Eggink *et al*., 1987a), and designated AlkJ (58 KDa), AlkK (59 KDa) and AlkL (20 KDa) (Kok *et al*., 1989b). Expression of *alk* genes affects the membrane morphology, and over expression of *alkB*, the membrane component, can have major effects on cell function. This is particularly interesting because the *alkB* gene is transcribed from its native *Pseudomonas* promoter (Maarten *et al*., 1993), which has no homology to *E. coli* promoter (Kok *et al*., 1989a). The *alkBFGHJKL* and *alkST* operons encode all of the enzymes that allow *P. putida* (later called as *oleovorans* and in this thesis the names are used interchangeably to reflect the names used by author at the time of publication) to metabolize alkanes (Van Beilen *et al*., 1992b). Both operons have been cloned and sequenced (Kok *et al*., 1989a; 1989b; Eggink *et al*., 1990; Van Beilen *et al*., 1992b). Figure 1.2 illustrates the postulated structure of the *P. oleovorans alk*-regulon (Eggink *et al*., 1984; 1988; 1990; Benson *et al*., 1977; 1979; Ichihara, 1969; Van Beilen *et al*., 1992b).

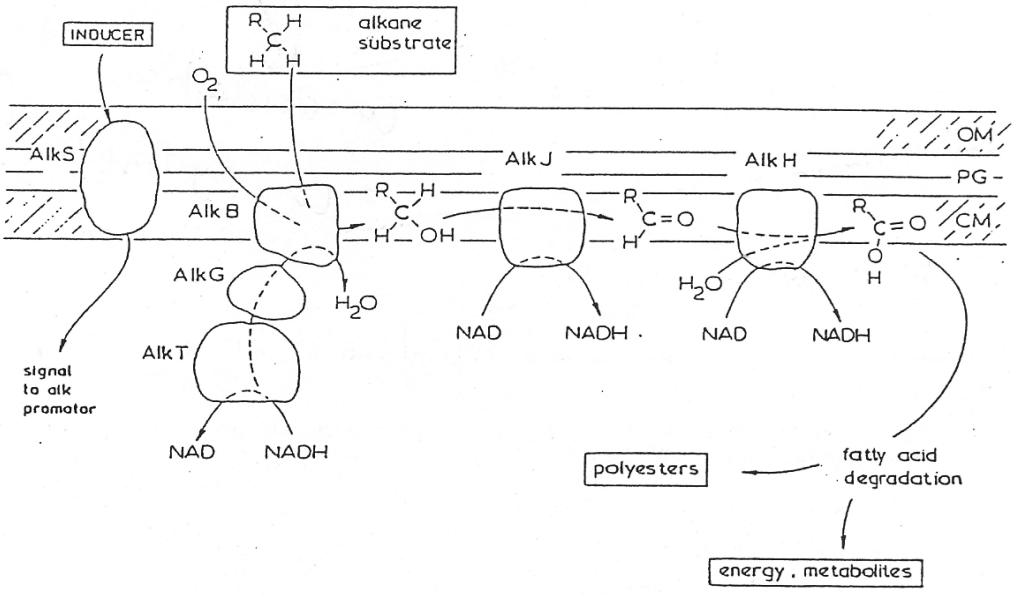
The enzymes responsible for the oxidation of *n*-octane to octanoic acid or beyond in *P. oleovorans* are octane inducible and are coded by genes borne on a transmissible extrachromosomal element (OCT-plasmid) encoded alkane hydroxylase (Chakrabarty *et al*., 1973). The DNA sequence determination and functional characterization of the OCT-plasmid-encoded *alk*JKL genes of *P. oleovorans* (Van Beilen *et al*., 1992b) provide the evidence for the role of the peptides in alkane oxidation. The G+C content of the *alk*JKL genes is 45%, identical to that of the *alk*BFGH genes, and significantly lower than the G+C content of the OCT-plasmid and *P. oleovorans* chromosome (Van Beilen *et al*., 1992a). Chakrabarty *et al*. (1973) suggested that the chromosome also carries genes coding octanol oxidation enzymes that, in contrast, are induced by octanol, not by octane. Grund *et al.* (1975) reported that the OCT plasmid codes for inducible alkane-hydroxylating and primary alcohol-dehydrogenating activities and that

the chromosome codes for constitutive oxidizing activities for primary alcohols, aliphatic aldehydes, and fatty acids. Grund *et al*., (1975) reported that the *alc*A locus is the chromosomal locus for octanol utilization and previously been called *ocl* or *ool* by Chakrabarty *et al*. (1973). The *alc*O locus is the plasmid locus, which permits growth on octanol in the presence of *alc*A⁻ mutation (Grund *et al*., 1975). The *alkBFGHJKL/alkST* (*alk*BAC/*alk*R) was the first *P. oleovorans* expression system to be described which is controlled identically in *Pseudomonas* and when cloned in *E. coli*. It was therefore an interesting candidate for a broad host range vector OCT or pLAFR1, with the added advantage of a well-regulated promoter, which can be induced with inexpensive aliphatic compounds such as octane. The cloned *alkBFGHJKL (alk*BAC) operon and the *alkST (alk*R) locus were used to construct several *alkBFGHJKL/alkST* (*alk*BAC/*alk*R) fusion plasmids, which are regulated and expressed very efficiently in both *E. coli* and *Pseudomonas* strains (Eggink *et al*., 1984; 1987a; 1987b).

1.2.1.3 Components of the alkane oxidation system

A model for the localization of the various gene products was described by Witholt *et al*.1990; Chen and Witholt (1995) and shown in Figure 1.4, which also shows the enzymatic reactions and cofactors associated with each of the alkane oxidation enzymes.

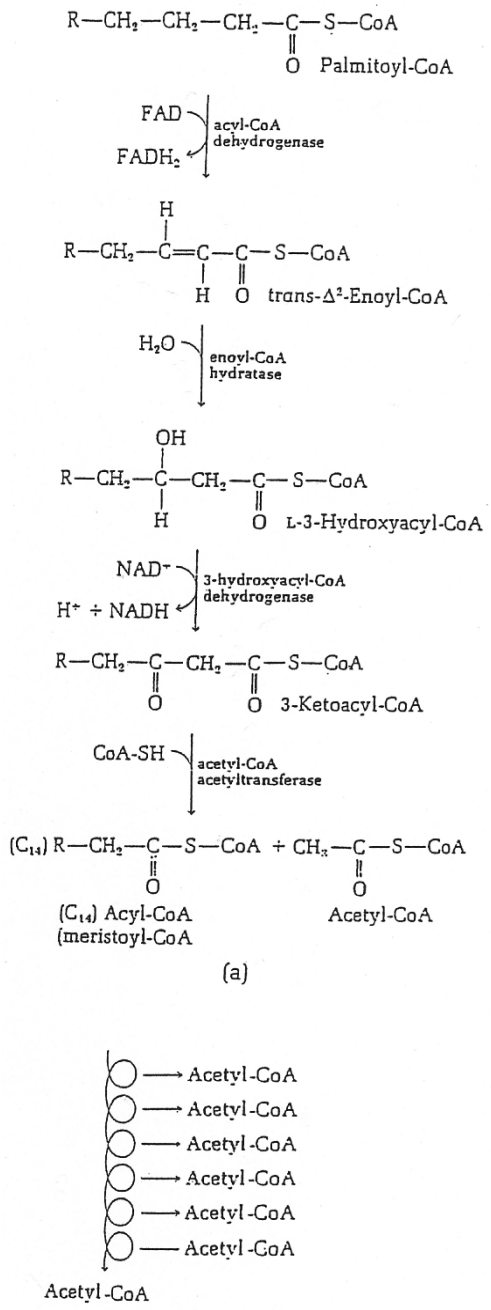
Figure 1.4: Model for the functions and cellular localization of alkane oxidation system in *P. oleovorans* (Witholt *et al*.1990; and Chen and Witholt, 1995).



OM, outer membrane; *PG,* peptidoglycan; *CM,* cytoplasmic membranene; *alkS,* positive regulator of the *alk* system; *alkB,* membrane-bound monooxygenase; *alkG,* rubredoxin; *alkT,* rubredoxin reductase; *alkJ,* alcohol dehydrogenase and *alkH,* aldehyde dehydrogenase.

The enzymes that convert *n*-alkanes to acyl coenzyme A are encoded by the *alk* genes, which are located on the OCT plasmid (Figure 1.2) (Eggink *et al*., 1987a; 1987b; 1988; 1990; Kok, 1988; Van Beilen *et al*., 1992b). The AlkB component of the alkane hydroxylase is a major membrane protein, which accounts for 25 to 30% of the total cytoplasmic membrane protein content of *P. putida* (also called as *oleovorans*) (Eggink *et al*., 1987a; Lageveen, 1986). The localization of AlkS, the positive regulator of the *alk* system, is speculative (Benson, 1979). When *alk*S encounters an inducer molecule (such as *n*-octane), it sends a second messenger (as yet unknown) that propagates a signal to the *alk* promoter, thus initiating transcription of the *alk* operon. The AlkK is an acyl-CoA synthetase, with specificity for medium-chain-length fatty acids as the other *alk*-system enzymes (Van Beilen *et al*., 1992b). There is approximately a ten-minute time lag between the cell being confronted with an alkane or alkanol and the full induction of the alkane hydroxylase complex. It has been determined that when the alkane oxidation system is fully expressed, as much as 9% of total cell protein is a product of the *alk*-regulon. The product of alkane oxidation by the hydroxylase complex, as shown in the Figure 1.4, is an alkanol (R-OH). The alkanol is then dehydrogenated by the 58 kDa NAD-dependent alkanol dehydrogenase, which dehydrogenates the substrate alkanol to form an aldehyde. In the final step of alkane oxidation, the terminal aldehyde group is converted into a carboxyl (R-COOH) group by the 49 kDa NAD-dependant membrane-bound aldehyde dehydrogenase. The fatty acid products made available to the cell *via alkane* oxidation enzymes are generally thought to enter cellular metabolism *via* the β-oxidation pathway (Chapman and Duggleby, 1967; Finnerty and Makula, 1975; McKenna and Kallio, 1965; Neider and Shapiro, 1975; Ratledge, 1978; Thijsse and Van der Linden, 1958; Van der Linden and Thijsse, 1965). The β-oxidation cycle (Figure 1.5) is a high-energy yielding pathway that produces one $FADH_2$ and one ($NADH + H^+$) for every acetyl-CoA group removed from the fatty acid by the enzyme acyl-CoA synthetase (Lehninger, 1986).

Figure 1.5: The fatty acid oxidation cycle (Lehninger, 1986).



This is a high-energy yield β-oxidation cycle and that it shows, as an example, the transformation of palmitic acid.

a) In one round of the cycle one acetyl residue is removed in the form of acetyl-CoA from the carboxyl end of palmitic acid (C_{16}), which enters as palmitoyl-CoA.

b) Six more rounds of the cycle take place, yielding seven more acetyl-CoAs, the seventh arising from the last 2-carbon atoms of the 6-carbon chain.

Note: $1FADH_2 = 2ATP$, $1NADH + H^+ = 3ATP$ (Lehninger, 1986).

1.2.2 Bioconversion of alkane into alkanoic acid

The alkane oxidation system of *P. oleovorans* is of interest in this respect because it converts medium-chain-length (C_6 – C_{12}) *n*-alkanes to fatty acids. Unfortunately these products are utilized as carbon and energy source by *P. oleovorans*, and so far not been able to find mutants which are blocked in the β-oxidation pathway. The genes enabling *P. oleovorans* to oxidize alkanes have been cloned into various *E. coli* strains (Favre-Bulle *et a*l., 1993). Since the β-oxidation system of *E. coli* is normally repressed and is not induced by medium-chain-length fatty acids, *E. coli* (*alk*$^+$) recombinants are potentially useful bioconversion strains which accumulates these fatty acids when the corresponding *n*-alkanes are present in the medium as a bulk apolar phase (Favre-Bulle and Witholt, 1992; Nunn, 1986). The recombinant *E. coli* [pGEc47] contains a plasmid with the alkane oxidation genes from *P. oleovorans* for the conversion of alkanes to the respective alkanoic acids (Favre-Bulle *et al*., 1991), which is able to convert octane to octanoate, and cannot oxidize octanoate further, when grown on defined medium with glucose as carbon source in batch and continuous culture (Rothen *et al*., 1998). The authors also reported that octanoate triggered changes in the composition of the cells, such as differences in fatty acid composition of the membranes seen on induction of alkane monooxygenase (Chen and Witholt, 1995; Nieboer *et al*., 1993). Carboxylic acids such as octanoic acid are well known to inhibit bacterial growth. Octanoic acid is a water-soluble product, which is toxic to bacteria at concentrations in excess of 15 or 20 mM, which clearly inhibited the growth of wild-type *E. coli* W3110 (no OCT plasmid) in batch cultures, and it affected the growth rate and the maximum cell density (Favre-Bulle *et al*., 1993).

1.3 Part B

1.3.1 Microbial production of *n*-hexanoic acid from glucose

Hexanoic acid is a six-carbon carboxylic acid i.e. $CH_3(CH_2)_4COOH$. Microorganisms that can make hexanoic acid are few in number, and species are mostly anaerobic bacteria. The advantage of using strictly anaerobic bacteria is that they have a highly efficient metabolism making them attractive candidates for biotechnological applications. Some of the strictly anaerobic bacteria that can produce *n*-hexanoic acid include *M. elsdenii, Eubacterium limosum, Clostridium scatolgenes* and *Clostridium ghoni* (Holdeman and Moore, 1975). *E. limosum* produces hexanoic acid as a minor by-product of the metabolism of C_1 compounds such as methanol, methane and carbon monoxide (Lindley *et al*., 1987; Pacaud *et al*., 1985; Zeikus, 1983) and was considered to be unsuitable for further investigation in this thesis. *M. elsdenii,* however, is fairly easy to work with, produces large amounts of *n*-hexanoic acid using simple carbohydrates as substrates and is relatively well studied when compared to the other two clostridia species. Hence *M. elsdenii* was the organism of choice and it has been the subject of preliminary investigations into the production of *n*-hexanoic

acid. It is firmly established in the litreature (Hino *et al*., 1991) that the major metabolic product of *M. elsdenii's* metabolism is *n*-hexanoic acid (Roddick and Britz, 1986). The organism was thus well suited for further investigation into the hyper-production of *n*-hexanoic acid. Hexanoic acid was known to inhibit further growth of *M. elsdenii* and production could be improved when the product was continually removed from culture media (Roddick and Britz, 1997). Using this approach, yields of 13 g/l of *n*-hexanoic acid have been achieved when loose beads of the ion-exchange resin Amberlite IRA-400, a strong anion exchanger that produces negatively charged counter ions, were placed in the growth medium. The components of the medium were being adsorbed onto the resin and OH^- ions were displaced and resulting the flux in pH of the culture (Roddick and Britz, 1997). It has also been found that by feeding glucose to immobilized cells in a fed-batch culture in the presence of the ion-exchange resin, Amberlite IRA-400, the production of *n*-hexanoic acid raised to an effective concentration up to 19 g/l with a 30% yield from glucose (Roddick and Britz, 1997).

1.3.2 *Megasphaera elsdenii*: taxonomy

M. elsdenii was isolated from the rumen of cattle in 1953 and was originally called organism LC; later its name was changed to *Peptostreptococcus elsdenii* in 1956 (Elsden and Lewis, 1953; Elsden *et al*., 1956). After doing a taxonomic survey of the organism (Rogosa *et al*., 1971), it was determined that *P. elsdenii* was significantly different from other members of the genus *Peptostreptococcus*. The organism was then reclassified into the new genus of *Megasphaera*, a genus that contained the only known species, *elsdenii* (Rogosa, 1971). *M. elsdenii* is a prominent rumen organism that has also been found in the human intestine and eye (Martin, 1994; Thiel and Schumacher, 1994). It occurs as pairs of diplococci, often arranged in such a fashion as to give the appearance of being in chains. It is a strictly anaerobic, non-motile, non-sporulating, Gram-variable macrococcus having a diameter of 2.4 to 2.6 μm and originally described as Gram-negative (Rogosa, 1971). It is surprising that *M. elsdenii* strains do not group with any of 200 Gram-negative eubacterial species investigated so far by 16S rRNA, however show a remote relationship to Gram-positive eubacteria of the '*Clostridium* Subdivision' (Stackebrandt *et al*., 1985). It grows between the temperatures of 25°C and 40°C with an optimum growth temperature of 37°C, the temperature of the rumen (Rogosa, 1971; Kreig and Holt, 1984). Its ability to utilize lactic acid is of major taxonomic importance and variation between rumen and pig strains in other characteristics does not appear to be very great (Vervaeke and Van Assche, 1975).

1.3.3 *Megasphaera elsdenii*: substrate preferences

The organism can utilize glucose, maltose, sucrose and lactate for growth in the medium. The utilization of sucrose is suppressed when either glucose or maltose is added but lactate does not inhibit sucrose utilization (Russell and Baldwin, 1978). Affinity of *M. elsdenii* for different substrates indicated that growth rate was lower for lactate than for glucose and was very low for maltose (Russell and Baldwin, 1979). The organism grows most rapidly upon glucose and maltose. Hence the substrate preferences of *M. elsdenii* are, in order; glucose, maltose, lactate and sucrose

(Russell and Baldwin, 1978; Russell *et al*., 1979). Generally if bacteria are exposed to glucose and a second sugar simultaneously, glucose is used preferentially and as a result growth is biphasic (diauxie) (Martin, 1987). Monod (1947) observed that glucose was the preferred sugar in enteric bacteria and he referred to this exclusion phenomenon as the "glucose effect". Later, Magasanik (1961) expressed the term "catabolite repression" to describe the phenomenon when it was discovered that substrates other than glucose could produce the same effect. Catabolite repression occurs at the level of transcription and is caused by the binding of a cyclic-AMP catabolite activating protein complex (CAP or CRP) to DNA (Martin, 1987). McGinnis and Paigen (1967, 1973) showed that substrate preferences could also be mediated at the level of transport. Two additional mechanisms have been proposed to explain sequential utilization not explained by catabolite repression. The first of these proposed mechanisms "catabolite inhibition," suggests that sequential utilization is mediated by an interference of preferred substrate with the enzymatic utilization of other substrates (Kundig *et al*., 1964; Kundig and Roseman, 1971). Catabolite repression, catabolite inhibition, and inducer exclusion (phosphotransferase system-mediated repression) collectively referred as "catabolite regulatory mechanisms" (Russell and Baldwin, 1978). Several ruminal bacteria (*Selenomonas ruminantium, Streptococcus bovis, Bacteroides ruminicola, M. elsdenii,* and *Butyrivibrio fibrisolvens*) exhibited substrate preferences or catabolite regulatory mechanisms when each organism was grown in batch culture (Russell and Baldwin, 1978).

In *M. elsdenii* B159, significant PEP-PTS (phosphoenolpyruvate [PEP]-dependent phosphotransferase system [PTS]) activity was observed (Martin, 1987). The lowest rates of glucose phosphorylation (either ATP- or PEP-mediated) were observed with *Bacteroides succinogenes* S85 and *M. elsdenii* B159, the species which grew the slowest, and it appeared that there was a general correlation between glucose phosphorylation and growth rate (Martin, 1987). The only report examining soluble carbohydrate transport in a ruminal bacterium indicated that glucose was transported by the PEP-PTS in *M. elsdenii* (Dills *et al*., 1981).

It has also been noted that the addition of acetate acts as a growth stimulator for *M. elsdenii* (Hino *et al*., 1991). In batch culture, addition of acetate stimulated the initial growth of *M. elsdenii* in medium containing glucose and trypticase, may indicate that acetate is not essential for the biosynthesis of cell components. As the initial concentration of acetate was increased, butyrate production increased. When acetate was used, the rate of butyrate production increased. Hydrogen production by the hydrogenase inhibitor carbon monoxide inhibited the growth of *M. elsdenii*, but this inhibition was cancelled by adding acetate (Hino *et al*., 1991). In continuous culture, where the growth rate was kept relatively low by limiting the glucose supply, adding acetate similarly increased butyrate production. It seems that acetate serves as an electron sink in *M. elsdenii* and electrons are consumed when acetate is metabolized to synthesize butyrate or caproate (Hino *et al*., 1991).

1.3.4 Hexanoate producing *M. elsdenii*: physiology

A major aspect of this project was to investigate the biochemical and genetic systems involved in *n*-hexanoic acid production. Roddick and Britz (1986) demonstrated that

as the concentration of both acetic and butyric acids increase in growth medium, the production of *n*-hexanoic acid increased. It is also known that if the pH of the growth medium is maintained around 7.0, the production of *n*-hexanoic acid is increased when compared to cultures where pH becomes more acidic during fermentation. Much higher yields of *n*-hexanoic acid were obtained in pH-regulated cultures and in ones in which *n*-hexanoic acid has been selectively removed from the culture medium by ion-exchange resins (Deguchi, 1994; Roddick and Britz, 1997). A potential difficulty is that *n*-hexanoic acid is toxic to the organism in high concentrations. Roddick and Britz (1986) reported that the degree of inhibition caused by acidic metabolic end-products increases with the number of carbons in the acid. Carboxylic acids can act as metabolic inhibitors because of their ability to interfere with the effective maintenance of proton gradients across the cellular membrane. Thus, makes the cell membrane increasingly permeable for longer chain carboxylic acids. This requires the cell to expend extra energy in maintaining proton gradients and hence reducing the amount of ATP formed by the cell from each molecule of glucose (Dawson, 1979; Foster and McLaughlin, 1974; Harold, 1986; Lehninger, 1986). These acids also interfere with substrate level phosphorylation and so further disrupt the cell's energy metabolism. Of its metabolic end-products, *n*-hexanoic acid causes the strongest inhibition of cellular metabolism (Roddick and Britz, 1986). Cell membranes are permeable to *n*-hexanoic acid, which means that the acid can freely diffuse into and out of the cell. Therefore, when high concentrations of *n*-hexanoic acid exist in the growth medium, similar concentrations will be found in the cell's cytoplasm (Walter and Gutknecht, 1984). There is a need to develop the mutants that have a high tolerance to *n*-hexanoate concentration in the fermentation medium. The resin was used to remove some of the *n*-hexanoic acid from the growth medium and so reduce the concentration of metabolically available *n*-hexanoic acid. Higher levels of *n*-hexanoic acid production need to be obtained, however, if economic viability is to be achieved.

1.3.4.1 Influence of carbon source

M. elsdenii is considered to be the predominant lactate-utilizing bacterium capable of fermenting up to 97% of rumen lactate. In addition to lactate fermentation *M. elsdenii* plays a major role in volatile fatty acids (VFA) production. Lactate utilization is not subject to catabolite regulation by glucose or maltose (Russell and Baldwin, 1978), and there was no inhibition of lactate transport when lactate-grown cells were incubated with excess glucose, sucrose or maltose (Waldrip and Martin, 1993). *M. elsdenii* NIAH 1102 utilized lactate in preference to glucose when both of them were present in the medium, even glucose grown cells switched substrate utilization from glucose to lactate (Hino *et al*., 1994). Major fermentation end-products could be manipulated by addition of glucose to lactate medium (Marounek *et al*., 1989). Hino and Kuroda (1993) have reported that *M. elsdenii* NIAH 1102 could not grow on acrylate; however, it could co-metabolize acrylate to propionate exclusively, while growing on glucose. The composition of fermentation end-products was not identical in media with glucose and lactate. The main metabolite of glucose in culture of *M.*

elsdenii was butyrate, whereas lactate was fermented to acetate and propionate (Marounek *et al*., 1989). The nutritional strategy of *M. elsdenii* is based on utilization of lactate and soluble products of polymer hydrolysis supplied by other bacteria (Marounek and Bartos, 1987). With increasing amounts of glucose to a medium with lactate, there was not only an increase in the production of butyrate and hexanoate but also of valerate, with a parallel decrease of propionate production (Marounek *et al*., 1989).

1.3.4.2 Nutrient transport and ionophores

Nutrient transport mechanism in most of the strains of *M. elsdenii* is poorly understood and therefore requires further research. Ionophores can inhibit substrate transport by disruption of cation gradients (Na^+, K^+) across bacterial membranes (Nicholls, 1982). However Waldrip and Martin (1993) observed that cations had little effect on lactate transport. *M. elsdenii*, a acid-tolerant ruminal bacteria allowed their intracellular pH to decline as a function of extracellular pH and did not generate a large pH gradient across the cell membrane until the extracellular pH was low as 5.2. This decline in intracellular pH prevented an accumulation of volatile fatty acid anion inside the cells (Russell, 1991; Miyazaki *et al*., 1991). Lactate transport was also inhibited (31%) by ATPase inhibitor N, N-dicyclohexylcarbodiimide (DCCD), however it is not known whether *M. elsdenii* possesses ATPase (Marounek *et al*., 1989). This suggests that protons may be involved in lactate uptake in *M. elsdenii*; the stimulation at low pH provides additional evidence that protons may be involved in the transport. Based on above findings, it appears that a proton motive force driven mechanism may be involved in lactate transport.

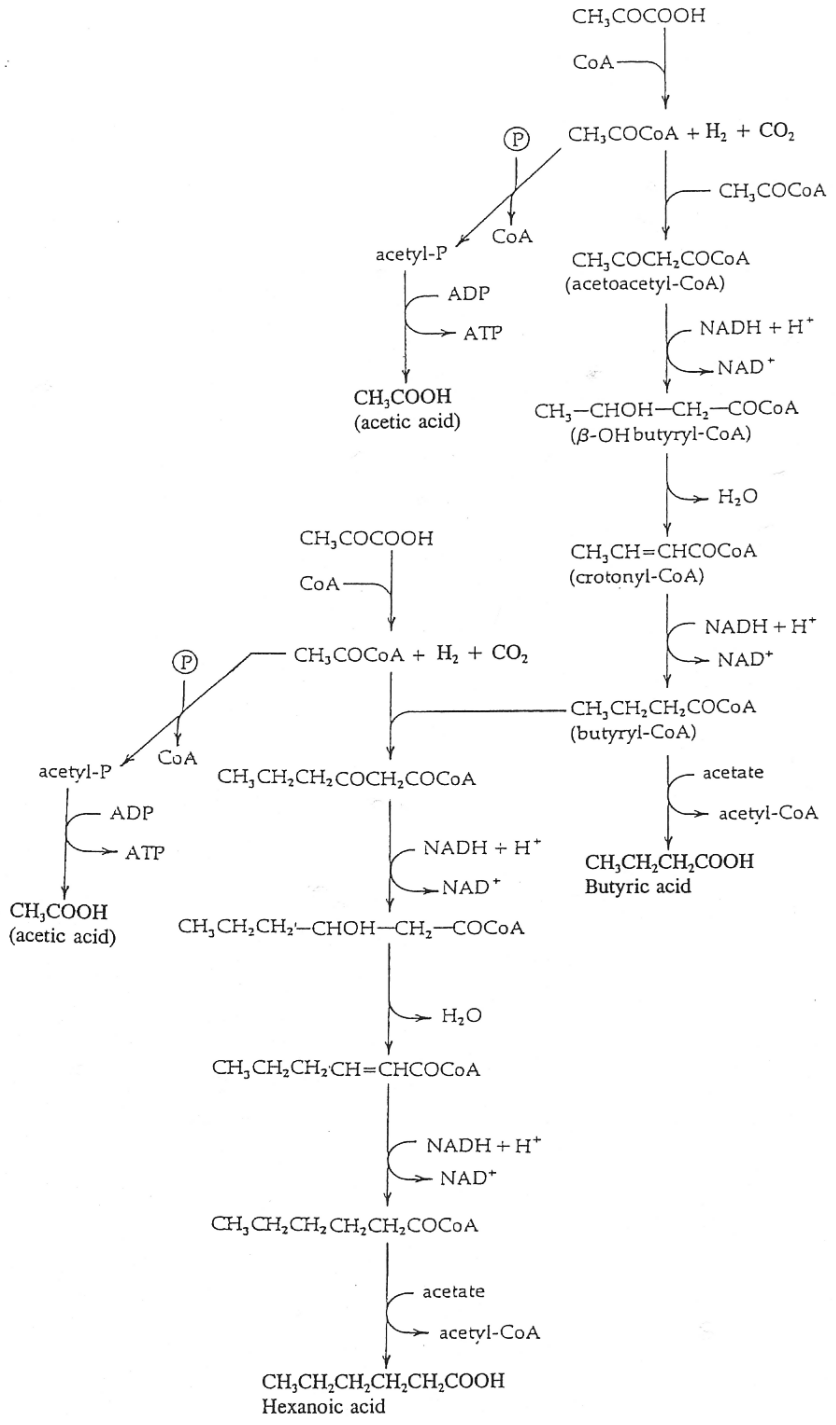
1.3.5 *Megasphaera elsdenii:* biochemistry

M. elsdenii has “a mixed acid metabolism”; as the bacterium converts its preferred substrates, glucose, maltose, lactate and sucrose into acetic, butyric and finally *n*-hexanoic acid *via* a series of condensations of acetyl-CoA derived from the metabolism of pyruvate to generate the extra reducing equivalents as $NADH_2$ (Pacaud *et al*., 1985; Roddick and Britz, 1986). The formation of the acetyl-CoA complex from the keto-acid pyruvate, and the reaction is effectively irreversible in physiological conditions (White *et al*., 1978).

$$CH_3\text{-}CO\text{-}COOH + NAD + CoA\text{-}SH + H_2O \rightarrow CH_3\text{-}CO\text{-}S\text{-}CoA + HCO_3 + NADH + H^+$$

The biochemical pathway shown in Figure 1.6 is speculative, as the specific biochemistry of pathway has not yet been extensively investigated. This is particularly true of the later part of the pathway, which involves the conversion of butyryl-CoA into *n*-hexanoic acid.

Figure 1.6: A biochemical pathway indicating the postulated steps that are involved in the production of butyric and hexanoic acid by *M. elsdenii*. The first part of the pathway, which involves the production of butyric acid *via* the condensation of two acetyl-CoA units, is probably correct, as the production of butyric acid in other bacteria has been examined, and the pathway determined. The second stage of the pathway, involves the production of *n*-hexanoic acid from butyric acid is hypothetical in nature (pathway partially derived from Stanier *et al*., 1984).



M. elsdenii is also capable of metabolizing lactate to propionate using the acrylate pathway (Ladd and Walker, 1965). Both acrylate and propionate are produced *via* the metabolic intermediate acrylyl-CoA. In the presence of 3-butynoic acid, acrylic acid is produced (Sanseverino *et al.*, 1989) and it is thought that the 3-butynoic acid acts as an inhibitor of the conversion of lactate to propionate *via* acrylyl-CoA, hence resulting in a cytosolic build up of acrylyl-CoA (see the hypothetical pathway below).

Lactate → Acrylyl-CoA → Propionate

↓

Acrylate

As the cell recycles its Co-A, it produces acrylic acid (Sanseverino *et al*., 1989).

1.3.5.1 Amino acid catabolism

M. elsdenii LC1 catabolized amino acid in an acid hydrolysate preferentially over enzymatic hydrolysate of casein. Further, threonine and serine were most actively degraded without any resultant increase in growth (Wallace, 1986). Also, more branched chain VFA were produced during stationary phase than during growth. It appears that amino acid catabolism is a major contributor to maintain energy rather than growth in *M. elsdenii.* It was reported that peptides are utilized preferentially over amino acids and could support growth. Increased in the production of amino acid 2-aminobutyrate was observed in *M. elsdenii* strains, grown on lactate based medium supplemented with threonine. However, serine, methionine or aspartate could not exhibit substantial increase, when replaced by threonine (Furtado *et al*., 1994; Soto *et al*., 1994). *M. elsdenii* is among poor ammonia producing strains of rumen bacteria (Cheng *et al*., 1988; Russell *et al*., 1988), this may be considered to be an advantage because amino acid deamination by rumen bacteria is nutritionally wasteful process that often yields more ammonia than can be used for microbial growth.

1.4 Lactic Acid Production

Lactate is an important end-product of bacterial fermentation of glucose and other carbohydrates. Lactic acid is formed *via* reduction of pyruvate by lactate dehydrogenase (LDH) for the regeneration of NAD^+ in lactic acid bacteria (Garvie, 1980). Homo-fermentative bacteria use the Embden Meyerhof pathway and in the final step convert pyruvate to lactate and regenerate nicotinamide adenine dinucleotide (NAD) from reduced nicotinamide adenine dinucleotide (NADH), which is formed at an earlier stage (Garvie, 1980). Interest in the production of L-(+)-lactic acid is presently growing in relation to its application in the synthesis of biodegradable polymer materials. In fact, lactic acid can be used for the synthesis of biodegradable polymeric materials and can be produced by microorganisms during fermentation. During typical lactic acid fermentation, the low pH (due to lactic acid production) has an inhibitory effect on the metabolic activities of the producing microbial cells (Porro *et al*., 1999). *M. elsdenii* posses an efficient lactate transport and metabolic machinery that can control lactic acidosis successfully (Kung and

Hession, 1995). *M. elsdenii* produced *n*-butyric and *n*-hexanoic acids were major fermentation products together with variable amounts of iso-butyric and iso-valeric acids. Production of *n*-valeric acid was enhanced by growth on sodium lactate. Lactic acid was formed in trace amounts by some strains of anaerobic streptococci; had characteristics similar to *M. elsdenii*, grown on glucose but apart from VFA no other soluble fermentation product was detected (Latham and Sharpe, 1979).

1.5 Sensitivity of Rumen Bacteria to End-Products and Antimetabolites

1.5.1 Antibiotics

Growth of *M. elsdenii* is inhibited by its end-products of glucose and lactate fermentation such as hexanoic and valeric acids, and in other anaerobic genera halogenated analogues of these are inhibitory. Flint *et al.,* (1988) reported the isolation of tetracycline-resistant strains from *M. elsdenii*. Studies on the resistance of rumen bacteria to antibiotics suggest that most of the common rumen species are sensitive to tetracycline (El Akkad and Hobson, 1966; Fulghum *et al*., 1968). Nagaraja and Taylor (1987) showed that *M. elsdenii* was totally resistant to eight antimicrobial feed additives. The susceptibility and resistance of *M. elsdenii* to thirteen other antibiotics have also been reported (El Akkad and Hobson, 1966; Wang *et al*., 1969). The report indicated that of five *M. elsdenii* isolates (Flint *et al.*, 1988), two had a plasmid of approximately 5-8 kilobase pairs (kbp) with a tetracycline (Tc) resistance marker.

1.5.2 Influence of halogen analogues of VFAs

3-Fluoropyruvate resistant mutants of *Clostridium acetobutylicum*, derived after nitrosoguanidine mutagenesis, accumulated more acetoin and lactate and could produce butanol in acidic pH, whereas fermentations of the wild-type ceased in the acidogenic range, and at the same time glucose uptake was increased (Junelles *et al*., 1987; El Kanouni *et al*., 1989). Fluoropyruvate sensitive mutants of *B. lactofermentum* exhibited a decrease in ratio of pyruvate dehydrogenase to pyruvate carboxylase, and therefore preferentially convert pyruvate to oxaloacetate (Satiawihardja *et al*., 1993). Pyruvate dehydrogenase complex of *E. coli* and L-lactate dehydrogenase of bakers yeast were also inactivated by fluoropyruvate (Urban, 1988; Flournoy, 1989). *M. elsdenii* mutants resistant to inhibition by 3-fluoropyruvic acids had altered fermentation profiles, which suggest that the flow of carbon *via* pyruvate as a key intermediate in intermediary metabolism has changed (Britz unpublished data). Acetate and butyrate halogen analogues have been used to select mutants of *C. acetobutylicum* resistant to these suicide co-substrates. Bromoacetate, choloroacetate, fluoroacetate, chlorobutyrate allowed the selection of acidogenic mutants but the strains selected with 2-bromobutyrate failed to produce acetone, whereas the synthesis of ethanol and butanol were unaffected (Junelles *et al*., 1987). The toxicity of fluoropyruvate perhaps depends on its ability to influence the pyruvate dehydrogenase complex and pyruvate carboxylase, which uses pyruvate as a substrate, so changes in these enzymes may be responsible for varied

product formation profiles. However, due to lack of genetic analysis, consistency in results and biochemical confirmation of the extent of inhibition, it would be difficult to define the basis and level of toxicity. Marounek *et al*. (1989) reported that *M. elsdenii* is relatively insensitive to many antimicrobial compounds. However, no reports on fluoropyruvate sensitivity were available in the literature searched on *M. elsdenii* and therefore part of this thesis was to study the effect of such analogues on *M. elsdenii* and mutants resistant to this.

1.6 End-Product Recovery

A wide range of pharmaceuticals and chemicals can be produced by fermentation. However, the accumulation of product in the fermentation broth inhibits its further production. The effects of product accumulation in the broth can be reduced by continuously removing product as it forms (Roffler *et al*., 1984). It is necessary to develop a means of continuously removing *n*-hexanoic acid from the growth medium during continuous fermentation. The economics of continuous fermentation process are more favorable than for batch systems, and if the product can be continuously removed from the growth medium, end-product inhibition can be avoided (Playne and Smith, 1983; Groot *et al*., 1984a, b). Various potential continuous extraction processes exist including, solvent extraction using a non-toxic organic solvent, pervaporation, or replaceable ion exchange resins, in a cartridge format. A major problem with the use of organic solvents in continuous extraction system is that they can inhibit bacterial growth by being toxic to the organisms, causing cell-to-cell aggregation at the liquid-liquid interface of the organic solvent and the growth medium (Playne and Smith, 1983). If a replaceable, reusable ion-exchange resin cartridge were used, the resin would have to preferentially bind *n*-hexanoic acid and be non-toxic to the organism. The literature also indicates the ion-exchange resin Amberlite IRA-400 could be used for this purpose (Roddick and Britz, 1986). Studies of bacterial physiology have provided new insights into the mechanisms of end-product inhibition and these may lead to reduced manufacturing costs for fermentation products such as ethanol, butanol, acetone and lactic acid (Herrero, 1983). Reported uses of ion-exchange resins in microbial cultures involve their action as scavengers of oxygen (Malin and Finn, 1951), as presumed binders of sporulation inhibitors (Clifford and Anellis, 1971), as solid supports for the adsorption of bacteria (Hattori and Hattori, 1981), as control of pH in liquid cultures of microorganisms (Styer and Durbin, 1982), to remove end-products of bioconversions from the medium (Demain, 1981). Many enzyme-catalyzed reactions are severely inhibited by high concentrations of the product. Likewise, microbial metabolism may be inhibited by shifts in equilibria as high concentrations of products accumulate or because the products are toxic to the cells. It may also be advantageous to remove the product from its site of production as quickly as possible since some products are subsequently degraded. For this reason it is often desirable to operate ‘extractive bioconversions’, i.e. processes with simultaneous bioconversion and product removal (Mattiasson, 1983).

1.6.1 On-line adsorption

By using adsorbents such as activated carbon, zeolites and polymeric resins, it is possible to reduce product inhibition in fermentation by continuous adsorption of the formed products. The adsorbent can be added directly to the fermentation broth, or can be applied in a way that avoids direct contact with the cells. When saturation of the adsorbent is complete it can be treated separately to liberate the adsorbed products. In a batch experiment it was demonstrated that product inhibition started later during the fermentation when pervaporation was used for product removal and, in a continuous process, a higher conversion of the substrate was observed when continuous removal of the product took place (Mattiasson and Larsson, 1985).

1.6.2 Ion-exchange resins

By adding anion-exchange resin directly to *P. aeruginosa* broth, salicylic acid production was increased to 30 g/l, over three times higher than normal (Tone *et al*., 1968). However, when resin was wrapped in a cellophane membrane before being added to the fermenter, there was a 5.5 times increase in salicylic acid production, indicating that direct contact between resin and cells had inhibited the microbes. However, ion exchange has rarely been used for the *in situ* recovery of ionic products, probably because of problems in using ionic resins with whole broth such as, the possible toxicity of the adsorbent to the microorganism and the non-specific adsorption of the by-products and broth components (Wang, 1981; Lencki *et al*., 1983). The ions that are released from an ion-exchange resin upon product adsorption may inhibit microbial growth, building up to toxic levels in a continuous process (Roffler *et al*., 1984). Addition of a strong-acid cation exchange resin in sodium form is found to control the water accumulation on the biocatalyst without stripping the essential water needed for the immobilized enzyme systems to function and substantial improvements in conversion are achieved (Mensah *et al*., 1998). In fermentative organisms, the production of acidic fermentation products and their consequent accumulation by cells leads to the failure of pH homeostasis (Baronofsky *et al*., 1984).

1.7 Fatty Acid Oxidation

An important source of energy in mammals especially during fasting and stress is fatty acids. Fatty acids are mainly metabolised in the mitochondria with some metabolism occurring in the peroxisomes as well (Wanders and Ijlst, 1992). The fatty acid oxidation cycle, or the β-oxidation cycle (Figure 1.5), is the metabolic pathway responsible for the release of energy contained in the fatty acids, and consists of successive steps of dehydrogenation, hydration, dehydrogenation and thiolytic cleavage (Bremer and Osmundsen, 1984; Sherratt, 1988).

1.7.1 Fatty acyl-CoA dehydrogenases

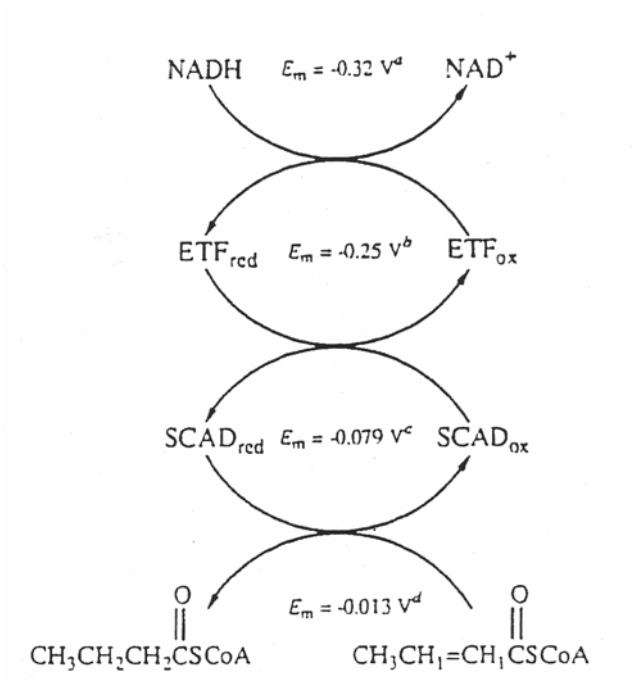
Mitochondrial fatty acid oxidation plays a major role in energy production during periods of fasting. The first step in the fatty-acid oxidation (β-oxidation) cycle is catalysed by a family of enzymes named fatty acyl-CoA dehydrogenases (Crane *et al*., 1956). Fatty acyl-CoA thioesters are oxidized to the corresponding trans-2, 3-

enoyl-CoA products with the reduction of enzyme-bound FAD. Reduced FAD is reoxidized by the FAD cofactor of electron transfer flavoprotein (ETF). The electrons are further transferred to the iron-sulfur cluster of ETF-ubiquinone oxidoreductase. The trans-2, 3-enoyl-CoA undergoes additional reactions, yielding acetyl-CoA that can re-enter the β-oxidation cycle (Djordjevic, 1994).

1.7.2 Short-chain acyl-CoA dehydrogenase from *M. elsdenii*

Short-chain acyl-CoA dehydrogenase (SCAD) also known as butyryl-CoA dehydrogenase (BDH) was first partially purified from *M. elsdenii* by Baldwin and Milligan (1964). BDH is the only fatty acyl-CoA dehydrogenase present in *M. elsdenii*, and it comprises about 2% of the dry weight of the bacteria (Engel and Massey, 1971; Engel, 1981), permitting high yields of the purified protein. In *M. elsdenii*, SCAD functions primarily as an enoyl-CoA reductase by reducing unsaturated short-chain (C_3-C_6) acyl-CoA thioesters as a means of disposing of excess reducing equivalents (Elsden and Lewis, 1953; Brockman and Wood, 1975). In *vitro*, however, *M. elsdenii* SCAD catalyses the oxidation of fatty acyl-CoA thioesters similar to mammalian SCAD with its optimum activity for butyryl-CoA. In the anaerobic bacterium *M. elsdenii*, ETF transfers two electrons simultaneously from NADH to SCAD after which SCAD reduces enoyl-CoA (Becker, 1994; Fink *et al*., 1986; Stankovich and Soltysik, 1987) (Figure 1.7). The primary structural information reported by Becker (1994) provides insights into the function of *M. elsdenii* SCAD. The amino acid sequence of BDH (Becker *et al*., 1993) bears 41% and 44% sequence identity to human medium and short chain acyl-CoA dehydrogenases, respectively.

Figure 1.7: Direction of electron flow from NADH to butyryl-CoA in *M. elsdenii*. The reduction potential values were determined at pH 7.0 (Becker *et al*., 1994).



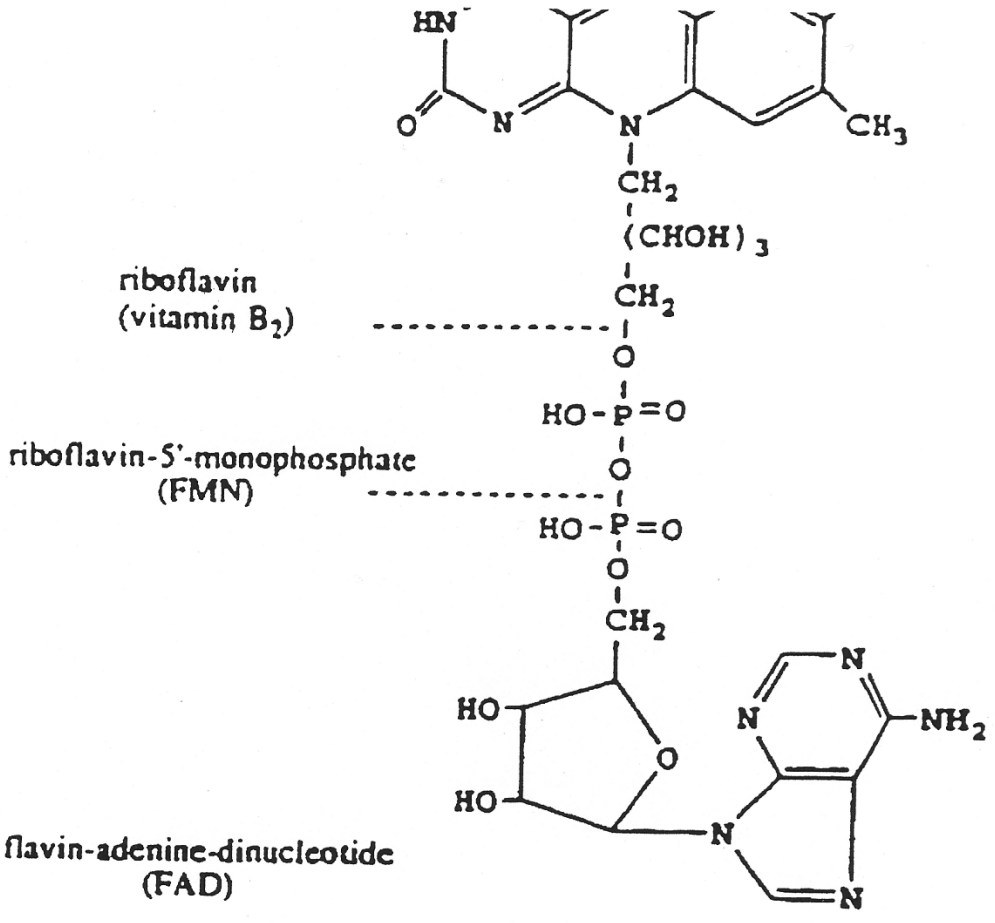
1.8 Flavin-containing Enzymes

Several hundred flavin-containing enzymes have been uncovered to date. Flavin is a versatile coenzyme that is involved in catalysis of a variety of reactions, ranging from the dehydrogenation of amino acids to 'DNA damage repair' and light emission. A common feature for all of these reactions is that at some point during the catalysis, an electron transfer occurs between the flavin molecule and the substrate. During a specific catalytic reaction, a flavin molecule is engaged in the transfer of either one or two electrons at a time. Versatility of the catalytic mechanisms is a distinct property of flavoenzymes, since; in general, most other cofactor-dependent enzymes catalyze a single type of reaction (Djordjevic, 1994).

1.8.1 Chemical properties of the flavin-containing enzymes

The structures of the flavoenzymes, riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) (Djordjevic, 1994) are shown in Figure 1.8.

Figure 1.8: The structures of the flavocoenzymes (Djordjevic, 1994).



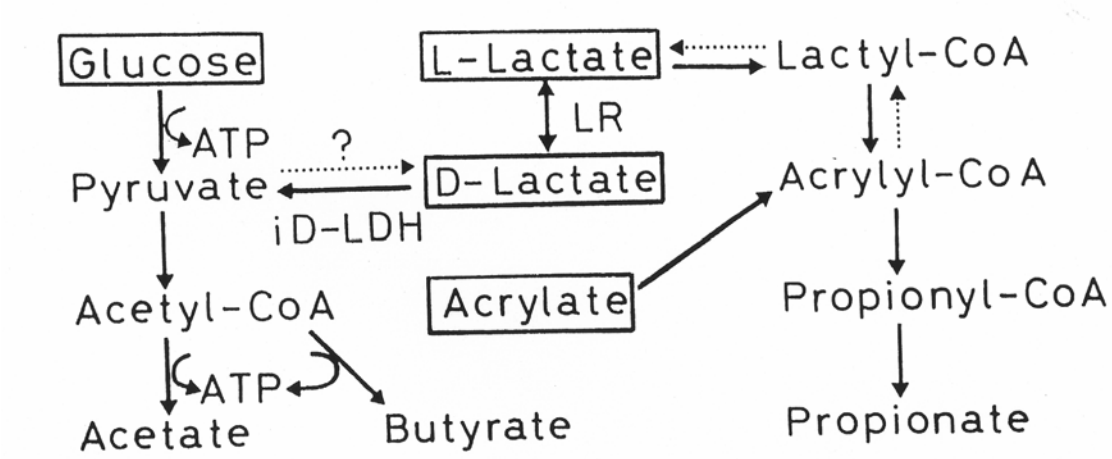
Riboflavin contains the structural components of the 7, 8-dimethylisoalloxazine, which is also the redox-active part of the flavin molecule, and the reduced form of D-ribose (Djordjevic, 1994). The original classification of flavoproteins was based on the type of reactions they catalyze with molecular oxygen as an electron acceptor (Singer and Edmondson, 1978). This criterion divides flavoenzymes into three groups such as; the oxidases, dehydrogenases and the oxygenases. Spectral evidence showed that D-lactate dehydrogenase, when reduced by D-lactate, was able to reduce butyryl-CoA dehydrogenase but only in the presence of the electron-transferring flavoprotein. Baldwin and Milligan (1964) established a path of electrons generated in the oxidation of pyruvate to α,β-unsaturated acyl-CoA). Electrons are transferred to ferredoxin and then to nicotinamide adenine dinucleotide (NAD). Reduced NAD (NADH) then reduces α,β-unsaturated acyl-CoA in a reaction requiring butyryl-CoA dehydrogenase. An electron-transferring flavoprotein which catalyses the reduction of butyryl-CoA dehydrogenase or dyes by NADH was isolated from *P. elsdenii* (Whitfield and Mayhew, 1974). Brockman and Wood (1975) reported that in *P. elsdenii*, a three-component flavoprotein electron transfer system catalyses the oxidation of lactate and the reduction of crotonyl-coenzyme A (CoA).

1.8.2 Lactate dehydrogenase (LDH) activity

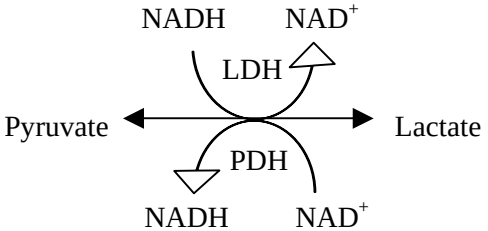
The first enzyme in lactate metabolism of the anaerobic bacterium *M. elsdenii* is a flavoprotein, D-lactate dehydrogenase (Baldwin and Milligan, 1964; Brockman and Wood, 1975). This enzyme belongs to a class of oxidation-reduction flavoproteins termed C-N transhydrogenase (Massey and Hemmerich, 1980) and has more than one metal-binding site (Morpeth and Massey, 1982). It catalyzes the transfer of reducing equivalents from carbohydrate substrates, D-lactate, to the flavin N-(1)-N (5) center of an electron transferring flavoprotein (Brockman and Wood, 1975; Whitfield and Mayhew, 1974). This electron transferring flavoprotein in turn passes the two reducing equivalents on to butyryl-CoA dehydrogenase, another flavoprotein (Engel and Massey, 1971). Rubredoxin (Mayhew and Peel, 1966) and flavodoxin (Mayhew and Massey, 1969) have been isolated from *P. elsdenii* and presumably can function in this reaction. The reducing equivalents from reduced ferredoxin may produce hydrogen gas or they may reduce NAD (Baldwin and Milligan, 1964). NADH can then donate to butyryl-CoA dehydrogenase *via* the FAD-containing electron transferring flavoprotein (Whitfield and Mayhew, 1974). The genes that encode the two different subunits of the novel electron-transferring flavoprotein (ETF) from *M. elsdenii* were identified by screening a partial genomic DNA library with a probe that was generated by amplification of genomic sequences using the polymerase chain reaction. It serves as an electron donor to butyryl-CoA dehydrogenase, and it also has NADH dehydrogenase activity. The ETF in *M. elsdenii* mediates electron transfer from the flavoprotein D-lactate dehydrogenase to a third flavoprotein, termed butyryl-CoA dehydrogenase (BCD) that functions physiologically by reducing enoyl-CoA to acyl-CoA (O'Neill *et al*., 1998). Hino and Kuroda (1993) reported the activity of D-lactate dehydrogenase (D-LDH) was shown not only in cell extracts from *M. elsdenii* grown on DL-lactate, but also in cell extracts from glucose-grown cells, although glucose-grown cells contained approximately half as much D-LDH as DL-lactate-grown cells. This indicates that the D-LDH of *M. elsdenii* is a constitutive

enzyme, and is synthesized at a constant rate regardless of conditions in the cell's environment. They also found that lactate racemase (LR) activity was present in DL-lactate grown cells, but was not detected in glucose-grown cells, suggesting that LR is induced by lactate. These results suggest that the primary reason for the inability of *M. elsdenii* in the strains studied to produce propionate from glucose is that cells fermenting glucose do not synthesize LR, which is induced by lactate. The fermentation pathways of glucose and lactate in *M. elsdenii* are shown in Figure 1.9, based on the knowledge reported so far (Hino and Kuroda, 1993). However, other publications show that propioinate can be produced from glucose growth in *M. elsdenii* (Elsden *et al.,* 1956). *M. elsdenii* has NAD-independent D-lactate dehydrogenase (iD-LDH) catalyzing the reaction from D-lactate to pyruvate (Brockman and Wood., 1975). In fact, the conversion of pyruvate to lactate was demonstrated in a cell extract from *M. elsdenii* (Ladd and Walker, 1959). The direction basically depends on the ratio of [pyruvate](electron donor) to [lactate](electron acceptor), where electron donor and acceptor mean reduced and oxidized forms of electron carrier, respectively. Even though the equilibrium position of the iD-LDH reaction in *M. elsdenii* strongly favours pyruvate formation, the reverse reaction should occur if the ratio of pyruvate to D-lactate becomes high enough under certain conditions. Such a situation may be brought about if D-lactate is readily metabolized to propionate (Hino and Kuroda, 1993) (Figure 1.9).

Figure 1.9: Possible metabolic pathways from glucose, lactate, and acrylate to main VFA in *M. elsdenii*. CoA, Coenzyme A (Hino and Kuroda, 1993).



Pyruvate is not the end-product of most fermentation, but is the common precursor to most end-products. In several bacteria, the homolactic fermentation converts all the pyruvate to lactate:



The enzymes involved are mainly soluble proteins found in the microbial cytoplasm or associated with the cytoplasmic membrane. A pyridine nucleotide independent D-lactate dehydrogenase has been purified (Olson and Massey, 1979) to apparent homogeneity from the anaerobic bacterium *M. elsdenii*. The enzyme has a molecular weight of 105,000 by sedimentation equilibrium analysis with a subunit molecular weight of 55,000 by sodium dodecyl sulfate gel electrophoresis and is thus probably a dimer of identical subunits. The enzyme is specific for D-lactate, and no inhibition is observed with L-lactate (Olson and Massey, 1979). The conversion of lactate to pyruvate is associated with a different, NAD-independent lactate dehydrogenase (iLDH), iLDH may be membrane-bound and linked to electron transport (Kemp, 1972; Kohn and Kaback, 1973). Among rumen bacteria, *M. elsdenii* possesses a constitutive D-iLDH involved in lactate oxidation (Brockman and Wood, 1975; Hino and Kuroda, 1993). Cell extracts of *S. ruminantium* mutant strain obtained from different stage of growth on glucose were assayed for all three LDH activities i.e. D-iLDH, L-nLDH and D-nLDH (Gilmour *et al*., 1994). Both nLDH activities peaked during exponential growth, when glucose was being converted to lactate, although activity could be detected throughout the stationary phase of growth (Gilmour *et al*., 1994).

1.8.3 Pyruvate dehydrogenase (PDH) activity

The enzymes of the pyruvate dehydrogenase complex catalyse the conversion of pyruvate to acetyl-CoA. This complex termed pyruvate dehydrogenase has three enzymes in *E. coli*, pyruvate decarboxylase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase. Many molecules of each of the three enzymes are organised into a giant enzyme complex termed as pyruvate dehydrogenase and is a site of multiple regulatory interactions. These regulatory responses of PDH are important also in the partitioning of pyruvate between conversion to acetyl-CoA for oxidation in the cycle and conversion to oxaloacetate to replenish cycle intermediates in E. coli (Zubay, 1988). The pyruvate dehydrogenase activity was measured in *Bacteroides fragilis* using methyl viologen (MV) as electron acceptor in enzymatic systems as described by Britz and Wilkinson, 1979. On reduction it forms a blue, stable; free-radical cation (MVH) with an absorption maximum in the region of 600 nm and activity was dependent upon coenzyme A, pyruvate and extract.

1.9 Aims of this Thesis

To investigate whether *P. putida* strains carrying plasmids that allow degradation of *n*-alkanes could accumulate adipic acid at any stage of growth, in the presence or absence of hexanoic acid.

To evaluate *M. elsdenii* strains for their capacity to hyper-produce hexanoic acid, which could than be used by *P. putida* strains for adipic acid synthesis, using fermentation approaches.

To evaluate antimetabolic resistant strains of *M. elsdenii* for their capacity to channel carbon to desired VFA end-products and characterise the changes in enzymology responsible for observed changes in product ratios.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Microbiological Methods

2.1.1 Bacterial strains and plasmids

Most of the bacteria, *P. putida*, *E. coli*, *M. elsdenii* strains and plasmids used in this study are shown in Table 2.1.

Table 2.1: List of strains and plasmids, and relevant genotype and or phenotype.

Strains/Plasmids	Relevant genotype/phenotype	Source/References
***Pseudomonas* strains**		
PpS145	*met*-145, (CAM-OCT)	Grund *et al.,* 1975
PpS201	*met*-145, *alcA*81 (CAM-OCT *alkB*201)	Benson *et al*., 1977
PpS205	*met*-145, *alcA*81 (CAM-OCT *alkB*205)	Benson *et al*., 1977
Gpo1	(OCT) prototroph	Schwartz and McCoy, 1973
Gpo12	Gpo1 cured of OCT	Kok, 1988
PpG1	Prototroph (wild type, no plasmid)	Chakrabarty *et al*., 1973
E. coli GEc137 (DH1, fadR) carrying Plasmids		Eggink *et al*., 1987b
pLAFR1	Tc, Tra$^-$, Mob, *cos*, RK2 replicon	Friedman *et al*., 1982
pGEc47	pLAFR1, *alkST/alkBFGHJKL*	Eggink *et al*., 1987b
pGEc41	pLAFR1, *alkST/alkBFGH*	Eggink *et al*., 1987b
***Megasphaera* strains**		
ATCC 25940	Wild type parental strain	American Type Culture Collection
ME5	Mutant of ATCC 25940	M. Britz lab. collection
ME7	Mutant of ATCC 25940	M. Britz lab. collection
B159	Wild type parental strain	M. Bryant lab. collection
ME8	Mutant of B159	M. Britz lab. collection
ME9	Mutant of B159	M. Britz lab. collection
ME10	Mutant of B159	M. Britz lab. collection
T-81	Wild type parental strain	M. Bryant lab. collection
ME12	Mutant of T-81	M. Britz lab. collection
ME14	Mutant of T-81	M. Britz lab. collection
28.7B	Wild type natural isolate	C. Beard lab. collection

[a]OCT and CAM-OCT are, respectively, the wild type octane plasmid (Chakrabarty *et al*., 1973) and the fused plasmid (Chakrabarty, 1973) carrying the replication, transfer, and camphor utilisation loci of the *P. putida* CAM plasmid (Rheinwald *et al*., 1973) and the alkane utilisation loci of the OCT plasmid (Chakrabarty *et al*., 1973), grown on carbon source when 0.01% methionine is added. The *alcA* locus is the chromosomal locus, which codes for growth on *n*-octanol (Grund *et al*., 1975). *fadR* is a chromosomal fatty acid degradation regulatory mutant and Tc (tetracycline resistance).

2.1.2 Chemicals, antibiotics and medium constituents

The sources of all chemicals, antibiotics and medium constituents are shown in appendix-I; abbreviations used for these are also listed (page.v). All other reagents, where not specified were analytical grade and supplied by Ajax Chemicals Ltd. (Australia), BDH Chemicals, and ICN Chemicals. All solutions and media were prepared in accordance with Sambrook *et al*. (1989) with distilled (dH_2O) or deionised water (Milli Q®) and sterilized by autoclaving at 121°C for 20 min and then kept at room temperature unless otherwise stated.

2.1.3 Media

2.1.3.1 Growth media for *E. coli* strains

The following media were used in this study:

LB (Luria Bertani) broth: chemicals including 10 g of bacto-tryptone (Oxoid; Unipath Ltd; Hampshire, England); 5 g of bacto-yeast extract (Oxoid) and 5 g of NaCl were dissolved in 800 ml of dH_2O and the pH was adjusted to 7.0 with 5 M NaOH. The volume was then made up to a litre with dH_2O (Sambrook *et al*., 1989).

Solid media: contained 1.5% (w/v) bacteriological agar (Oxoid).

Antibiotics preparation and storage:

Ampicillin stock: ampicillin was dissolved in sterile dH_2O at a concentration of 25 mg/ml. The solution was filter-sterilized, aliquots poured into Eppendorf tubes and stored at –20°C (Sambrook *et al*., 1989).

Chloramphenicol stock: chloramphenicol was dissolved in methanol at a concentration of 25 mg/ml. The solution was filter-sterilized, aliquots poured into Eppendorf tubes and stored at –20°C (Sambrook *et al*., 1989).

Tetracycline stock: Tetracycline was dissolved in 70% ethanol at a concentration of 25 mg/ml. The solution was stored at –20°C in a dark place (Sambrook *et al*., 1989).

Kanamycin stock: kanamycin was dissolved in sterile dH_2O at a concentration of 25 mg/ml. The solution was filter-sterilized, aliquots poured into Eppendorf tubes and stored at –20°C (Sambrook *et al*., 1989).

LB agar (LBA) with antibiotics: an amount of 15 g of bacteriological agar was added to 1 litre of LB media and the pH was adjusted to 7.5 with NaOH, autoclaved and cooled to 50°C using a water bath. The cooled media was supplemented with 2 ml of ampicillin (25 mg/ml). The medium was poured into Petri-dishes (30-35 ml) and the agar allowed to harden before stored at 4°C (Sambrook *et al*., 1989).

2.1.3.2 Maintenance and propagation of *E. coli* strains

For long-term storage, bacterial strains were maintained as glycerol stocks. A single colony was inoculated into LB medium (supplemented with antibiotics, if required), and then incubated with shaking at 200 rpm overnight at 37°C. A 2 ml of overnight

culture was pelleted by centrifugation (12,000 x g, 1 min) and resuspended in 30% glycerol (in LB medium). The glycerol stocks were maintained at –80°C.

For general use, *E. coli* strains were maintained on solid LB medium. Inoculated plates were incubated overnight at 37°C for cell growth and then stored at 4°C. Strains were subcultured onto fresh medium at regular intervals.

Strains were propagated by inoculating a single colony into LB medium and incubating with shaking at 37°C overnight. The culture medium of strains transformed with recombinant plasmids carrying the ampicillin or kanamycin resistance gene was supplemented with 50 μg or 25 μg ampicillin or kanamycin to maintain selection for the plasmid.

2.1.3.3 Growth media for *Pseudomonas* strains

The following media were used in this study:

E2 media: chemicals including $NaNH_4HPO_4.4H_2O$, 3.5 g; $K_2HPO_4.3H_2O$, 7.5 g; KH_2PO_4, 3.7 g; were dissolved in one litre dH_2O, supplemented with 10 ml of 100 mM $MgSO_4.7H_2O$ and 1 ml of MT (Metallothionein) microelements stock solution contain $FeSO_4.7H_2O$, 2.78 g; $MnCl_2.4H_2O$, 1.98 g; $CoSO_4.7H_2O$, 2.81 g; $CaCl_2.2H_2O$, 1.47 g; $CuCl_2.2H_2O$, 0.17 g; $ZnSO_4.7H_2O$, 0.29 g were dissolved in one litre1 N HCl (Lageveen *et al*., 1988).

LB (Luria Bertani) broth: same as in section 2.1.3.1.

Organic solvents for growth: Octane or Octanol

After preparation, all media were autoclaved for 15 min with a steam pressure of 180 to 200 kPa at 121°C. When required, ampicillin was added to 50 μg/ml, chloramphenicol was added to 20 μg/ml and kanamycin was added to 25 μg/ml to the liquid medium after autoclaving. For solid medium containing agar, after autoclaving, the medium was allowed to cool to 55°C prior to addition of antibiotics and amino acids if required.

2.1.3.4 Maintenance and propagation of *Pseudomonas* strains

For long-term storage, *Pseudomonas* strains were maintained as glycerol stocks. A single colony was streaked onto LB agar plates, saturated with octane or octanol depending on the growth condition of the strains by placing the drops of these organic solvents in the lid of the Petri dishes on Whatman filter paper and then incubated at 28°C for 3-5 days. Heavy inoculum was collected with sterile loop and resuspended in 30% glycerol (in LB medium). The glycerol stocks were maintained at –80°C.

For general use, *Pseudomonas* strains were maintained on solid LB medium in the presence of organic solvents described above. Strains were subcultured onto fresh agar plates at regular intervals.

All growth tests were carried out on E2 medium contained 1.5% agar. Hydrocarbon substrate was added by placing a few drops on a piece of filter paper in the lid of each Petri dish and was incubated at 28°C for 3-5 days (Eggink *et al*., 1987b).

2.1.3.5 Growth media for *M. elsdenii* strains

The following media were used in this study:

40% Glucose solution: 40 g anhydrous D-glucose was dissolved in 100 ml of dH_2O and then autoclaved before use.

40% Lactate solution: 47 ml of 85% lactic acid was dissolved in 100 ml of dH_2O. The pH was adjusted to 7.0 with 10 M NaOH and then autoclaved before use.

Todd Hewitt Blood Agar Glucose (THBAG): chemicals including 36.5 g of Todd-Hewitt Basal medium (BBL) (Oxoid) and 15 g of bacteriological agar were dissolved in 910 ml of dH_2O, autoclaved and cooled to 50^oC using a water bath. To the cooled media, 20 ml of sterile 40% glucose, filtered sterile (Millipore membrane, 25 mm diameter, type HA, 0.45 μm) 10 ml of 5% (w/v) cysteine hydrochloride (cysteine-HCl) and 60 ml of sterile defibrinated horse blood were added. The components were mixed well and poured into Petri dishes and stored at 4^oC after setting and drying.

Todd Hewitt Blood Agar Lactate (THBAL): chemicals including 36.5 g of Todd-Hewitt Basal medium (BBL) and 15 g of bacteriological agar were dissolved in 910 ml of dH_2O, autoclaved and cooled to 50^oC using a water bath. To the cooled media, 20 ml of sterile 40% lactate, filtered sterile 10 ml of 5% (w/v) cysteine-HCl and 60 ml of sterile defibrinated horse blood were added. The components were mixed well and poured into Petri dishes and stored at 4^oC after setting and drying.

VPI salts solution: VPI (Virginia Polytechnic Institute) salts solution (Holdeman and Moore, 1972) was prepared as follows: $CaCl_2$ (anhydrous), 0.2 g; $MgSO_4.7H_2O$, 0.2 g; KH_2PO_4, 1.0 g; K_2HPO_4, 1.0 g; $NaHCO_3$, 10 g; NaCl, 2 g. The solution was prepared by adding the $CaCl_2$ and $MgSO_4$ to 300 ml distilled water and mixing until dissolved. The rest of the components were dissolved separately in 500 ml distilled water then the two solutions were mixed and made up to one litre with distilled water and stored at 4^oC.

Resazurin solution: 0.1 g resazurin was dissolved in 100 ml distilled water and stored at 4^oC as a stock solution.

4 g Tris [hydroxymethyl] aminomethane, 0.2 g glucose and 0.1% (w/v) aqueous resazurin from above stock were dissolved in 19 ml distilled water, boiled the solution until decolorized and transferred to the anaerobic chamber as working solution (Levett, 1991).

PYG broth: The PY (Peptone, yeast-extract) broth contained 10 g of bacto-yeast extract; 10 g of bacteriological peptone, and 40 ml of VPI salts solution in 930 ml dH_2O. The PY broth was autoclaved and cooled down to 50^oC. To the cooled media, 20 ml of 40% glucose, 10 ml of 5% cysteine-HCl and 160 μl of resazurin solution (5 mg/ml) were added to PY broth aseptically.

PYL broth: The PY (Peptone, yeast-extract) broth contained 10 g of bacto-yeast extract; 10 g of bacteriological peptone, and 40 ml of VPI salts solution in 930 ml dH_2O. The PY broth was autoclaved and cooled down to 50^oC. To the cooled

media, 20 ml of 40% lactate, 10 ml of 5% cysteine-HCl and 160 µl of resazurin solution (5 mg/ml) were added to PY broth aseptically.

PYGL broth: The PY (Peptone, yeast-extract) broth contained 10 g of bacto-yeast extract; 10 g of bacteriological peptone, and 40 ml of VPI salts solution in 910 ml of dH_2O. The PY broth was autoclaved and cooled down to 50^oC. To the cooled media, 20 ml of 40% glucose, 20 ml of 40% lactate and 10 ml of 5% cysteine-HCl and 160 µl of resazurin solution (5 mg/ml) were added to PY broth aseptically.

Culture storage broth: chemicals including 0.5 g of bacto-tryptone (Oxoid), 0.25 g of NaCl, 0.15 g of beef extract (Lab-Lemco, Oxoid), 0.25 g of bacto-yeast extract (Oxoid), 0.02 g of cysteine hydrochloride, 0.05 g of glucose, 0.2 g of Na_2HPO_4 and 7.5 ml (v/v) of glycerol and made up to 50 ml with dH_2O. After mixing, aliquots of 2 ml were dispensed into Bijou bottles, then sterilized by autoclaving and stored at -20^oC (for working stocks) and -80^oC (for the long-term storage stocks)(Narayan, 1998).

2.1.3.6 Use of anaerobic chamber

An anaerobic chamber, equipped with glove ports and a rigid airlock for transfer of materials as shown in Figure 2.1, provided an oxygen-free environment in which conventional bacteriological techniques can be applied to the isolation and manipulation of obligate anaerobes in conditions of strict and continuous anaerobiosis.

Figure 2.1: Anaerobic chamber used for the studies of anaerobic bacteria *M. elsdenii* (Kaltec Anaerobic System, Kaltec Pty. Ltd. Edwardstown, SA, Australia).

Anaerobiosis in these cabinets was achieved with palladium catalyst pellets encased in wire gauze and a non-explosive gas mix (10% hydrogen, 10% carbon dioxide and 80% nitrogen) maintained at a slight positive pressure. The interchange is programmed automatically to evacuate and flush with gas mix prior to access being gained to the main working chamber; traces of oxygen entering the cabinet from the interchange are rapidly removed by catalysis. The whole working area was maintained at an incubation temperature of 37^{o}C. The anaerobic cabinet was used with a redox indicator such as resazurin in order to monitor the current status of the chamber atmosphere (Levett, 1991).

2.1.3.7 Long-term storage of *M. elsdenii* strains

The media for freezing the strains of *M. elsdenii* was prepared as described in section 2.1.3.5. The strains were grown on THBAG plates (2-3 plates per strain per bottle) using normal streaking after incubation for 24-36 h at 37^{o}C in anaerobic jars. The growth was scraped from the plates in an anaerobic chamber and made into an even suspension (no clumps) in the storage broth and the culture bottles were placed at –20^{o}C. For recovering, fresh culture plates were prepared, dried and the bottle removed from the freezer and thawed out in the anaerobic chamber. The culture was mixed by rotating gently and a loop full of the suspension removed and placed on appropriate plates and incubated. The storage culture was returned to the freezer.

2.1.3.8 Freeze-Drying of *M. elsdenii* strains

Freeze drying ampoules (Samco) were approximately 7 mm x 10 cm. Labels were prepared and inserted into the ampoules, plugged up with a non-absorbent cotton wool for sterilization, autoclaved and then dried in a 60^{o}C oven. The media were prepared for freeze-drying the strains of *M. elsdenii* consisted of 7.5% (w/v) D-glucose and 50% (v/v) horse serum (defibrinated, CSL and filter sterilized with a sterile 0.45 μm filter, Sartorius). This was dispensed into 3 ml sterile Bijou bottles and then transferred into the anaerobic chamber. The strains were grown on THBAG plates (2-3 plates per strain per bottle) using normal streaking after incubation for 24-36 h at 37^{o}C in anaerobic jars. The growth was scraped from the plates in an anaerobic chamber and an even suspension made in above broth (Mist-dessicans). The cell density in the Mist-dessicans was greater than 10^{12} cells/ml. Using a sterile Pasteur pipette, 150 μl of the suspension was dispensed into each ampoule (5 mm of culture in the bottom of the ampoule). The ampoules were removed from the anaerobic chamber and the extra cotton plug trimmed leaving about 8-9 mm of cotton from the top of ampoules. The ampoules were transferred to –80^{o}C freezer for 30 min to stop the foaming in the culture during centrifugation. After freezing, ampoules were quickly transferred to the freeze-drier centrifuge (less than 30 min) to reduce the thawing in the vacuum chamber. Centrifugation was switched off after 30 min and the ampoules left overnight to dehydrate the culture completely. After overnight drying, the ampoules were partially constricted using an Air/Gas Burner as narrow as possible without completely sealing the ampoules and they were placed onto a vertical manifold for the final freeze-drying overnight. The vials were sealed under vacuum (< 10^{-1} Torr). Finally, the vacuum within the sealed vials was tested using a Tesla coil and stored at –20^{o}C, the ampoules without vacuum were discarded.

2.1.3.9 Maintenance and propagation of *M. elsdenii* strains

For long-term storage, *Megasphaera* strains were maintained as glycerol stocks and freeze-dried culture under anaerobic conditions as described in the section 2.1.3.5. Freeze-dried culture was obtained from frozen ampoules and grown in MacCartney (20 ml capacity) bottles with 10 ml PYG media, in standard Oxoid anaerobic jars containing active palladium catalysts pellets (reactivate by heating at 160^{o}C for 90 min), disposable methylene blue indicator strips (Oxoid) and in the presence of Oxoid gas generating kit sachet (code no: BR38, contains tablets of sodium borohydride, tartaric acid and sodium bicarbonate) for producing anaerobic conditions (1800 ml hydrogen and 350 ml carbon dioxide). The cultures were incubated overnight at 37^{o}C without shaking, and used as inocula for large cultures and for streaking the THBAG plates. The working stock cultures were prepared by streaking single colony of cells onto the entire surface of THBAG plates. After 36-48 h incubation in Oxoid anaerobic jars as described above, plates were taken to the anaerobic chamber (section 2.1.3.6) and growth was harvested into glycerol storage broth. 20 µl of glycerol broth was used to inoculate 10 ml PYG media and loop full glycerol broth was used for streaking on THBAG plates to get single colonies. To maintain the culture in stock, freeze-drying of all *M. elsdenii* strains was used routinely in this study.

2.1.3.10 Gram staining of *M. elsdenii* strains

A single colony was picked from a plate using a sterile loop and emulsified with the drop of water. The slide was air dried and fixed the smear on Bunsen burner briefly. Stained the culture with crystal violet for 1 min and washed with tap water to remove the excess stain. Following, the smear was stained with Gram's iodine for 2 min and washed the slides gently with a mixture (70% ethanol, 30% acetone) along the edges of the slide for decolourisation. Before performing secondary staining, rinsed the slide with running tap water, and stained with safranin for 30 s, following the washing with running tap water to remove excessive stain. Finally, air-dried the slide, placed a drop of oil immersion and observed under light microscope.

2.2 Determination of Minimal Inhibitory Concentrations (MICs) of End-products and Antimetabolites using THBAG and THBAL Plates

The minimal inhibitory concentration (MIC) was defined as the concentration of antimetabolites that significantly inhibited the growth of bacteria.

2.2.1 Stock solution of end-products and antimetabolites used for MICs

The stock solutions of following antimetabolites were made as 100 mg/ml in Milli-Q water or other wise indicated. Pyruvic acid (PA), 3-fluoropyruvate (3-FP), hexanoic acid (HA, neutralized), 6-bromohexanoyl chloride (6-BHC), acetic acid (AA), bromoacetic acid (BAA), butyric acid (BA), 2-bromobutyric acid (2-BBA), 4-

chlorobutyryl chloride (4-CBC), acrylic acid (Acryl. A), 2-bromomethyl acrylic acid (2-BMAA), propionic acid (Prop. A, dissolved in ether), 2-bromoproponyl bromide (2-BPB), 3-chloropropionic acid (3-CPA) and valeric acid (VA).

2.2.2 Working concentration of end-products and antimetabolites used for MICs

These were: pyruvic acid (PA), 0.5-3.0 mg/ml; 3-fluoropyruvate (3-FP), 0.5-2.0 mg/ml; hexanoic acid (HA), 0.05-3.0 mg/ml; 6-bromohexanoyl chloride (6-BHC), 0.1-5.0 mg/ml; acetic acid (AA), 0.5-5.0 mg/ml; bromoacetic acid (BAA), 0.05-0.50 mg/ml; butyric acid (BA), 0.05-5.0 mg/ml; 2-bromobutyric acid (2-BBA), 0.05-5.0 mg/ml; 4-chlorobutyryl chloride (4-CBC), 0.1-5.0 mg/ml; acrylic acid (Acryl. A), 0.05-4.0 mg/ml; 2-bromomethyl acrylic acid (2-BMAA), 0.5-2.5 mg/ml; propionic acid (Prop. A), 0.05-5.0 mg/ml; 2-bromoproponyl bromide (2-BPB), 0.5-2.0 mg/ml; 3-chloropropionic acid (3-CPA), 0.05-5.0 mg/ml and valeric acid (VA), 0.05-5.0 mg/ml.

The THBAG and THBAL agar plates (section 2.1.3.5) were prepared on the day of use with above antimetabolite concentrations. The appropriate working concentrations were added into 25 ml of THBAG and THBAL agar, mixed thoroughly, the plates poured, left to set in a laminar flow hood, and then taken to anaerobic chamber for replica plating. The plates of THBAG and THBAL without any antimetabolite compound were used as a control.

2.2.3 Replica plating for the determination of MICs

A replica plating technique (Linnane *et al*., 1970) was used to determine the minimal inhibitory concentration of antimetabolites (section 2.2.2) on parent strain of *M. elsdenii* (ATCC 25940, B159 and T-81) and strains previously isolated as $3FP^r$ mutants (ME5, ME7, ME8, ME9, ME12, ME14 and 28.7B). For this purpose, a metal multi-pronged replicator and a matching Perspex block having 37 wells were used. All these strains grown overnight in 10 ml PYG or PYL media were subjected to measure the absorbance at 600 nm to standardize the inoculum and diluted this in a standard fashion to get a “light” inoculum. To each selective well was added 150 µl sterile PYG (section 2.1.3.5) or PYL media (section 2.1.3.5) for THBAG or THBAL plates respectively (section 2.1.3.5). The multi-pronged replicator was used to transfer samples of bacterial suspensions to appropriate solid medium plates of different compounds with varying concentrations in an anaerobic chamber. The positions of the strains applied on the plates are shown in the appendix-2 Figure A-1. Plates were incubated at 37^{o}C for 48 h in Oxoid anaerobic jars and checked the sensitivities of antimetabolites of different concentrations against all the strains given above. The plates of THBAG and THBAL without any antimetabolite were used for control experiments and the growth of all the strains studied is shown in the appendix-2 Figure A-2.

2.3 Culture and Analyses used for *Pseudomonas* Experiments

2.3.1 Batch culture conditions for *Pseudomonas* strains

To obtain cells for biotransformation experiments, a single colony was inoculated to 20 ml of E2 medium containing *n*-octanol or *n*-octane as the sole carbon source provided as the vapour. One to two millilitres of this starter culture was then used to inoculate 75 ml of E2 medium in 125 ml Erlenmeyer flasks with *n*-octanol or *n*-octane supplied as the vapour. Cultures were incubated in an orbital shaker (model 013422, Panton Scientific Pty, Ltd) at 28^{o}C. Six strains of *Pseudomonas* (PpS201, PpS205, PpG1, Gpo12, Gpo1 and PpS145) were used throughout the experiments. Experiments were designed in three groups for individual strain with appropriate controls: 1) 75 ml E2 medium in Erlenmeyer flask, inoculated with 10% pre-induced bacterial culture, and added *n*-octanol or *n*-octane in a glass tube inserted in to the flask to provide vapour; 2) 75 ml E2 medium inoculated with 10% pre-induced bacterial culture, 0.4% neutralized *n*-hexanoic acid, and added *n*-octanol or *n*-octane as described in experiment one; 3) 75 ml E2 medium, inoculated with 10% pre-induced bacterial culture, and 0.4% neutralized *n*-hexanoic acid only. All the flasks were sealed with suba-seal rubber stopper and were run in an incubator on 100 rpm at 28^{o}C. The samples were collected periodically under strict sterile conditions into microcentrifuge tubes and kept at -20^{o}C for chemical analysis. The growth of bacteria was monitored by following the change in absorbance at 600 nm using a LKB Ultraspec plus 4054 UV/Visible spectrophotometer in samples prior to freezing.

2.3.2 GC analysis of methanol-treated alkanoic acid

Analysis of products made during growth of *Pseudomonas* strains on different substrate was accomplished by gas chromatography (GC) on a Varian Star 3400 gas chromatograph equipped with a flame ionization detector (FID), after chemical conversion of compounds to their methyl esters. The extraction and methylation procedures were made according to Rizzo (1980) with some modifications. For this purpose 1 ml of culture was treated with 400 μl of 50% H_2SO_4 in 2 ml of methanol using *n*-heptanoic acid (20 mM *n*-heptanoic acid dissolved in diethyl ether) as an internal standard. After 30 min incubation at 55^{o}C, 1 ml of water was added, followed by 0.6 ml of chloroform. The samples were mixed by gentle inversion 50 times, centrifuged briefly to break the emulsion and 1 μl of the organic phase was injected onto a BP21 column (25 meter, ID. 0.53 mm, film thickness 0.5 micron) (SGE) with the GC programmed from 80 to 131^{o}C at 50^{o}C/min and holding for 22 min, injector temperature 200^{o}C, detector temperature 280^{o}C, with a nitrogen carrier gas pressure of 3.0 psi using 15:1 split ratio. Octanoic acid standards (reagent grade) were dissolved in fermentation media and treated similarly. Standards were injected under the same conditions as the fermentation samples, and the retention times were compared and the standard chromatogram is shown in the appendix-2 Figure A-3.

2.3.3 Gas chromatography-mass spectrometry (GC-MS) for the detection of *n*-octanoic acid

The *Pseudomonas* culture was analysed for the confirmation of *n*-octanoic acid on a gas chromatograph-mass spectrometer (Hewlett Packard 5890 series II) equipped with a Supelcowax 10 fused silica capillary column (30 meter, ID. 0.32 mm, film thickness 0.25 μm). The standard *n*-octanoic acid was also run under the same conditions as samples (section 2.3.2), and the retention times compared. The operating conditions were as follows: column temperature, 45°C for 3 min to 120°C at 5°C/min followed by further increase to 250°C at 10°C/min; and ionising source temperature, 280°C. Helium was used as a carrier gas at a flow rate of 1 ml/min.

2.4 Culture and Analyses used for *M. elsdenii* Fermentation

2.4.1 Batch culture conditions for *M. elsdenii*

The equipment used most for this project consisted of Applicon glass fermenters (maximum volume 1 litre) as shown in Figure 2.2. Alkaline pyrogallol (150 ml of KOH [300 g/l], 15 g pyrogallol dissolved in 20 ml dH_2O) was used to absorb any oxygen in the nitrogen gas. PY medium (680 ml) was added to each fermenter and two to three drops of anti-foaming solution was added before autoclaving at 121°C for 20 min. 4% glucose (80 ml of 40% glucose) was added aseptically to the autoclaved medium with 1.0 ml of resazurin solution. To obtain cells for biotransformation experiments, a single colony was inoculated in to 40 ml of PYG medium and incubated overnight at 37°C and used as a starter culture to inoculate 160 ml of PYG medium, which was grown overnight at 37°C.

Figure 2.2: Photograph of Applicon glass fermenters used in the fermentation studies for the growth of anaerobic bacteria *M. elsdenii* with pH controlled and stirred conditions under strictly anaerobic atmosphere.

Cultures were centrifuged at 3,000 rpm for 10 min and cells were collected and resuspended in 40 ml PYG medium in an anaerobic chamber to prevent oxidation. Once the fermenters were ready, a 50 ml syringe was used to inoculate 40 ml of cells into PYG medium (made up to 800 ml) in a 1litre capacity Applicon fermenter under all possible anaerobic conditions provided. Then the fermenters were attached to the automated pH (6.5) using 2 M NaOH and temperature (37^{o}C) controlled fermentation system with continuous stirring (100 rpm) under nitrogen flow. Nitrogen flow was stopped once growth was sufficient to maintain a positive gas pressure. Samples were removed periodically under nitrogen flow and analyzed for VFAs, glucose and cellular protein concentrations. For resin experiments, the cultures in the fermenters were grown for 24 h at 37^{o}C before the addition of appropriate resin.

2.4.2 Gas chromatographic analysis of volatile fatty acids (VFAs) from *M. elsdenii*

GC analyses of VFAs were performed on a Varian Star 3400 gas chromatograph equipped with FID using BP21 (25 meters, ID. 0.53 mm, film thickness 0.5 micron) capillary column (SGE). The oven temperature for the BP21 column was programmed at 135^{o}C for 23.90 min, followed by a linear increase of 10^{o}C/min to 136^{o}C, and then holding at 136^{o}C for 1 min. Injector and detector temperatures were set to 200^{o}C and 300^{o}C respectively, with a nitrogen carrier gas pressure of 2.0 psi using 8:1 split ratio. Concentrations of VFAs in media were measured after extraction into diethyl ether. For this purpose, 1 ml sample was treated by adding 200 µl of 50% H_2SO_4 in a sealable tube containing 0.4 g of NaCl and 50 µl of *n*-heptanoic acid was added from the stock solution of 400 mM to give a final concentration of 20 mM in the extracted sample. One ml of HPLC grade diethyl ether was added. The samples were mixed by gentle inversion 50 times, centrifuged briefly to break the emulsion and 1 µl of the organic phase was injected onto the column. All results represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.

2.4.3 Resin conditioning, sterilization, addition and recovery

2.4.3.1 Conditioning of the resins

The resins Amberlite IRA-93 and Amberlite IRA-400 were conditioned by a modified version of the procedure described by Kawabata and Ohira (1979).

The resin, Amberlite IRA-93 (200 g, trimethylammonium on polystyrene, free base form, anion exchange resin, BDH), was placed in a beaker and initially washed with distilled water and the large floating particles and suspended fines in the supernatant were removed by decantation by repeating the step 4-6 times. Step 1: The resin was washed with ethanol twice (1 bed volume, where bed volume is the volume of the resin). Step 2: The resin was washed (2 bed volume) with dH_2O three times. Step 3: The resin was washed once (1.5 bed volume) with 2 M NaOH. Step 4: The resin was washed with dH_2O twice (2 bed volume). Step 5: Then washed the resin with 2 M HCl (1.5 bed volume) once. Step 6: The resin was washed three times with dH_2O (2

bed volume). Step 7: Steps 3 to 6 were repeated three times. Step 8: The resin was placed in a glass column (5 cm ID, 35 cm height fitted with sintered glass disc and stopcock) and 1 M NaOH passed through the column until chloride ion was no longer detected in the eluate (as checked by loss of white precipitate when tested by two to three drops of 0.1 M $AgNO_3$ after the addition of five to six drops of concentrated HNO_3). Step 9: The resin was washed in the column with dH_2O until the pH of the eluate was less than 9.5. Step 10: The water was removed by gravity filtration through a sintered glass funnel and the moist resin was stored in a sealed bottle.

The resin, Amberlite IRA-400 (200 g, trimethylammonium on polystyrene, chloride form, a strong base anion exchange resin, BDH), was placed in a beaker and initially washed with distilled water and the large floating particles and suspended fines in the supernatant were removed by decantation by repeating the step 4-6 times. Step 1: The resin was washed with ethanol twice (1 bed volume). Step 2: The resin was washed (2 bed volume) with dH_2O three times. Step 3: The resin was washed (1.5 bed volume) with 2 M NaOH. Step 4: The resin was washed with dH_2O twice (2 bed volume). Step 5: Then washed the resin with 2 M HCl (1.5 bed volume) once. Step 6: The resin was washed three times with dH_2O (2 bed volume). Step 7: Steps 3 to 6 were repeated three times. Step 8: The resin was placed in a glass column (5 cm ID, 35 cm height fitted with sintered glass disc and stopcock). Step 9: The resin was washed in the column with dH_2O until the pH of the eluate was greater than 4.5. Step 10: The water was removed by gravity filtration through a sintered glass funnel and the moist resin was stored in a sealed bottle.

2.4.3.2 Resin sterilization and addition

The resin was added after the cells were grown for 24 h fermentation time in fermenters (section 2.4.1). Prior to addition, the conditioned ion exchange resin (section 2.4.3.1) was placed in a beaker and dH_2O added such that the resin was completely covered. It was then autoclaved (15 min at 121^{o}C), quickly cooled, the water decanted and the resin added aseptically to the fermenters *via* a sterile funnel, under nitrogen flow. The amount of resin added to fermentation was equivalent to 25% of the volume of the fermentation broth (800 ml). The resin was measured by volume under water in the chloride form for Amberlite IRA-400 and as the free base form for Amberlite IRA-93.

2.4.3.3 Procedure for adsorption of volatile fatty acids (VFAs) by ion exchange resin Amberlite IRA-93 and Amberlite IRA-400

The resin was collected at the end of the fermentation, washed over glass wool with PYG to remove cells, then eluted by serial washing in 3x 160 ml 2 M NaOH, 3x 160 ml dH_2O and 3x 160 ml 2 M HCl (for every 100 g of resin) and then washings pooled for measurement of VFAs.

2.4.3.4 Methods for esterification of resin-adsorbed VFAs

Esterification of VFAs adsorbed to ion exchange resin was carried out as follows. After adsorption, the solution was filtered off using a sintered glass funnel and aspirator. The resin to which the acids were adsorbed (about 5 g wet) was dried

under aspiration until the particles no longer adhered to each other on the sintered glass funnel. The dried resin was placed in a 50 ml Greiner tube and the methanol-sulfuric acid mixture was added. The methanol-sulfuric acid mixture was prepared by weighing approximately 50 ml methanol (BDH) into a beaker and then adding sulfuric acid (BDH) equivalent to 10% of the weight of methanol. After standardisation of methanol volume used for the esterification of hexanoic acid recovered from desorption of resins, methanol-sulfuric acid mixture (27 ml) was transferred to a 50 ml Greiner tube that was capped and shaken in a 55^{o}C water bath at 140 rpm for 2 h and used the aqueous solution for the determination of VFAs through Gas chromatography (GC) as described in the section 2.4.4.

2.4.4 Gas chromatographic analysis of methanol-treated fatty acids from *M. elsdenii*

GC analyses of VFAs were performed on a Varian Star 3400 gas chromatograph equipped with FID using BP1 (25 meters, ID. 0.32 mm, film thickness 0.5 micron) capillary column (SGE). The oven temperature for the BP1 column was programmed at 115^{o}C for 10 min, followed by a linear increase of 10^{o}C/min to 140^{o}C, holding for 7.5 min. Injector and detector temperatures were set to 200^{o}C and 300^{o}C respectively, with a nitrogen carrier gas pressure of 3.2 psi using 20:1 split ratio.

Methyl esters (method as described in section 2.4.3.4) were analyzed as follows: 1ml of water was added in 1 ml of methylated sample, followed by 1 ml of chloroform, and then 50 µl of n-heptanoic acid was added from the stock solution of 400 mM to give a final concentration of 20 mM in the extracted sample as an internal standard. The samples were mixed by gentle inversion 50 times, centrifuged briefly to break the emulsion and 1 µl of the organic phase was injected onto a BP1 column (SGE). Standards (see section 2.4.4.1 and 2.4.4.2) were injected under the same conditions as the fermentation samples, the retention times were compared and amounts of unknowns calculated from peak areas adjusted for variations in internal standard area.

2.4.4.1 Determination and identification of standard volatile fatty acids compounds using retention time

A typical chromatogram was obtained from the mixture of different volatile fatty acids compounds in the concentration of 20 mM each from the stock solution of 400 mM prepared in diethyl ether. Mixed standard solutions were prepared by mixing 50 µl of each acetic acid, 50 µl of propionic acid, 50 µl of 2-methyl propionic acid (isobutyric acid), 50 µl of butyric acid, 50 µl of 2-methyl butyric acid (iso-valeric acid), 50 µl of 2-propenoic acid (acrylic acid), 50 µl of pentanoic acid (n-valeric acid), 50 µl of 2-methyl pentanoic acid (iso-hexanoic acid), 50 µl of hexanoic acid, 50 µl of heptanoic acid and made up to 1 ml with diethyl ether and 1 µl was injected into BP21 capillary column by using the temperature program described in section 2.4.2. A typical chromatogram is shown in the appendix-2 Figure A-4 with respective retention time for each compound.

2.4.4.2 Preparation of standard curve for esterified heptanoic acid

Standard curve was generated by preparing standard solution containing 5 mM, 10 mM, 15 mM, 20 mM, and 25 mM from the 400 mM stock solution and treated as described in section 2.3.2. 1 μl sample from each concentration was injected into the gas chromatograph described in section 2.3.2 and typical standard curve is shown in appendix-2 Figure A-5.

2.4.4.3 Preparation of standard curve for esterified hexanoic acid

Standard curve was generated by preparing standard solution containing 5 mM, 10 mM, 15 mM, 20 mM, and 25 mM from the 400 mM stock solution and treated as described in section 2.3.2. 1 μl samples from each concentration were injected into the gas chromatograph described in section 2.3.2 and typical standard curve is shown in appendix-2 Figure A-6.

2.4.4.4 HPLC analysis of organic acids

HPLC analyses were performed using a Varian liquid chromatographic system containing a solvent delivery system (Varian 9012), automated injection system (Varian 9100), variable wavelength 410 UV/Vis detector (Varian 9050) controlled by Star chromatography software version 4.02. Level of organic acids was measured using an Aminex HPX-87H ion exchange column – 300 x 7.8 mm (Bio-Rad, North Ryde, New South Wales, Australia) and guard column with disposable 2 micron filters. The elements were heated to 65-66^{o}C with a Bio-Rad HPLC column heater. The mobile phase was prepared by dissolving H_2SO_4 in deionised water and degassing for 1 h. After many assays under different conditions, the concentration of 0.0075 N H_2SO_4, and in the mobile phase, a flow rate of 0.7 ml/min, and detection of 210 nm wavelengths was used. An injection volume of 10 μl was used for the standard lactic acid and pyruvic acids. Standard curves for lactic and pyruvic acids were prepared using pure acids (Sigma chemicals Co., St. Louis, USA) of known concentrations and shown in appendix-2 Figure A-7 and Figure A-8 respectively. Quantification of lactic and pyruvic acid was carried out by the external standard method. Linear regression curves (R2 > 0.999) based on peak height were calculated for the organic acids after duplicate injections of two aqueous standard solutions covering 4-20 mM range of concentrations. The standards were very stable under refrigeration. Both standard solutions were injected in triplicate to verify the reproducibility of the method. Identifications were based on matching retention times of standards. All results represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.

2.5 Biochemical Methods

Lowry Reagent A: (Lowry *et al*., 1951) Reagent A consisted of 2% Na_2CO_3 in 0.1 M NaOH. The solution was stored at room temperature.

Lowry Reagent B: (Lowry *et al*., 1951) Reagent B consisted of 0.5% $CuSO_4.5H_2O$ in 1% Na-K-tartrate. The pH was adjusted to 9.0 and the solution was stored at 4^{o}C.

Lowry Reagent C: (Lowry *et al*., 1951) Reagent C was prepared by mixing Lowry reagent A with Lowry reagent B at a ratio of 50:1. Lowry reagent C was prepared immediately before protein assays and discarded after one day.

Bovine Serum Albumin (BSA): The stock solution of bovine serum albumin (1 mg/ml) was diluted in 0.45M NaH_2PO_4 to achieve a concentration of 0-100 μg. The BSA solution was stored at 4°C.

0.45 M Phosphate solution: Phosphate solution was prepared by dissolving $NaH_2PO_4.2H_2O$ (70.2 g) in one litre of dH_2O. The solution was sterilised by autoclaving and stored at room temperature.

2.5.1 Protein concentration assay

Bacterial biomass was determined by measuring protein concentration according to the methods described by Lowry *et al*., 1951. Bacterial cells were harvested from duplicate cultures by centrifugation (Hettich Universal, HD Scientific, Melbourne) for 10 min at 4,000 rpm/4°C, and the cell pellet was resuspended in 1 ml of 4.6 M NaOH and held overnight. The suspension was heated at 90°C for 10 min, and 50 μl of heated sample was ten-fold diluted in 0.45 M NaH_2PO_4 buffer (450 μl), and then mixed briefly by vortexing for 10 s. To 0.5 ml of protein sample, 2.5 ml Lowry C reagent (Lowry reagent A [2% Na_2CO_3 in 0.1 M NaOH] plus Lowry reagent B [0.5% $CuSO_4.5H_2O$ in 1% Na-K-tartrate] in the ratio of 50:1) was added, followed by vortexing and then standing at room temperature for 10 min. After adding 0.25 ml of diluted Folin-Ciocalteau reagent (2:3 ratio in MilliQ water), the contents were mixed thoroughly and allowed to stand at room temperature for another 30 min. The absorbance at wavelength of 750 nm was recorded using LKB ultraspec plus 4054 UV/Visible spectrophotometer. Standards, ranging from 0 to 100 μg, were treated the same as samples and prepared from 1 mg/ml BSA solution as shown in appendix-2 Figure A-9.

2.5.2 Glucose concentration assay

The concentration of glucose in the fermentation broth was measured by the DNS method described by Sumner (1921). The DNS reagent was prepared by dissolving 5 g DNS (3'5'-Dinitrosalicyclic acid) in 200 ml 1 M NaOH. Sodium potassium tartrate-$4H_2O$ (150 g) was dissolved in 200 ml dH_2O. The DNS solution was mixed well with the Na-K-tartrate solution and made up to 500 ml with dH_2O. The solution was stored in the dark bottle and made fresh each month. A stock glucose solution (2 g/l) was made in distilled water in a volumetric flask. Working standard glucose solution were prepared by diluting stock solution with distilled water to give a duplicate series of test tubes containing 1 ml volumes of 0.5, 1.0, 1.5, and 2.0 g/l glucose. The fermentation broth was centrifuged and 50 μl diluted to 1 ml with dH_2O in duplicate. One millilitre of DNS reagent was added and placed in a boiling water bath for 5 min. The samples were removed and placed in ice to cool them down and then 5 ml distilled water was added to each sample. The contents were mixed well and absorbance was measured at 570 nm. The concentrations of glucose were taken from a linear standard curve (R^2>0.99) for glucose (concentration range of 0-100 μg) as shown in appendix-2 Figure A-10.

2.5.3 Enzymatic activities

2.5.3.1 Preparation of cell suspensions and extracts

The preparation of cell suspensions and extracts of *M. elsdenii* strains by a method modified from those was described by Mink (1981). Growing cell cultures were harvested by centrifugation (4,000 x g, 10 min, and 4^{o}C). Each volume was washed twice with half volumes of TKD (50 mM Tris-HCl, 1% KCl, and 1 mM dithiothreitol, pH 7.4) buffer by centrifugation. Washed pellets were frozen by suspension in liquid nitrogen for 10 s and stored for up to 6 days at -80^{o}C before enzymatic assays were performed. Sometimes freshly washed cell pellets were also used for the enzyme assays. When preparing for analysis, frozen or fresh pellets were resuspended in TKD buffer in an anaerobic chamber to 1% of their original volume and stored anaerobically on ice until use (up to 12 h). These cell suspensions were used for protein determinations (section 2.5.1) and preparation of cell-free extracts. Cell-free extracts were prepared using homogenizer (B. Braun Biotechnology International GmbH, Type 853022, Melsungen Ag, Germany) by homogenizing the cell suspensions in a 12 ml vial (B. Braun Biotechnologgy) in the presence of an equal amount of glass beads (Glasper len, 0.25 – 0.30 mm, B. Braun Biotechnology) with 15 s bursts two times under anaerobic conditions (glass beads were added in cell suspensions in anaerobic chamber). Homogenates were held on ice throughout this procedure. After centrifugation (4,000 x g, 10 min) at 4^{o}C, the cell-free extracts were transferred anaerobically into microcentrifuge tubes and centrifuged (15,000 x g, 20 min) at 4^{o}C. The supernatant (final cell-free extract) was stored on ice in the anaerobic chamber until used (1 to 4 h).

2.5.3.2 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity in cell-free extracts was measured by a method modified from the Mink (1981). The LDH activity in cell-free extracts was measured by monitoring the rate of NADH oxidation at 340 nm with a spectrophotometer (Pharmacia LKB Novaspec® II) at room temperature in the anaerobic chamber as shown in appendix-2 Figure A-11. The absorbance was monitored for 5 min and the decrease in NADH concentration was calculated from the decrease in initial absorbance. The reaction mixture contained 25 mM Tris-HCl (pH 7.0), 0.2 mM NADH and 5.2 mM sodium pyruvate. The reaction mixture was prepared in cuvettes by mixing 1.8 ml of 25 mM Tris-HCl (pH 7.0), 0.1 ml of 4 mM NADH and 0.025 to 0.05 ml of a 1:10 dilution (in TKD buffer) of cell-free extract (5-50 mg protein). This mixture was allowed to equilibrate, and the reaction was then initiated by the addition of 0.1 ml of 0.1 M sodium pyruvate. Separate controls with NAD in place of NADH, and reaction mixtures without pyruvate or NADH, were run to correct for background activity which amounted to less than 5% of total activity in all cases. One unit of LDH was defined, as the amount of enzyme required oxidizing one nmol NADH per min. Specific activity was calculated as units per gram of protein in extracts.

2.5.3.3 Pyruvate dehydrogenase assay

Assay of pyruvate dehydrogenase activity was similar to that described by Britz and Wilkinson (1979) with slight modifications. Reaction mixture (50 ml) contained 2 mM sodium pyruvate, 0.075 mM HS-CoA and 6 mM methyl viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride) in phosphate buffer (0.1 M, pH 7.0) with 250 μM 2-mercaptoethanol (P-ME buffer). Reactions were started in the anaerobic chamber by addition of cell free extract (10 to 500 μl), and make the final volume up to two ml with reaction mixture in the cuvette at zero time at 30^{o}C. Changes in absorbance were monitored at 30 s intervals using a spectrophotometer (Pharmacia LKB Novaspec® II) at 600 nm. The increase in absorbance was observed for 11 min due to reduced methyl viologen as shown in appendix-2 Figure A-12. Enzyme activity was expressed in milli-International Units (M.I.U.) directly as an increase in absorbance at 600 nm per minute per mg of protein. The molar absorption of methyl viologen was 6300 M^{-1} cm^{-1}.

2.5.4 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.5.4.1 Gel preparation

SDS-PAGE was performed essentially as described by Laemmli (1970) using a Mini-PROTEAN 3 system (Bio-Rad). The electrophoresis gel was cast between two glass templates of 10.1 x 8.3 cm in size. The gel consisted of two parts, a resolving gel at the bottom and a stacking gel at the top into which the gel comb was inserted. As much as 12 ml resolving gel mixture was prepared consisting of 0.1% (w/v) SDS, 0.375 M Tris-HCl pH 8.8, 15% (w/v) acrylamide: NN'-bis methylene acrylamide (30:0.8), 0.04% (w/v) ammonium persulphate (APS) and 0.075% (N,N,N',N'-tetramethylethylenediamine) (TEMED). The solution was degassed under vacuum for at least 15 min, then APS and TEMED added and poured the plates. The gel was immediately overlaid with water-saturated *n*-butanol (50 ml *n*-butanol, 5 ml dH_2O) and allowed to polymerize for 45 min. The gel surface was rinsed completely with distilled water. The 7 ml stacking gel solution was prepared consisting of 0.35% (v/w) SDS, 0.125 M Tris-HCl pH 6.8 and 6% (w/v) acrylamide: NN'-bis methylene acrylamide (30:0.8), 0.04% (w/v) ammonium persulphate (APS) and 0.075% (N,N,N',N'-tetramethylethylenediamine) (TEMED). The solution was degassed under vacuum for at least 15 min, then APS and TEMED added, and the solution poured onto the top of resolving gel. After inserting the gel comb (10 wells), the gel was allowed to set for 30-45 min. While still in the casting sandwich, the gel was then assembled into the running apparatus, followed by addition of running buffer (0.1% (w/v) SDS, 0.35 M glycine and 0.05 M Tris base (pH 8.3) into the apparatus. Any air bubbles formed around the gel edges were carefully removed. Apart from the samples (preparation in section 2.5.4.2), high molecular weight (HMW) standards (Bio-Rad) were used as markers. The gel was electrophoresed at 200 V until the dye front reached the bottom part of the gel. Once electrophoresis was completed, the gel was analyzed to determine the location of the separated proteins by staining with silver and Coomassie Blue. After staining, the gel was photographed, preserved and dried using a GelAir Dryer (Bio-Rad) for a record of the position and intensity of each band.

2.5.4.2 Sample preparation for SDS-PAGE

Added 12 µl of 2x sample buffer [0.25 M Tris-HCl (pH 6.8); 4% (w/v) SDS; 20% (v/v) Glycerol; 0.02% bromophenol blue and 5% (v/v) 2-mercaptoethanol] with equal volume (12 µl, 10 µg) of each protein sample in 1.5 ml microcentrifuge. Mixed by vortexing briefly and placed immediately in a boiling water bath for 3 min. Samples were cooled down to room temperature and 12 µl of each sample was loaded on a gel. The 5 µl of high molecular weight markers (BENCHMARK^{TM} Protein Ladder, 0.5 µg/µl) was loaded directly on a gel without boiling.

2.5.4.3 Coomassie Brilliant Blue Staining

Gels were stained as described by Weber and Osborne (1969) in a solution consisting of 50% (v/v) methanol, 9.2% (v/v) acetic acid, 0.025% (w/v) Coomassie Brilliant Blue R250 (Sigma) for 2-4 h at room temperature. Removal of background stain was achieved by repeated washing in a destaining solution (40% (v/v) methanol, 7.5% (v/v) acetic acid). After destaining, the gels were transferred into a preserving solution containing (10% (v/v) glycerol, 10% (v/v) acetic acid and 35% (v/v) methanol). The gel was preserved in a GelBond film (Pharmacia) and dried using a GelAir Dryer (Bio-Rad).

2.5.4.4 Silver staining

The silver staining method of Merril *et al*., 1984 was used for the visualisation of low amounts of protein in the range of 1-5 µg of protein. The gels were transferred into a fixing solution (40% (v/v) absolute ethanol, 10% (v/v) glacial acetic acid) for 30 min. Gels were treated with sensitising solution (30% (v/v) ethanol, 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulphate and 0.125% (v/v) glutaraldehyde) for 30 min. After sensitisation, the gels were washed three times with distilled water for 15 min and placed in silver solution (0.25% (w/v) silver nitrate, 0.015% (v/v) formaldehyde) for 40 min with gentle shaking. The gels were then transferred into developing solution (2.5% (w/v) sodium carbonate, 0.0074% (v/v) formaldehyde), and left until all protein bands were clearly visible. Development was ceased by removal of developer and washing with distilled water and placed the gels in stop solution (1.5% (w/v) Na_2EDTA (ethylenediaminetetraacetic acid) disodium salt) for 5-10 min. The gels were washed twice with distilled water for 5-10 min with shaking and then finally placed in preserving solution (30% (v/v) ethanol, 4% (v/v) glycerol). The gel was washed in Milli-Q water with one change for 5-10 min then preserved in a GelBond film (Pharmacia) and dried using a GelAir Dryer (Bio-Rad).

2.6 DNA Methodologies

The following reagents were used in this study:

100 mM Tris-HCl: 15.76 g of tris (hydroxymethyl) methylammonium chloride was dissolved in 800 ml of dH_2O and the pH was adjusted to 8.0 with 5 M NaOH. The volume then was made up to 1 litre with dH_2O (Sambrook *et al*., 1989).

0.5 M EDTA (pH 7.5, 8.0 and 9.0): The stock solution was prepared by dissolving 93.05 g of the disodium ethylenediaminetetra-acetate into 400 ml of dH_2O, using

stirrer and the pH was adjusted to the appropriate pH with 10 M NaOH. The volume was then made up to 500 ml with dH_2O. The stock solution was sterilized by autoclaving and stored at room temperature (Sambrook *et al*., 1989).

TE buffer (pH 8): the buffer was prepared by mixing 10 ml of 100 mM Tris-HCl (pH 8.0) and 0.2 ml of 0.5 M EDTA. The volume then was made up to 100 ml with dH_2O. The stock solution was stored at room temperature (Sambrook *et al*., 1989).

50x TAE buffer: (Smabrook *et al*., 1989) Tris base (242.5 g), glacial acetic acid (57.1 ml) and 0.5 M EDTA (pH 8) (100 ml) were added to 842.9 ml of water. The stock solution was stored at room temperature (Sambrook *et al*., 1989).

STE buffer: 87.65 g of NaCl and 44.1 g of sodium citrate were dissolved in 400 ml of dH_2O. The pH was adjusted to 7.0 with the addition of 5 M NaOH. The volume then was made up to 500 ml with dH_2O.

DNase-free RNase: 10 mg/ml stock solution was prepared by dissolving Ribonuclease A from bovine pancreas into 10 mM Tris-HCl pH 7.5, and 15 mM NaCl, then heating the mixture at 90-95^oC for 15 min to denature DNase. The solution was cooled slowly to room temperature, dispensed into aliquots of 100 μl and then stored at –20^oC.

20% SDS: 20 g of electrophoretic grade SDS was dissolved in 80 ml of H_2O. The solution was heated to 68^oC to assist solubilization. The pH was adjusted to 7.2 with HCl. The volume was adjusted to 100 ml with dH_2O and stored at room temperature (Sambrook *et al*., 1989).

Proteinase K solution: Proteinase K was dissolved in TE (pH 8) to achieve a final concentration of 20 mg/ml. Proteinase K solution was prepared immediately prior to use.

10 M NaOH: 40 g of sodium hydroxide was dissolved and made up to 100 ml with dH_2O. The stock solution was stored at room temperature.

5 M NaCl: 29.22 g of sodium chloride was dissolved and made up to 100 ml with dH_2O. The stock solution was sterilized by autoclaving and stored at room temperature.

Ethidium Bromide: 100 mg of ethidium bromide was dissolved and made up to 10 ml with dH_2O. The solution was stored in an amber bottle at 4^oC.

40% Glucose solution (w/v): 400 gm of glucose was dissolved and made up to 1 litre with dH_2O. The stock solution was sterilized by autoclaving and stored at 4^oC.

Chloroform: isoamyl alcohol (24:1; v/v): 1 ml of isoamyl alcohol; was added to 24 ml of chloroform and stored in an amber bottle.

Phenol-chloroform-isoamyl alcohol (25:24:1; v/v): Solid phenol was melted first at 65^oC for 1 h. An equal part of TE buffer and the melted phenol was then mixed in an amber glass bottle, to which 8-hydroxyquinoline at a concentration of 0.1% (w/v) was added, to inhibit oxidation and to identify the organic phase with a bright yellow color. The two phases were allowed to separate overnight. One part of the lower phase

(phenol phase) was taken, mixed with one part of chloroform: isoamyl alcohol (24:1) and stored in an amber glass bottle at 4°C.

3 M Sodium acetate (pH 5.2): 24.61 g of sodium acetate salt (anhydrous) was dissolved in 80 ml dH_2O, the pH was adjusted to 5.2 with glacial acetic acid and the volume made to 100 ml with dH_2O and was autoclaved.

5 M Potassium acetate (pH 4.8-5.2): 29.5 ml glacial acetic acid mixed with water and pH was adjusted with KOH pellets to 4.8 (several) and final volume made up to 100 ml with dH_2O and stored at room temperature.

MOPS X 10: The buffer constituted of 200 mM of 3-[N-Mopholino] propane sulfonic acid, 80 mM sodium acetate and 10 mM EDTA (The pH was adjusted to 7.0 with 2 M NaOH), filters sterilized and was stored in an amber bottle at room temperature.

IPTG stock solution (0.8 M): isopropyl β-D-thiogalactopyranoside (IPTG) was dissolved and made up to 10 ml with dH_2O. Following filter-sterilization, the stock solution was dispensed into 1 ml aliquots and stored at –20°C.

X-Gal stock solution (20 mg/ml): an amount of 40 mg of 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) was dissolved in a final volume of 2 ml with N, N-dimethylforamide. The stock solution was stored in an amber bottle at –20°C.

2.6.1 Isolation and quantification of DNA

2.6.1.1 Small-scale preparation of plasmid DNA from bacteria

For routine use, plasmid DNA was isolated from *E. coli* using a modified alkaline lysis procedure (Sambrook *et al*., 1989). A 1.5 ml aliquot of a 10 ml overnight culture was harvested by centrifugation (12,500 x g, 3 min). After removing the supernatant by aspiration, the pellet was resuspended in 100 µl of Solution I (25 mM Tris-HCl pH 7.5, 10 mM EDTA, and 15% sucrose) and incubated on ice for 5 min. Cells were lysed by adding 200 µl of Solution II (0.2 M NaOH, 1% SDS) followed by gentle inversion and incubating on ice for a further 5 min. Then, 150 µl of Solution III (3 M sodium acetate, pH 4.8) was added to the mixture, and following gentle inversion the mix was incubated on ice for 5 min. After centrifugation (12,500 x g, 15 min, 4°C), the resultant supernatant was extracted with an equal volume of phenol/chloroform (1:1) and centrifuged (12,500 x g, 5 min, 4°C). Plasmid DNA was precipitated by addition of 2 volumes of absolute ethanol and standing at room temperature for 5 min. Following centrifugation (12,500 x g, 10 min, 4°C), the pellet was washed with 70% ethanol, dried (*in vacuo*) using a speed Vac concentrator (Selby), resuspended in 50 µl sterile dH_2O and stored at –20°C.

2.6.1.2 Genomic DNA preparation from *M. elsdenii*

The *M. elsdenii* genomic DNA was purified by the method as described by Becker *et al*., 1993. Frozen *M. elsdenii* cells (0.5 g) were ground in a mortar cooled with liquid nitrogen, and dispersed in a 10 ml solution containing 50 mM Tris-HCl, pH 8.5, 100 mM EDTA, and 200 mM NaCl. The cells were lysed by the addition of 0.5 ml of 10% SDS and 2.5 mg of proteinase K followed by incubation at 50°C for several hours.

The homogenate was extracted with an equal volume of phenol saturated with 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA (TE buffer), using a wrist action rocker for 15 min. The phases were separated by centrifuging the mixture at 3,000 rpm for 10 min at 10°C. This extraction was repeated once with a 1:1 phenol-chloroform-isoamyl alcohol) and then extraction was repeated one more time with chloroform-isoamyl alcohol alone. The aqueous phase was then adjusted to 0.3 M with sodium acetate, pH 5.2. The DNA was precipitated by the addition of 2-propanol (0.8 volume). Next, the DNA pellet was removed with a plastic pipette and transferred to 70% ethanol, spun down, dried and resuspended in 0.5 ml of TE buffer to which RNase A was added (55 μg/ml). The yield of *M. elsdenii* genomic DNA was approximately 1 mg of DNA/g of bacteria.

2.6.1.3 Genomic DNA preparation from *Pseudomonas* strains

The *P. putida* genomic DNA was isolated as described previously (Ausubel *et al*., 1994) with some modifications. A 10 ml culture of each strain was grown to saturation. The cells were centrifuged (4,000 x g, 10 min) and resuspended gently in 567 μl TE buffer. The cells were lysed by the addition of 30 μl of 10% SDS and 30 μl of 20 mg/ml proteinase K in a microcentrifuge tube, followed by incubation at 37°C for 1 h. After incubation, 100 μl of 5 M NaCl was added, mixed thoroughly and then 80 μl of CTAB/NaCl solution (4.1 g NaCl in 80 ml water and slowly add 10 g CTAB [hexadecyltrimethyl ammonium bromide] while heating and stirring, made up to 100 ml with water) added, thoroughly mixed followed by incubation at 65°C for 10 min. The homogenate was extracted with an equal volume of chloroform-isoamyl alcohol thoroughly. The phases were separated by centrifuging the mixture in a microcentrifuge for 5 min at room temperature. The aqueous phase was transferred to a fresh microcentrifuge tube and re-extracted with a 1:1 phenol-chloroform-isoamyl alcohol by mixing thoroughly, then centrifuging for 5 min. The aqueous phase was transferred to a fresh microcentrifuge tube to precipitate DNA with 0.6 volumes of isopropanol, spinning down the pellet for 5 min, and washing the DNA with 1 ml of 70% ethanol (-20°C), and centrifuged for 5 min at room temperature. The supernatant was removed and the DNA pellet was air dried and resuspended in 100 μl TE buffer.

2.6.1.4 Wizard Mini-prep for preparation of plasmid DNA from bacteria

This method was used only when extremely pure plasmid DNA was required (for such purposes as DNA sequencing) using a commercially available kit (Promega Wizard Minipreps DNA Purification System). An aliquot (10 ml) of overnight bacterial culture was harvested by centrifugation (10,000 x g, 5 min). After removing the supernatant, the cells were resuspended by vortexing in 250 μl Cell Resuspension Solution (supplied with kit) and completely resuspend the cell pellet by vortexing well. To the suspension, 250 μl of Cell Lysis Solution (supplied with kit) was added; the microcentrifuge tube was then inverted several times to gently mix the content. After the mixture became almost clear, added 10 μl of Alkaline Protease Solution (supplied with kit) and mixed by inverting the tube four times and incubated for 5 min at room temperature. Neutralizing Solution 350 μl (supplied with kit) was added and the

contents mixed by inverting several times. The bacterial lysate was centrifuged (14,000 x g, 10 min) at room temperature. The 850 μl clear lysate was transferred into the Wizard™ Plus SV Minipreps Spin Column inserted into a 2 ml collection tube and centrifuged (14,000 x g, 1 min) at room temperature. The Wizard™ Plus SV Minipreps Spin Column was removed from the tube and the flow-through discarded from the collection tube. Column Wash Solution 750 μl (supplied with kit) was added to the minicolumn and centrifuged (14,000 x g, 1 min) at room temperature. Once again, the column was removed from the tube and the flow-through discarded from the collection tube and the column finally rewashed with 250 μl of Column Wash Solution by centrifuging (14,000 x g, 2 min) at room temperature. The column was transferred to a clean, sterile 1.5 ml microcentrifuge tube and the plasmid DNA finally eluted by adding 100 μl of Nuclease-Free Water (supplied with kit) to the column. The column was centrifuged at 14, 000 x g for 1 min at room temperature in a microcentrifuge tube and eluted DNA stored at –20^{o}C.

2.6.1.5 Determination of quality and quantity of DNA by spectrophotometry

DNA concentrations were determined using the spectrophotometric method as described by Sambrook *et al*., 1989. Amounts of synthesized oligonucleotides were determined using the spectrophotometric method. Isolated nucleic acid samples and synthesized oligonucleotides were diluted 1:50 (for DNA) or 1:100 (for oligonucleotides) in distilled water and absorbance readings were taken at wavelengths of 260 nm and 280 nm, and the A_{260}/A_{280} ratio provided an estimation of the purity of samples. Nucleic acid concentration was determined according to the equation: 1 A260 unit (1 cm light path) = 50 μg/ml solution for double-stranded DNA and 33 μg/ml solution for single-stranded oligonucleotides.

2.6.1.6 Quantification of DNA using the agarose plate method

Agarose plate method was used as described by Sambrook *et al*., 1989. A 1% agarose solution containing ethidium bromide (2 μl) was prepared in 1x TAE (Tris-acetate-EDTA) buffer (50 ml). DNA samples were prepared in sterile distilled water (5 μl) containing loading buffer (2 μl) and DNA (3 μl). The samples (10 μl) were loaded into the gel and run for one hour at 80 V with 1x TAE as the running buffer. DNA concentration standards (varying from 1-20 μg /ml) were run in parallel with the DNA samples. The quantity of DNA was estimated after photography of the gel by comparing the intensity of the sample DNA with the DNA markers.

2.6.2 DNA Manipulations

2.6.2.1 Restriction enzyme digestions

Restriction enzyme digestion was conducted for several different purposes, including preparing plasmid or DNA fragments for ligation, linearizing plasmids for DNA quantitation etc. The reactions were carried out in the buffer systems and at the temperature recommended by the manufacturers. A two-to-five time excess of enzyme was used (considering that one unit of enzyme digests 1 μg of DNA in 1 h) to digest DNA (Sambrook *et al*., 1989). Generally the mixture was incubated for at least

2 h before complete digestion of the DNA was confirmed by agarose gel electrophoresis (section 2.6.2.2). A large-scale digest was stopped by either heating to 68°C for 5 min to inactivate the enzyme or by phenol extraction according to the enzyme used (Sambrook *et al*., 1989). Various commercially available molecular weight standards were used to compare the size of the DNA fragments. They are described for each experiment in the results sections.

2.6.2.2 Agarose gel electrophoresis of DNA fragments

Agarose gel electrophoresis was routinely used to fractionate DNA fragments following enzyme digestion, PCR (polymerase chain reaction), etc. The separation was carried out using horizontal agarose mini-submarine gel apparatus (Bio-Rad). Gels were cast by pouring a molten solution of electrophoresis grade agarose dissolved in 1x TAE buffer running normally at 80 V for 1-2 h or until the dye ran near the bottom of the gel, whilst 15-20 V was used for large gels in 1x TAE buffer overnight for genomic digests. Generally, agarose concentrations ranged from 0.8 to 1.5% (w/v) depending on the size range of the DNA to be separated (Sambrook *et al*., 1989). Samples mixed with loading buffer (and RNase if required) were loaded into wells of the gel submerged under 1x TAE buffer in the electrophoresis tank along with approximately 0.5-1.0 μg of *Eco*RI/*Hind*III pre-digested DNA (Progen) or SPP1-bacteriophage DNA pre-digested with *Eco*RI. Gels were then stained by soaking in ethidium bromide solution (2 μg /ml) for 20 min followed by washing in distilled water for 20 min. DNA fragments were visualized by UV illumination (LKB 2011 Macrovue Transilluminator) at a wavelength of 320 nm. A permanent record of results was obtained by photography of the illuminated gel with a Polaroid MP-4 Land camera through a Kodak 23A filter using Polaroid 667 (positive) or 665 (negative) films.

2.6.2.3 Recovery of DNA fragments from low melt agarose gels

Following electrophoresis to fractionate DNA fragments, the electrophoresis gel was stained with ethidium bromide and the DNA bands were visualized. The gel containing DNA fragment of interest was sliced out and placed in a 1.5 ml microcentrifuge tube. After melting the gel slice on a heating block (at 65°C to 70°C), 1 ml of Wizard PCR Prep Purification Resin (Promega, Cat. No. A718A) was immediately added. The mixture was quickly mixed by pipetting up and down several times before it was passed through a Wizard Minicolumn (Promega, Cat. No. A721B). After washing the column with 2 ml 80% isopropanol, the DNA was eluted from the column with warm sterile dH_2O.

2.6.2.4 Ligation of DNA fragments

Ligation of DNA fragments was performed essentially as described by Sambrook *et al*., 1989. Linearized vector DNA and insert DNA were prepared as described (Sections 2.6.2.1). The ligation mixture contained 100-500 ng of vector DNA and a 3 fold relative molar excess of insert DNA in ligation buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 4 mM DTT, 0.5 mM ATP) together with 1 unit of T4 ligase (Promega, TM042) in a final volume of 10 μl. After incubation at 4°C overnight, the mix was transformed into competent *E. coli* cells (Section 2.6.3).

2.6.3 Preparation of competent bacterial cells

Competent bacterial cells of DH5αF' *E. coli* were prepared as follows (Hanahan, 1985). Cells were pre-cultured in a 10 ml LB medium and incubated with shaking at 37°C overnight. An aliquot (1 ml) of this pre-culture was used to inoculate a 100 ml LB medium in a 2 litre flask, followed by incubation with moderate agitation at 37°C and periodically checked the cell density by taking absorbance at 600 nm until it reached to 0.55 (usually for 3.5 to 4 h). The cells were then harvested by centrifugation (3000 rpm, 12 min) at 4°C. After thorough removal of the supernatant, the pellet was resuspended in 33 ml of filter sterilised RF1 solution (100 mM RbCl, 50 mM $MnCl_2.4H_2O$, 30 mM potassium acetate, 10 mM $CaCl_2.2H_2O$, 15% (w/v) glycerol, pH 5.8). The suspension was incubated on ice for 1 h, and the cells were then pelleted as before. This pellet was eventually resuspended in 7 ml of RF2 solution (10 mM RbCl, 10 mM MOPS, 75 mM $CaCl_2.2H_2O$, 15% (w/v) glycerol, pH 6.8). The cell suspension was aliquot into chilled 1.5 ml microcentrifuge tubes, flash frozen in a dry-ice/ethanol bath, and stored at –80°C.

2.6.4 Bacterial transformation

DNA to be transformed was mixed with 100 μl of competent *E. coli* cells (section 2.6.3) in a sterile microcentrifuge tube and incubated on ice for 30 min. The transformation mix was then heat shocked by transferring the tube to a 42°C water bath (without shaking) for 45 s. Following addition of I ml LB medium, the mixture was incubated with shaking at 37°C for I h. Cells (100 μl aliquots) were then spread onto selective solid LB medium plates containing ampicillin (100 μg /ml). If required, the β-galactosidase inducer IPTG and the chromogenic substrate X-gal were added prior to plating at concentrations of 0.3 mM and 0.3 mg/ml respectively. The plates were then incubated at 37°C overnight.

2.6.5 Amplification of specific DNA sequences by Polymerase Chain Reaction using Expand™ Long Template PCR System

Specific DNA sequences were enzymatically amplified by the method of Polymerase Chain Reaction. Generally the method required the design and synthesis of 2 oligonucleotide primers of 18-24 nucleotides in length, which would anneal to that portion of the DNA strand adjacent to the 5' end of the target sequence in either strand of a double stranded template.

The Expand™ Long Template PCR system (Boehringer Mannheim, Cat. No. 1681834) consisted of a unique enzyme mixture containing thermostable Taq and Pwo DNA polymerases to give a high yield of PCR products from genomic DNA. Two separate master mixes were prepared according to the company's manual that circumvents the need of "hot start" and in addition avoids interaction with primers or template without dNTPs, which could lead to a partial degradation of primer and template through the 3'-5' exonuclease activity of *Pwo*. Master mix 1 (300-500 ng of genomic DNA, 50 ρmol of each of the oligonucleotide primers, 10 mM of dNTPs mix,

sterile milliQ water) was added to a sterile 0.2 ml thin-walled plastic snap-cap tube. Master mix 2 (PCR buffer 1 comprises 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol [DTT], 0.1 mM EDTA, 0.5% [v/v] Tween® 20, 0.5% [v/v] Nonidet® P40, 50% glycerol [v/v], 17.5 mM $MgCl_2$), the enzyme mix supplied by the manufacturer and MilliQ water was added to another sterile 0.2 ml thin-walled plastic snap-cap tube. On ice, 25 μl master mix 1 and 25 μl master mix 2 were mixed by pipetting in a thin-walled PCR tubes shortly before cycling to a final volume of 50 μl. The amplification reaction was carried out in a Peltier thermal cycler (PTC-200) to provide the following conditions: First cycle to denature template for 2 min at 94°C, 10 cycles (denaturation at 94°C for 10 s, annealing 55°C for 30 s, elongation at 68°C for 90 s), 15-20 cycles (denaturation at 94°C for 10 s, annealing 55°C for 30 s, elongation at 68°C for 45 s to 30 min plus cycle elongation for more yield of 20 s for each cycle). For last 1 cycle used a prolonged elongation time up to 7 min at 68°C. The PCR products were analyzed by agarose gel electrophoresis and stored at –20°C. The PCR conditions were used for LDH gene amplification as described by Garmyn *et al*., 1995.

2.6.6 Purification of PCR products

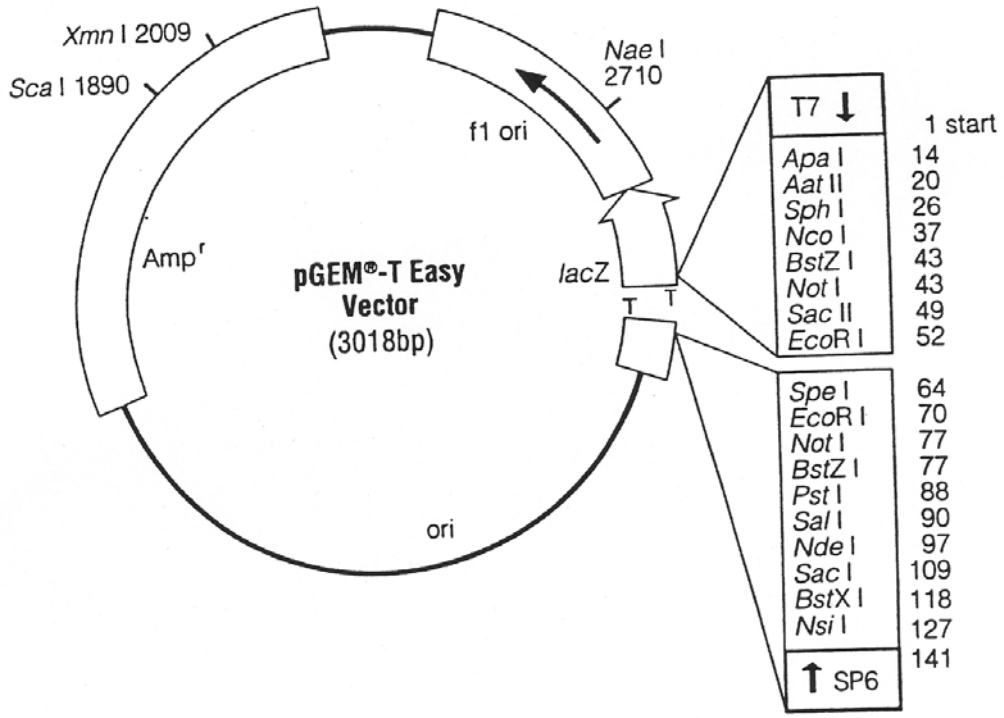
To purify PCR products from non-specific amplification products such as primer-dimers and amplification primers, the Wizard™ PCR Preps DNA Purification System (Promega, Cat. No. A7170) was used following the manufacturer's instructions. For each complete PCR reaction, the entire contents (approximately 95 μl) was added to a 1.5 ml microcentrifuge tube containing 100 μl of Direct Purification Buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.8], 1.5 mM $MgCl_2$, 0.1% Triton® X-100) and then vortexed briefly to mix. Subsequently, 1 ml of PCR Prep DNA Purification Resin was added and vortexed briefly three times over a one-minute period. One Wizard™ PCR preps Mini-column was prepared for each PCR reaction and followed the procedure given in the Manual. Subsequently, the column was washed with 2 ml of 80% isopropanol. The column was centrifuged (10,000 x g) for 2 min to dry the resin. To remove DNA, 50 μl of sterile MilliQ water was added to the column with incubation at room temperature for 1 min. The column was centrifuged (10,000 x g) for 20 s to elute the bound DNA fragment, and the purified DNA was stored at –20°C.

2.6.7 Optimizing insert: vector molar ratios

The pGEM®-T Easy Vector System (Promega, TM042) was used for the cloning of purified PCR fragments. The pGEM®-T Easy Vector (Figure 2.3) used in this study was approximately 3 kb and is supplied at the concentration of 50 ng/μl. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, the following equation was used, with 3:1 insert: vector molar ratio.

$$\frac{\text{ng of vector} \times \text{size (kb) of insert}}{\text{Size (kb) of Vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Figure 2.3: pGEM® –T Easy Vector circle map.



2.6.8 Ligation using pGEM®-T Easy Vector

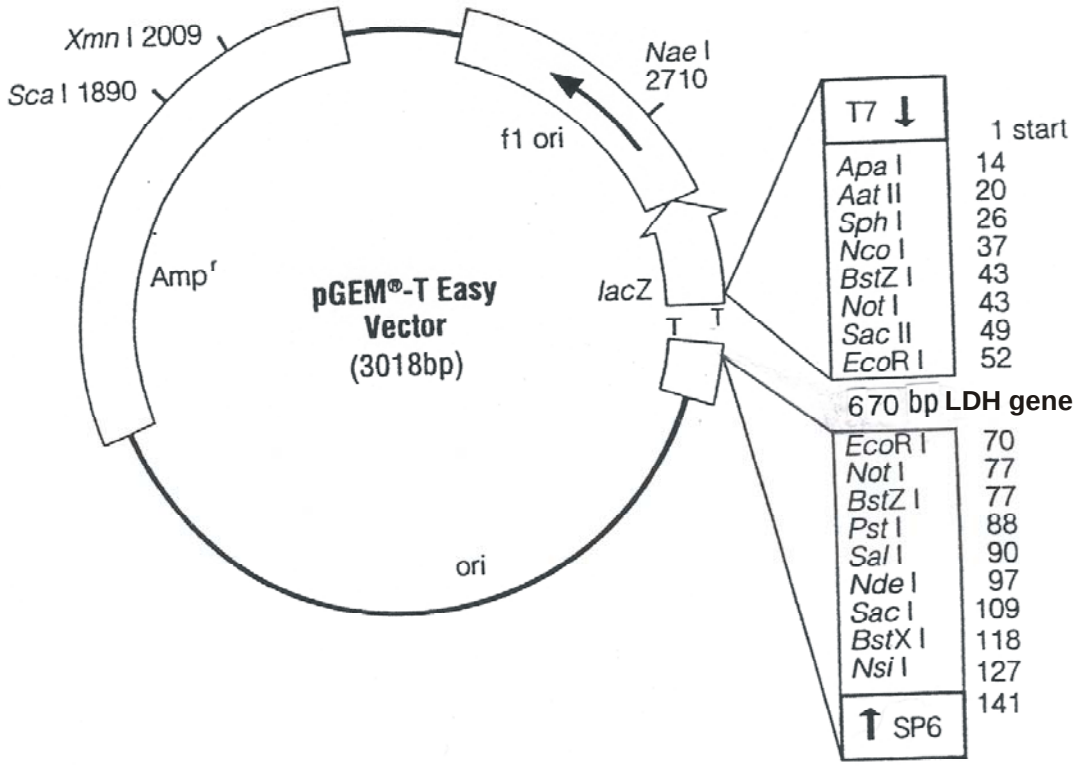
Before setting up a ligation reaction, the pGEM®-T Easy Vector and control insert microcentrifuge tubes were briefly centrifuged to collect contents at the bottom of the tube. Ligation reaction mixture contained 1 μl of T4 DNA Ligase buffer (10x), 1 μl of pGEM®-T Easy Vector (50 ng), 1 μl of PCR product, and 1 μl of T4 DNA ligase (3 Weiss units/μl). The final volume of the reaction was made up to 10 μl with deionized water. The contents of the microcentrifuge tube were mixed by pipetting, and the reaction was incubated overnight at 4°C. Appropriate positive and background controls were used according to manual instructions (Promega, TM042).

2.6.9 Cloning into pGEM®-T Easy Vector

In order to facilitate further gene manipulation of the constructs, the PCR fragments were cloned into the pGEM®-T Easy Vector. This vector system is designed for direct cloning of PCR products by taking advantage of the fact that during PCR the Taq DNA polymerase catalyses the addition of a nucleotide, almost exclusively adenosine (A), in a non-template dependent manner to the 3'-termini of the double stranded product (Clark, 1988, Promega TM042). The vector, therefore, is designed to possess thymidine (T) overhangs on both its 3'-strand ends. These T-overhangs facilitate direct ligation of PCR product into the vector by complementary base pairing.

A schematic diagram of the introduction of the 665 bp PCR product into the pGEM®-T Easy Vector is shown in Figure 2.4. Following ligation of the PCR product to the pGEM®-T Easy Vector, the ligation mixture was then used to transform competent *E. coli* (strain DH5αF'). Then transformants were plated on solid LBA medium supplemented with 0.3 mM IPTG and 0.3 mg/ml X-gal.

Figure 2.4: pGEM® –T Easy Vector circle map with 665 bp PCR LDH gene product.



Selection of transformants was based on blue/white colony formation. As shown in Figure 2.4, the PCR product containing the LDH gene was inserted into a site within the *lacZ* gene of pGEM®-T Easy Vector. This means that the recombinant vectors (bearing insert) were ampr *lacZ*$^-$, while the non-recombinant vectors (with no insert) were amp^r *lacZ*$^+$. As X-gal was included in the selective LBA medium, the recombinant clones containing the LDH gene were white, while the non-recombinant ones were blue. The IPTG was added to include the *lacZ* gene. This so-called blue/white selection technique (Brown, 1991, Promega, TM042) allowed the identification of the colonies harboring the pGEM®-T Easy Vector inserted with the LDH gene from the very first appearance of the transformants on the selective medium.

The presence of the LDH gene in the recombinant clones was further confirmed with restriction enzyme digestion of DNA. DNA isolated from the putative positive clones was subjected to *Eco*RI digestion, under conditions as described in section 2.6.2.1. The digestion products were then analyses by agarose gel electrophoresis. Cloning of the 665 bp second step PCR into pGEM®-T Easy Vector will yield DNA that upon digestion with *Eco*RI generates a 3,065 bp (vector) fragment and a 665 bp fragment (LDH gene). To definitively confirm the successful incorporation of the insert, the plasmid DNA from the positive clones was subjected to sequencing analysis as described in section 2.6.12 by using T7 and SP6 forward and reverses primers respectively (Promega, TM042).

2.6.10 Transformation

High efficiency competent *E. coli* cells (strain DH5αF') were used for transformation as described in the section 2.6.3. The tubes of frozen competent cells were removed from -80°C storage and placed them in an ice bath until just thawed. The cells were gently flicked and 50 µl was transferred into each tube containing 2 µl ligation reactions and to the tube containing uncut plasmid as a control. The tubes were gently flicked to mix and placed them on ice for 20 min. The cells were heat shocked for 45-50 s in a water bath at 42°C. The tubes were returned immediately to ice for 2 min. Finally 300 µl of LB medium was added to the tubes containing ligation reactions and were incubated at 37°C for 1.5 h with shaking at 150 rpm. The 100 µl of each transformation culture cells were plated out onto duplicate LB plates with ampicillin/IPTG/X-Gal (100 µg/ml/0.5 mM/80 µg/ml respectively). The plates were incubated overnight at 37°C. To facilitate the blue/white screening, plates were incubated at 4°C for couple of hours before examining the colonies.

2.6.11 Southern hybridisation for the analysis of genomic DNAs

2.6.11.1 Southern blotting (DNA transfer)

After electrophoresis, agarose gels were capillary blotted to Hybond™-N^+ membrane (Amersham) (Sambrook *et al*., 1989) as follows. DNA in the gel was firstly depurinated by immersing the gel in 0.25 M HCl for 10-15 min, and then the gel rinsed in dH_2O and placed in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 30 min at room temperature with gentle shaking. The gel was rinsed in dH_2O and finally neutralized by soaking in 0.5 M Tris-HCl pH 7.2, 1.5 M NaCl and 0.001 M EDTA for 30 min at room temperature with gentle shaking. The DNA transfer from the gel to the Hybond™-N^+ membrane was performed by capillary action and blotting was carried out for 16 h using 20x SSC (3 M NaCl, 0.3 M trisodium citrate).

2.6.11.2 α[^{32}P] CTP labelling of DNA

The Boehringer Mannheim High Prime kit (Cat. No. 1585592) was used to label 25 ng of DNA to highest specific activities (2 x 10^9 dpm/µg) according to manufacturer instructions. The DNA probe was denatured at 95°C for 10 min in a heating block (Ratex Instruments) than chilled on ice for 2 min. The reaction mixture was set up to contain the denatured DNA in TE buffer, 4 µl of 5x High Prime reaction mix (1 U/µl Klenow polymerase, labelling grade, 0.125 mM dATP, 0.125 mM dGTP, 0.125 mM dTTP, and 5x stabilized reaction buffer in 50% (v/v) glycerol), 5 µl of α[^{32}P]dCTP (3,000 Ci/mmol, Bresatec) made up to 25 µl with dH_2O. The labelling reaction was performed at 37°C for 10 min. The labelling reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8). Transfer RNA (2 µl of tRNA stock solution, 5 µg /ml) and TE buffer (75 µl) were added to the labelled samples and centrifuged for 5 s at 14,000 g. Samples were placed on ice while Sephadex G50 columns were prepared.

The G50 columns consisted of syringe barrels (1 ml, Terumo Medical Corporation, USA) plugged with sterile glass wool. G50 in TE buffer was added to the column and

placed in disposable microcentrifuge tubes (10 ml) and centrifuged for 30 s (Spinette centrifuge, International Equipment Company, USA). STE buffer (100 μl) was added to the column and centrifuged for 30 s. Washed the columns with TE buffer (100 μl) and centrifuged as above. The G50 columns were placed inside the microcentrifuge tubes, the labelled probes were added to the columns and centrifuged for 30 s and collected in a new microcentrifuge tube. TE buffer (200 μl) was added to the probes and the samples were denatured at 95°C for 10 min in a heating block (Ratek Instruments) and used for hybridization immediately.

2.6.11.3 DNA: DNA hybridisation with ^{32}P-labelled probes

After transfer of DNA, the membranes were placed in hybridization bags with pre-heated (65°C) pre-hybridization solution (6x SSPE [1.08 M NaCl, 0.06 M sodium phosphate, 0.006 M EDTA, pH 7.7], 7% SDS, 0.1% non-fat powdered milk) added at the rate of 150 μl pre-hybridization solution per cm^2 of Hybond™-N^+ membrane. Membranes were incubated at 65°C overnight (Extron HI2001, Bartelt Instruments Pty Ltd) in a sealed bag with a shaking platform. The hybridization reaction was initiated by addition of denatured radiolabelled probe DNA (section 2.6.11.2) and radiolabel led SPP-1 (bacteriophage DNA restricted with *Eco*RI) in a sealed bag and allowed to proceed overnight at 65°C. After hybridization, membranes were washed briefly in 2x SSC and then gently shaken for 15 min in each of the following solutions: 2x SSC/0.1% SDS, 0.5 x SSC/0.1% SDS and 0.1x SSC/0.1% SDS.

2.6.11.4 Detection of hybridisation signals

After washing out the non-specific radio labelling, the membrane were air dried for 5 min, wrapped in SaranWrap and were autoradiographed (Fuji-RX X-Ray film) with an intensifying screen (DuPont) at –80°C for 6-24 h or at room temperature without using any intensifying screen depending on the strength of signals. The film was developed in the dark, under safety lights as described in section 2.6.13 and air-dried.

2.6.12 DNA Sequencing

2.6.12.1 Sequencing using the automated dye terminator system

Automated sequencing was performed using the ABI-PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). A reaction mixture of final volume of 20 μl was made by mixing 8 μl of Terminator Ready Reaction Mix (provided with the kit, which contained A-Dye Terminator, T-Dye Terminator, C-Dye Terminator, G-Dye Terminator, dITP [deoxyinosine triphosphate], dATP, dCTP, dTTP, Tris-HCl [pH 9.0], $MgCl_2$, pyrophosphatase, AmpliTaq DNA Polymerase) with 350 ng of template DNA and 3.2 ρmol of sequencing primer. This mixture was over layered with 40 μl paraffin oil and subjected to 25 cycles of PCR under the following conditions: denaturation (96°C, 30 s), annealing (50°C, 15 s) and elongation (60°C, 4 min).

2.6.12.2 Purification of automated dye terminator sequencing product

After removing the paraffin oil, the product of the sequencing reaction (section 2.6.12.1) was mixed with 2.0 μl of 3 M sodium acetate, pH 5.2 and 50 μl chilled ethanol in a 1.5 ml microcentrifuge tube. The mixture was vortexed, and then incubated at –20^{o}C to allow precipitation of nucleic acids. Following centrifugation at 12,000 x g for 30 min at 4^{o}C, the supernatant was removed, the pellet was then washed with 250 μl of 70% ethanol, re-centrifuged at 12,000 x g for 15 min and air-dried the pellet. The sequencing reaction pellet was resuspended in 4 μl of de-ionized formamide, 50 mM EDTA and pH 8.0 (5:1). After heating to 90^{o}C for 2 min, the samples were then loaded onto an Applied Biosystem 373A DNA Sequencer (Perkin Elmer, at Dept. of Microbiology, Monash University, Clayton, Australia) that utilizes a polyacrylamide gel for separating the samples.

2.6.13 Photography

Photographs of agarose gels, minimal inhibitory concentration plates and SDS-PAGE were taken with a Polaroid MP-4 Land Camera with black and white positive (Polaroid Polapan 667) or positive/negative (Polaroid Polapan 665) FILM using a yellow filter. Agarose gels were placed on a trans-illuminator (302 nm) (LKB 2011 Macrovue) and photos were taken with an orange filter in the dark. The camera shutter speed was 1-1/8 s and the f-stop was 5.6 for black and white positive film (667).

Negatives were immediately placed in water to remove the film lining. Once the black lining was removed, the negatives were washed in 18% Na_2SO_3. Negatives were fixed in Kodak Photoflow 600 solution (1/600 dilution in dH_2O) for 10 s and hung up to dry. X-ray films were developed in AGFA Gevaert G150 manual X-ray developer (1/5 dilution in dH_2O) for 3-5 min. Films were rinsed in H_2O for 1 min followed by fixing in Kodak Photoflow 600 solution (1/600 dilution in dH_2O) for 1-5 min. Films were rinsed in running water for 5 min and hung up to dry. The size of restriction or hybridization bands was determined from the positive and X-ray films by comparing the bands with the molecular size markers. The photographs were scanned on Hewlett Packard Officejet 6110.

CHAPTER 3

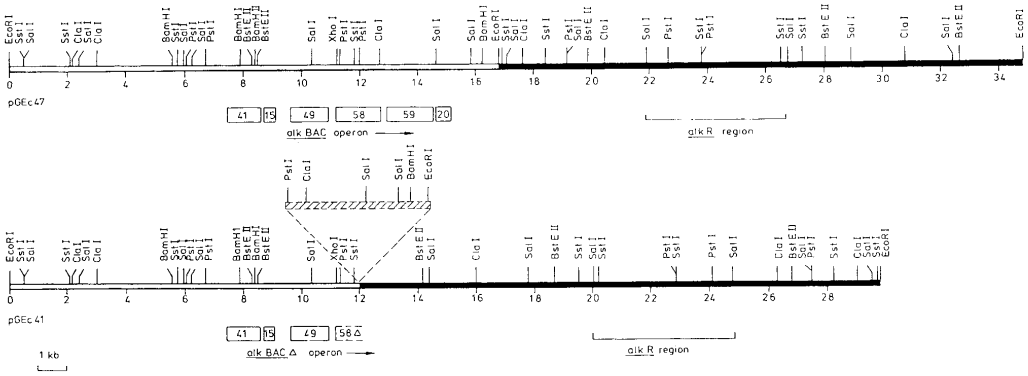
3 INVESTIGATION ON THE POSSIBLE FORMATION OF ADIPIC ACID FROM HEXANOIC ACID BY *PSEUDOMONAS* STRAINS

3.1 Introduction

The primary aim of the research presented in this chapter was to investigate the microbial conversion of hexanoic acid to adipic acid through a biochemical process called ω-oxidation and/or the effect of hexanoic acid on the bioconversion of alkanes into alkanoic acids by *Pseudomonas* strains. Several strains of *Pseudomonas* are able to grow in the presence of *n*-alkanes of 6 to 10 carbon atoms as the sole source of carbon and energy by virtue of the transmissible OCT plasmid (Baptist *et al*., 1963; Chakrabarty *et al*., 1973; Nieder and Shapiro, 1975). *n*-Alkanes are utilized in two distinct stages: they are first taken up and oxidized to carboxylic acids *via* the corresponding 1-alkanols and 1-alkanals, and then the acids are degraded in the main metabolic routes of the cell (Eggink *et al*., 1987) which involves β-oxidation. Alkane oxidation takes place by alkane hydroxylase regulated by the *alk* system, which is located on the OCT plasmid. The OCT plasmid encodes inducible alkane-hydroxylating and primary alcohol-dehydrogenating activities while the chromosome encodes for constitutive oxidizing activities for primary alcohols, aliphatic aldehydes, and fatty acids (Grund *et al*., 1975). Two operons are located on this plasmid; *alkBFGHJKL* (Eggink *et al*., 1987a; Kok *et al*., 1989b) which codes for most of the enzymes in the alkane oxidation pathway, and *alkST* (Eggink *et al*., 1988; 1990), which codes functions that allow *P. putida* to metabolize alkanes (Van Beilen, 1992), of which *alkS* positively regulates the expression of *alkB-L* (Fennewald *et al*., 1979; Eggink *et al*., 1987a; Eggink *et al*., 1988; Nieboer *et al*., 1993). The induction characteristics of *P. oleovorans* cells possessing OCT and CAM (Camphor degrading) plasmids suggest that the enzymes specified by the octane genes are induced by octane and repressed by *n*-octanol. There is a second octanol dehydrogenase, presumably specified by a chromosomal gene, which is induced only by octanol but not by octane (Chakrabarty and Gunsalus, 1971). Several chromosomal loci have been identified as being involved in alkanol, alkanal, and fatty acid oxidation: *alcA*, *aldA*, and *oic* (Grund *et al*., 1975; Eggink *et al*., 1987b). The octanol to octanoate conversion proceeds by two DPN-specific dehydrogenases-one for alcohol and the second for aldehyde (Peterson *et al*., 1966; Boyer *et al*., 1971; Chakrabarty *et al*., 1973). DPN reduction is clearly dependent on the presence of octanol, and it is apparent that TPN cannot substitute for DPN (Baptist *et al*., 1963; Gholson *et al*., 1963). The *alkBAC and alkR* sequences have been cloned in various combinations in suitable broad host range vector pLAFR1 (derived from the plasmid RK2 and cloned in *E. coli* GEc137 strain) (Eggink *et al*., 1987b), represented as pGEc47 and pGEc41 and their restriction maps are shown in Figure 3.1. Plasmid

(pGEc47) contains all *alkB-L* and *alkST* sequences and encodes the entire alkane-to-alkanoic acid oxidation pathway. Structural components are encoded on the 7.5-kilobase pair *alkBAC* operon, whereas positive regulatory components are encoded by *alkR*.

Figure 3.1: DNA inserts in pLAFRI carrying *alkBAC* and *alkR* sequences.



Open lines represent DNA sequences derived from the 16.9 kb *Eco*RI fragment containing *alkBAC* sequences. Solid lines represent DNA derived from the 18 kb *Eco*RI fragment which contains *alkR*. pGEc47 consists of pLAFRI, the 16.9- and 18- kb *Eco*RI fragment carrying the *alkBAC* operon and the *alkR* locus, resepectively. pGEc41 consists of pLAFRI and a 30- kb *Eco*RI fragment carrying the *alkR* locus and part of the *alkBAC* operon. The dashed area down in the middle part of the figure refers to the down stream deletion of the *alkBAC* operon. The boxes represent size and position of the proteins encoded by *alkBAC*, and the numbers in the boxes refer to the molecular masses in kilodalton. The region encoding the *alkR* activity is represented by a thin line. Note that the *alkR* sequence has the same orientation relative to the *alkBAC* sequence in pGEc41, whereas its orientation is reversed in pGEc47 (Eggink, *et al.*, 1987b).

The *alkR* locus was strictly necessary in *E. coli* as well as in *P. putida* for expression of the *alkBAC* operon (Eggink *et al*., 1987b). Another plasmid (pGEc41) which consists only *alkBFG(H)* and *alkST* sequences and therefore encodes only alknae hydroxylase activity rather coding for an entire alkane-to-alkanoic acid oxidation pathway. However, alkanol can be oxidized further by the chromosomally encoded alcohol dehydrogenase. But in *P. putida* PpS81, which has an *alcA* mutation in the chromosome, and there is neither plasmid nor chromosomal alcohol dehydrogenase and as a result, alkanol is not oxidized to alkanal (Eggink *et al*., 1987b). In this study, *Pseudomonas* and recombinant *E. coli* strain used has no OCT plasmid or OCT plasmid with genetic modification and though codes for different enzymes involved in the bioconversion of alkanes into alkanoic acids.

The general aim of research reported in this chapter was to investigate the accumulation of adipic acid at any stage as a metabolic intermediate. In particular, the specific aims were to:

1. observe the growth of *Pseudomonas* strain on *n*-octane or *n*-octanol in the presence and absence of *n*-hexanoic acid for the validation of their phenotype;
2. investigate the transient accumulation of adipic acid (in particular) or any other alkanoic acids in general, at any stage of growth on alkane or alkanol as an intermediate product, targeting use of long-term cultures (metabolically remain active in stationary phase of growth);
3. determine the effect of *n*-octane or *n*-octanol on the induction of plasmid and chromosomal borne genes, encoding alkane oxidation in the *Pseudomonas* strains in the presence of *n*-hexanoic acid; and
4. determine the effect of *n*-hexanoic acid on the bioconversion products during long-term incubations of *Pseudomonas* cultures in the presence of *n*-octane or *n*-octanol vapours.

3.2 Results

3.2.1 Growth of *Pseudomonas* strains on specific media: validation of phenotype

Pseudomonas strains with or without recombinant *alk* plasmids (in *E. coli*) were grown on E2 medium agar plates and tested for growth on *n*-octanol or *n*-octane vapours as described in Materials and Methods. Table 3.1 shows the results obtained for strain PpS145, (*met*-145, *alcA*$^{-}_{81,}$ CAM-OCT *alk*$^{+}$ *alcO*$^{+}$), PpS201 (*met*-145 *alcA*$^{-}_{81}$ CAM-OCT *alkB*201), PpS205 (*me*t-145, *alcA*$^{-}_{81,}$ CAM-OCT *alkB*205), PpG1 (Wild type, *alcA*$^{+}$, no plasmid), Gpo1 (OCT), and Gpo12 (Gpo1 cured of OCT). Strain PpS145 has the alkane utilization loci of the OCT plasmid that permits growth on *n*-octane vapours when methionine is added on E2 agar plates and this was observed when tested. Strain PpG1 and Gpo12 grew on solid E2 media due to the presence of the *alcA* and *AldA*$^{+}$ loci on the chromosome respectively, which was induced by *n*-octanol vapours and are involved in alkanol, alkanal, and fatty acid oxidation. Strain PpS201 and PpS205 grew on E2 agar plates in the presence of *n*-octanol vapours when provided with methionine due to mutation in *alkB* gene of the *alkBAC* operon. Strain Gpo1 shows growth on E2 agar plates in the presence of *n*-octane vapours as sole carbon and energy source without amino acid supplements, consistent with the presence of OCT in a prototrophic background. Plasmid pGEc47 consists of pLAFRI, the 16.9- and the 18-kb *Eco*RI fragment carrying *alkBAC* operon of OCT and the *alk*R locus, respectively. Plasmid pGEc41 hosted in pLAFRI consists of a 30-kb *Eco*RI fragment carrying the *alk*R locus and *alkBFGH* of the *alkBAC* operon. Both plasmids allowed growth on E2 agar plates in the presence of *n*-octane vapours when thiamine was added and thus shows only the alkane hydroxylation system is necessary for growth on octane.

Table 3.1: Growth of *P. putida* and *E. coli* transformed with OCT-related plasmids on different media.

Strains/ Plasmids	Relevant genotype or phenotype	Growth on				Reference
		E2A with		LA with		
		Octane vapours	Octanol vapours	Octane vapours	Octanol vapours	
Pseudomonas putida						
PpG1	Wild type, *alcA* (no plasmid)	-	+	+	+	Chakrabarty *et al*., 1973
PpS145	*met*145, *alcA81* (CAM-OCT *alk*$^+$ *alcO)*[a]	+	+	+	+	Grund *et al.,* 1975
PpS201	*met*145, *alcA81* (CAM-OCT *alkB*201)[a,b]	-	+	+	+	Benson *et al*., 1977
PpS205	*met*145, alcA81 (CAM-OCT *alkB*205)[a,b]	-	+	+	+	Benson *et al*., 1977
Gpo1	Prototroph (OCT)	+	-	+	+	Schwartz and McCoy, 1973
Gpo12	Gpo1 cured of OCT, *AldA*	-	+	+	+	Kok, 1988
E. coli GEc137 (DH1, fadR) carrying plasmids						Eggink *et al*., 1987b
pLAFRI	Tc, Tra$^-$, Mob$^+$, *cos*, RK2 replicon	+	-	+	-	Friedman *et al*., 1982
pGEc47	pLAFR1, *alkST/alkBFGHJKL*[c]	+	-	+	-	Eggink *et al*., 1987b
pGEc41	pLAFR1, *alkST/alkBFGH*[c]	+	-	+	-	Eggink *et al*., 1987b

[a]OCT and CAM-OCT are, respectively, the wild type octane plasmid (Chakrabarty *et al*., 1973) and the fused plasmid (Chakrabarty, 1973) carrying the replication, transfer, and camphor utilisation loci of the *P. putida* CAM plasmid (Rheinwald *et al*., 1973) and the alkane utilisation loci of the OCT plasmid (Chakrabarty *et al*., 1973), grown on carbon source when 0.01% methionine is added. The *alcA* locus is the chromosomal locus, which codes for growth on *n*-octanol (Grund *et al*., 1975). The *alc*O locus is the plasmid locus, which permits growth on octanol in the presence of an *alc*A$^-$ (lack an alcohol dehydrogenase activity) mutation. The *aldA* locus is the chromosomal locus, which codes for growth on aliphatic aldehydes. *fadR* is a chromosomal fatty acid degradation regulatory mutant. [b]Grown on *n*-octanol vapour. [c]Grown on *n*-octane vapour in the presence of 0.001% thiamine. E2A (E2 agar), LA (Luria agar), + (growth), - (no growth), Tc (tetracycline resistance), Tra$^-$ (RK2 transfer function negative), Mob$^+$ (efficiently mobilized from *E. coli* to *Pseudomonas*),

3.2.2 Utilization of *n*-octanol or *n*-octane and *n*-hexanoic acid for growth

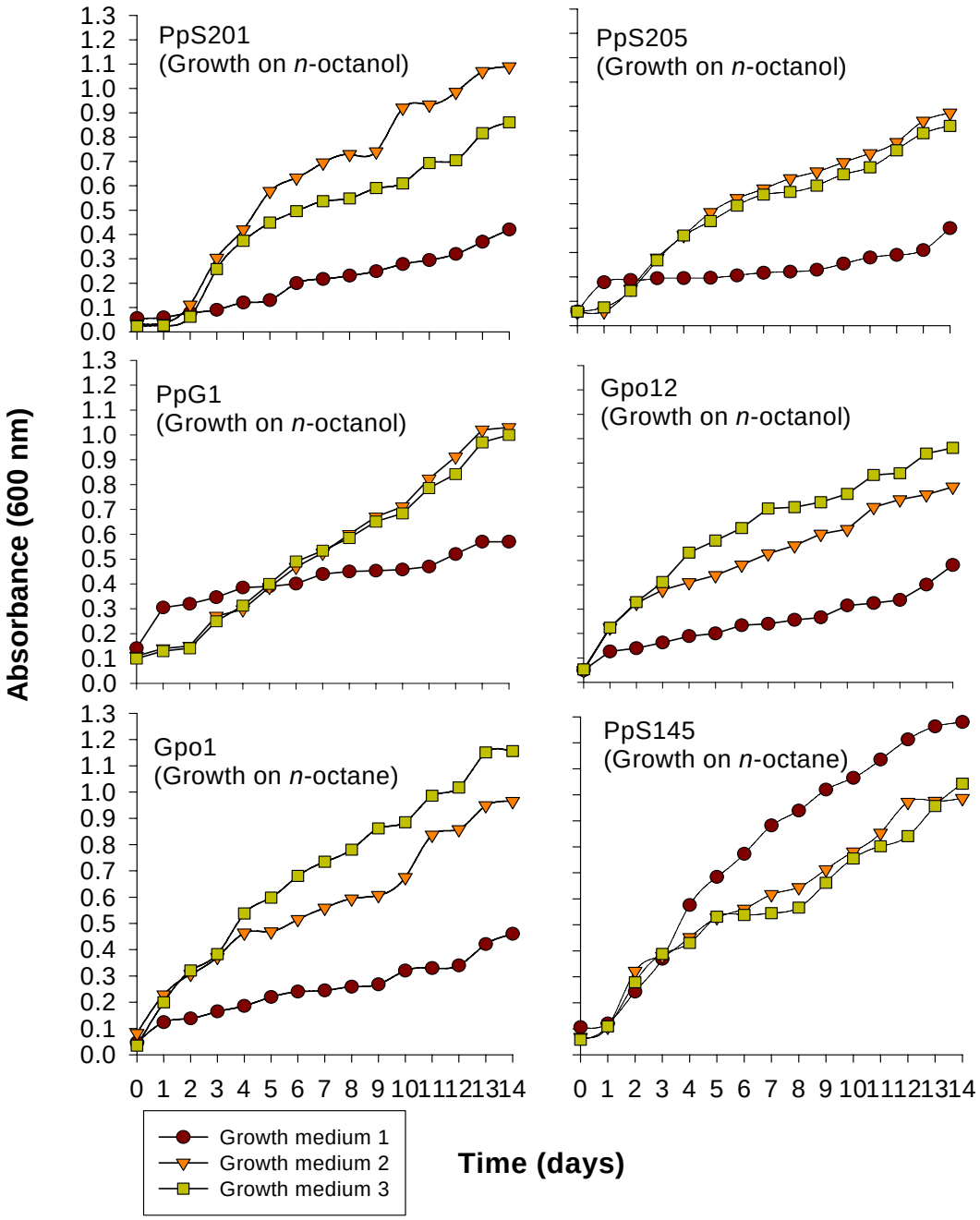
A time course of growth by *Pseudomonas* strains with three different treatments was undertaken in flasks with constant shaking. Samples (1 ml) were withdrawn at the indicated times and were used to measure growth (A_{600}) for PpS201, PpS205, PpG1, Gpo12, Gpo1, and PpS145 (Figure 3.2). Growth of all the strains studied in the presence of *n*-octanol, or *n*-octane vapours, was very low compared to those supplemented with neutralized *n*-hexanoic acid, or on neutralized *n*-hexanoic acid alone, except for strain PpS145. Strains (PpS201, PpS205 and PpG1) showed maximum growth with *n*-octanol vapours in the presence of neutralized *n*-hexanoic acid, while strain (Gpo12) showed maximum growth with neutralized *n*-hexanoic acid in the absence of *n*-octanol vapours. Strain PpS145 shows maximum growth in the presence of *n*-octane vapours alone, while strain Gpo1 comparatively showed better growth with neutralized *n*-hexanoic acid in the absence of *n*-octane vapours.

3.2.3 Detection and identification of acidic metabolic product

Pseudomonas strains were tested for their ability to accumulate adipic acid on three different treatments for the period of 52 weeks. The aqueous phase was tested for the presence of adipic acid. Samples (1 ml) were withdrawn, centrifuged and the aqueous supernatant was analysed by gas chromatography (GC) and mass spectroscopy (MS) as described in Materials and Methods. Adipic acid could not be found in any of the samples studied, although the low level of sensitivity detectable easily for standard adipic acid was 0.2 mM accomplished by gas chromatography as shown in appendix-2 Figure A-13. However, one peak was found in the chromatogram of the methanolized aqueous sample. This peak had a retention time of 10.8 min, which corresponds to that found for the chemically synthesized *n*-octanoate methyl ester (Figure 3.3a and b). The molecular weight and the structure of the GC peak component were further confirmed by GC-MS. The electron ionization spectra of both standard and culture samples are identical and showing major peaks at 127, 115, 101, 87, and 74 m/z (Figure 3.3a and b). Gas chromatogram for standards such as adipic acid, *n*-octanoic acid and other volatile fatty acids is shown in appendix-2 and the results are summarized in Table 3.2. Strain PpS201, PpS205, PpG1 and Gpo12, were grown very slowly in E2 medium containing *n*-octanol vapours. However, strain PpS145 tested on *n*-octane vapours in E2 medium showed better growth but failed to produce any *n*-octanoic acid in the conditions provided as shown in Figure 3.2. Strain PpS201 and PpS205 showed no *n*-octanoate accumulation after two weeks with *n*-octanol vapours alone, compared with those incubated with *n*-octanol vapours in the presence of neutralized *n*-hexanoic acid. Also, the amount of *n*-octanoate produced by PpS201 and PpS205 in the presence of *n*-octanol vapours alone was lower compared to those grown in the presence of *n*-octanol vapours supplemented with 0.4%-neutralised *n*-hexanoic acid. By contrast, strain PpG1 and Gpo12 produced significant amounts of *n*-octanoic acid when they were grown on *n*-octanol vapours alone compared to those grown on *n*-octanol in the presence of neutralized *n*-hexanoic acid. Although all the strains in first two weeks of incubation, the most of *n*-octanoic acid been produced with *n*-octanol vapours supplemented with neutralized *n*-hexanoic acid. No *n*-octanoic acid was produced by strains PpS145 and Gpo1 under any growth conditions tested.

Controls containing no bacteria showed no *n*-octanoic acid accumulation indicating that the reaction was cell dependent and did not occur spontaneously.

Figure 3.2: Growth of *Pseudomonas* strains in three specialised growth media.



Growth medium 1: E2 medium containing *n*-octanol/*n*-octane vapours; Growth medium 2: E2 medium containing *n*-octanol/*n*-octane vapours supplemented with 0.4% neutralised *n*-hexanoic acid; and Growth medium 3: E2 medium supplemented with 0.4% neutralised *n*-hexanoic acid.

Figure 3.3: GC/MS spectra of methyl esters of *n*-octanoic acid standard (a) and *n*-octanoic acid produced by the *Pseudomonas* strain from *n*-octanol utilization (b). The retention time for *n*-octanoic acid was 10.8 min. The electron ionization spectra for the standard and sample were identical.

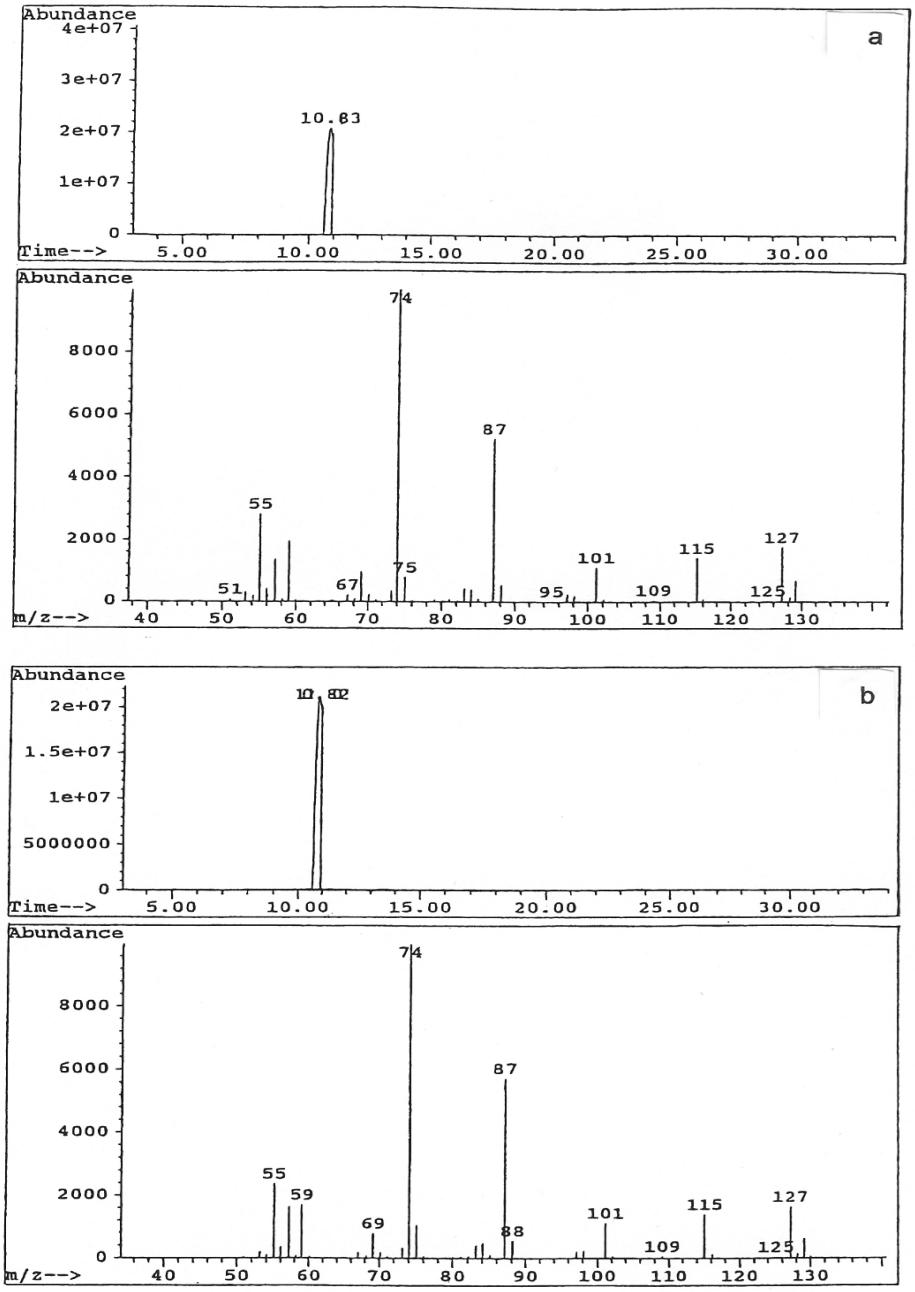


Table 3.2: Bioconversion of *n*-octanol into *n*-octanoic acid by alkanol induced strains of *P. putida*.

Strains/ Plasmids	Production of Octanoic Acid (mM)					
	E2 growth medium containing *n*-octanol vapours			E2 growth medium containing *n*-octanol vapours supplemented with 0.4% *n*-hexanoic acid		
	Time (Weeks)					
	2	16	52	2	16	52
PpS201 *met*145 *alcA*81 (CAM-OCT *alkB*201)	0 ± 0	4.35 ± 0.18	10.65 ± 0.82	2.47 ± 0.41	7.43 ± 0.43	20.90 ± 0.85
PpS205 *met*-145 *alcA*81 (CAM-OCT *alkB*205)	0 ± 0	4.62 ± 0.29	15.33 ± 0.50	2.42 ± 0.43	6.63 ± 0.48	23.97 ± 0.41
PpG1 Wild type, *alcA*$^+$ (no plasmid)	2.26 ± 0.28	7.70 ± 0.51	37.96 ± 0.82	2.32 ± 0.47	5.66 ± 0.71	23.67 ± 0.62
Gpo12 Gpo1 cured of OCT	0 ± 0	3.91 ± 0.49	18.78 ± 0.59	2.38 ± 0.48	3.12 ± 0.25	7.80 ± 0.63

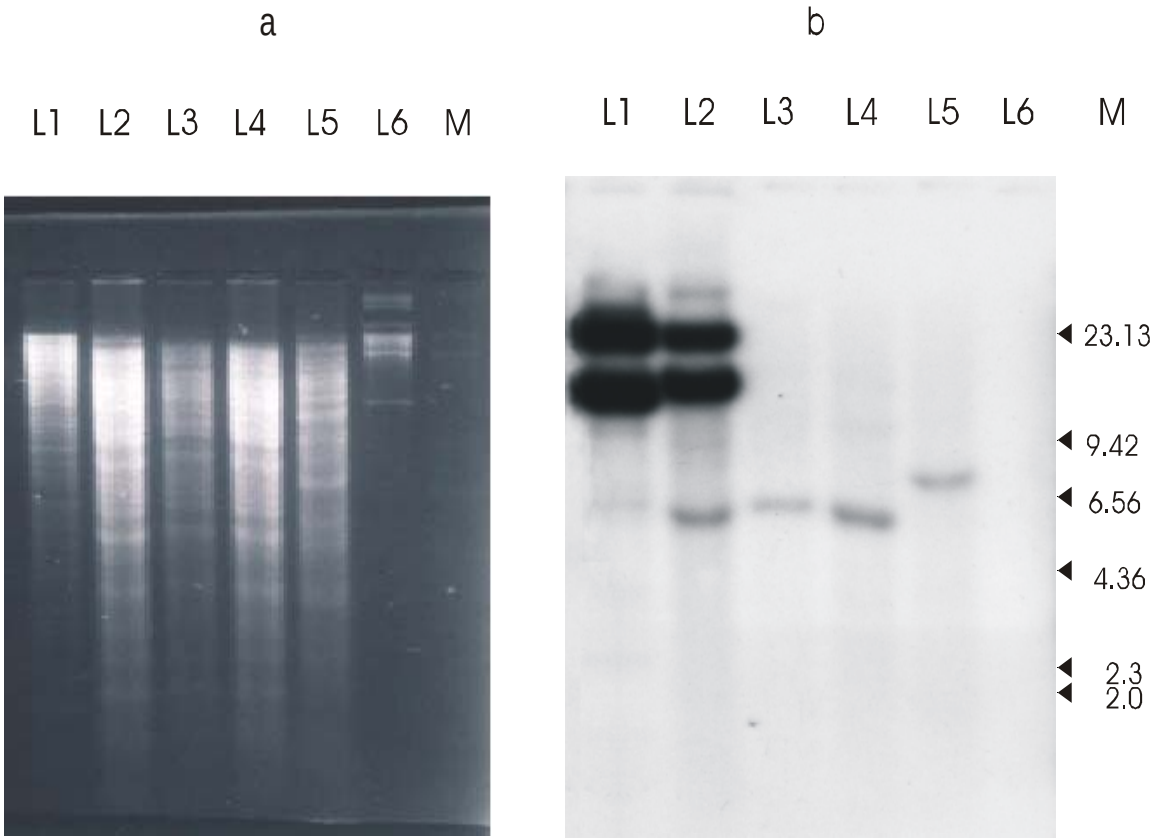
Values are means ± standard deviations of triplicate determinations. No variations in the liquid volume (hence in the determination of the true concentrations) over the 52 weeks. A control sample which contained growth medium with all the supplements was incubated under the same conditions without any inoculums was not able to produce any *n*-octanoic acid.

3.2.4 Detection of *alkBAC* genes using Southern blot analysis

As discussed in the introduction to this chapter, Eggink *et al*. (1987) used a *Bst*EII digest of pGEc47 to confirm the presence of the *alkBAC* operon (see Figure 3.1). This thesis used the same approach develop by these authors to confirm the presence of *alkBAC* genes using *Bst*EII digested genomic DNA of *Pseudomonas* strains. Multiple restriction sites for *Bst*EII were present in 16.9 Kb *Eco*RI fragment containing *alkBAC* sequences and 18 Kb *Eco*RI fragment which contained *alkR* sequences (Eggink *et al*., 1987). The restriction enzyme *Bst*EII was also used for the characterization of the OCT plasmid encoding alkane oxidation and Mercury resistance in *P. putida* (Harder and Kunz, 1986). Figure 3.4 shows the digested genomic DNA of *Pseudomonas* strains and that was run on TAE agarose gel at low voltage. The amounts of digested genomic DNA loaded were identical for all the samples, as indicated by ethidium bromide staining prior to blotting. In Figure 3.4a, the genomic DNA of *Pseudomonas* strains PpS145 (L1), PpS201 (L2), PpS205 (L3), PpG1 (L4), and Gpo1 (L5) shows complete digestion with *Bst*EII enzyme, while

Gpo12 (L6) shows incomplete digestion, transferred to nylon membrane and hybridized with ~4.0kb *Xho*I fragment of plasmid pGEc47 (Figure 3.4b).

Figure 3.4: Gel electrophoresis of *Bst*EII digested genomic DNA of *Pseudomonas* strains and their Southern blot analysis.



Panel a: The agarose gel of the digested genomic DNA of *Pseudomonas* strains PpS145 (L1), PpS201 (L2), PpS205 (L3), PpG1 (L4), Gpo1 (L5) and Gpo12 (L6). M is the molecular weight marker of the various fragments was determined by using λ phage DNA that had been digested with *Hind*III.

Panel b: The Southern blot analysis of *Bst*EII digested genomic DNA of Pseudomonas strains as indicated in panel a. DNA probe was derived from 4.0-kb *Xho*I fragment of pGEc47 plasmid DNA, and labeled as described in Materials and Methods. The sizes of the bands are indicated in kilobasis.

The 49 kDa peptide is soluble component of the alkane hydroxylase complex and was located between the *Hind*III and *Xho*I sites of a 16.9 kb *Eco*RI fragment containing the complete *alkBAC* operon (Eggink *et al*., 1987). The restriction enzyme *Xho*I has unique restriction site and was used to get the fragment of *alkBAC* operon for hybridization. The Southern blot (Figure 3.4b) of strain PpS145 (L1) and PpS201 (L2) showed hybridization of a 21 kb, 17 kb, and ~6.0 kb *Bst*EII fragments when probed with the 4.0 kb *Xho*I fragment of pGEc47 probe. Strain PpS205 (L3) and Gpo1 (L5) show ~6.0 kb hybridized band only, indicating the loss of plasmid during storage or DNA preparation, whereas PpG1 (L4) (prototroph, no plasmid), shows very weak hybridization signal of a ~6.0 kb *Bst*EII fragment, indicating the presence of a chromosomal gene that reacts slightly with the probe. While Gpo12 (L6) (cured of OCT) did not digest with the *Bst*EII as mentioned above, and therefore could not show any signal with this probe. The presence of hybridization signals of ~6.0 kb *Bst*EII by PpG1 (prototroph, no plasmid) indicate cross reactivity with chromosomal genes.

3.3 Discussion

Adipic acid was not detected as an intermediate at any stage of growth, including very old cultures (metabolic enzymes activities remain active in static culture), in contrast to previous reports (Kunz and Weimer, 1983; Thijsse, 1964) by using whole cells and crude cell extracts of alkane-utilizing pseudomonads, studies on adipic acid production by *Pseudomonas* strains was not pursued further in this thesis.

The conversion of *n*-octanol into *n*-octanoic acid by the *Pseudomonas* strains occurred in all of the cultures where octanol was added as substrate (Table 3.2). Since most *Pseudomonas* strains grow on octanol, and since the octanol-degrading enzymes appear to be governed by chromosomal genes, the plasmid-borne genes for the synthesis of octanol-degrading enzymes are presumably redundant (Chakrabarty *et al*., 1973). Cell-free, soluble enzyme extracts of *P. oleovorans* strains catalyzed the oxidation of radioactive octane to radioactive octanoic acid (Baptist *et al*., 1963). Favre-Bulle *et al*., 1993 reported the continuous bioconversion of *n*-octane to octanoic acid by recombinant *E. coli* (alk^+) grown in a two-liquid-phase chemostat. Rothen *et al*., 1998 also reported the biotransformation of octane by *E. coli* HB101 [pGEc47] to octanoate, was grown on defined medium with glucose as carbon source in batch and continuous culture. The plasmid and chromosomal loci of *P. putida* strains involved in the oxidation of alkanes (Grund *et al*., 1975). The product of the *alcA* locus on the *P. putida* chromosome is likely an *n*-octanoic acid, produced by the PpG1 and Gpo12 in the absence of OCT plasmid (Table 3.2). The *alcA* gene is involved in alkanol oxidation as well as *alkC* that encode a membrane-bound alkanol dehydrogenase (Grund *et al*., 1975; Benson *et al*., 1979). The partial involvement of the chromosomal *alcA* allele in the assimilation of ethylbenzene was also suggested (Fukuda *et al*., 1989). The inability of *n*-hexanoic acid alone grown cells to biotransform *n*-octanol indicated that this reaction was cell dependent and did not occur spontaneously.

The time courses of growth of *Pseudomonas* strains on three different treatments are described in the text. Strain PpS201 and PpS205 expressing CAM-OCT plasmid show maximum growth and production of *n*-octanoic acid in the presence of *n*-octanol vapour supplemented with neutralized hexanoic acid (Figure 3.2 and Table 3.2 respectively). While strain PpG1 and Gpo12 grew well in the presence of *n*-hexanoic acid with *n*-octanol vapours, but produce a maximum concentration of *n*-octanoic acid in the absence of *n*- hexanoic acid provided with *n*-octanol vapours (Figure 3.2 and Table 3.2 respectively). All *Pseudomonas* strains grew well on hexanoic acid alone, but no traces of *n*-octanoic acid were detected in the culture fluid. The reason to follow up the growth pattern for only two weeks of incubation was the small-volume culture used. The growth of strains on *n*-octanol vapours was quite stationary after a couple of days but *n*-octanoic acid concentration increased gradually up to the duration of the studies. This study provides the first report of significant amount of *n*-octanoic acid production in *Pseudomonas* strains culture with and without OCT plasmid. These are potentially useful bioconversion strains, which accumulate these fatty acids when the corresponding alkanols are present in the medium as a bulk gaseous phase.

The accumulation of *n*-octanoic acid was observed when all the studied organisms were grown on *n*-octanol vapours only. The strain Gpo1 and PpS145 grown on *n*-octane (Figure 3.2), did not show any sign of *n*-octanoate accumulation probably due to OCT plasmid loss or inactivation of alkane hydroxylase activity (Eggink *et al*., 1990) which converts *n*-octane into *n*-octanol, further brings the substrates under chromosomal genes activation for the complete conversion of *n*-octane into *n*-octanoic acid. The accumulation of *n*-octanoic acid was highest in *n*-octanol induced cells of PpG1 (wild type, no plasmid) within two weeks of incubation. The strains PpS201, PpS205, and Gpo12 were grown in the presence of *n*-octanol vapours did not produced *n*-octanoic acid within two weeks time, while strain PpG1 showed a significant amount of *n*-octanoic acid. In contrast, all the strains grown in the presence of *n*-octanol vapours supplemented with hexanoic acid produced significant amount of *n*-octanoic acid in two weeks incubation. The strains PpS201 and PpS205 with CAM-OCT plasmid produced more *n*-octanoate in the presence of *n*-octanol vapours with hexanoic acid, showing that hexanoic acid facilitates the production of octanoic acid in conjunction with *n*-octanol vapours, when compared with growth on *n*-octanol vapours only. Strains PpG1 and Gpo12, without OCT plasmid, produced more *n*-octanoate in the presence of *n*-octanol vapours without hexanoic acid supplement, showing that hexanoic acid may play a role in the repression of *alcA* chromosomal gene.

Cell suspensions grown on *n*-octanol vapours in the presence and absence of neutralized *n*-hexanoic acid converted *n*-octanol into *n*-octanoic acid as high as 38 mM in concentration when incubated for longer time periods (Table 3.2). Proof of the identity of *n*-octanoic acid in these incubations was provided by gas chromatography-mass spectroscopy of reaction product derivatized as their methyl esters. Both biologically formed and authentic *n*-octanoic acids gave the same retention times on gas chromatography (10.8 min) and also gave identical fragmentation pattern on GC-MS spectra (Figure 3.3a and b).

The production of *n*-octanoic acid at such high concentration by these strains shows that they are blocked or repressing the β-oxidation pathway (Nunn, 1986), and keep on accumulating *n*-octanoic acid in long-term incubating cultures. Genomic DNA isolated from *Pseudomonas* strains were digested with *Bst*EII and equal amounts analyzed by Southern blotting, with radiolabeled OCT gene probe. Strain [PpS201, PpS205 (CAM-OCT)] showed hybridization of 20-kb, 14-kb, and 7-kb *Bst*EII fragments with the OCT probe, whereas PpG1 (prototroph), and Gpo12 (cured of OCT) showed only the hybridization of a 7-kb *Bst*EII fragment with the 4-kb *Xho*I fragment of pGEc47 plasmid probe, indicating a minor cross-reactivity with the probe (Figure 3.4b).

Continued production of octanoic acid over extended periods showed that cells or enzymes remained active over very long period (up to 52 weeks) as evidenced by continued production of octanoic acid. This was probably only possible due to the lack of β-oxidation activity, as this activity would normally utilise octanoic acid *via* energy metabolism. This is an interesting phenomenon in terms of demonstrating the total metabolic capabilities of these *Pseudomonas* strains.

CHAPTER 4

4 CHARACTERIZATION OF *MEGASPHAERA ELSDENII* STRAINS ISOLATED AS 3-FLUOROPYRUVATE RESISTANT (3FP^r) MUTANTS

4.1 Introduction

M. elsdenii metabolises glucose to acetic, butyric and hexanoic acids and lactate to propionic acid *via* the acrylate pathway then to valeric acid, with acetic and butyric acids as minor products. The range of acids produced, which could be used as chemical feedstock for conversion into dicarboxylic acids, alkanes, ketones and esters or used directly, makes it an interesting model for studies on regulation of intermediary metabolism. Microorganisms are generally sensitive to the environment to which they are exposed and so the conditions for handling the microorganisms and for fermentations require stricter control than for conventional chemical reactions. It is therefore necessary to optimise the conditions for growth of cells and for consequent product synthesis in order to increase the productivity of the volatile fatty acids. For such reactions, it has been found that maintenance of aseptic conditions, and control of temperature and pH are necessary since these factors have a major influence on the productivity of microorganisms.

The organism can utilize glucose; maltose, sucrose or lactate as sole carbon source and when glucose, maltose and lactate occur together in a growth medium the organism uses all three (Russell and Baldwin, 1978). The substrate preferences of *M. elsdenii* are, in order; glucose, maltose, lactate and sucrose (Russell and Baldwin, 1978; Russell *et al*., 1979). Incubations with *M. elsdenii* indicated that affinity was lower for lactate than for glucose and that affinity was very low for maltose (Russell and Baldwin, 1979). The major volatile fatty acids of glucose fermentation by the strictly anaerobic *M. elsdenii* strain ATCC 25940 are hexanoic and butyric acids (Roddick and Britz, 1997).

Growth of *M. elsdenii* is inhibited by its end-products of glucose and lactate fermentation, and some halogenated analogues of these are inhibitory. A series of 3FP^r mutants of *M. elsdenii* parent strain ATCC 25940, T-81, and B159 have been isolated (Margaret Britz personal communication). Preliminary tests showed that these mutants had been altered the ratio of end-products suggesting that their intermediary metabolism had been altered, which suggested that the flow of carbon *via* pyruvate as a key intermediate in intermediary metabolism had changed (Britz unpublished data).

The first part of this chapter describes the characterization of these mutants to:

a) validate their phenotype from stored cultures (freeze-dried);

b) determine their sensitivity to 3FP and other antimetabolites (halogenated end-products and their unhalogenated form); and

c) determine the changes in end-product formation in controlled fermentation systems operated under optimum pH and temperature conditions, using single and mixed carbon sources.

The toxicity of 3-fluoropyruvate perhaps depends on its ability to influence pyruvate dehydrogenase complex and pyruvate carboxylase in sensitive strains; changes in these enzymes may be responsible for varied product formation profile. It was observed that in 3-fluoropyruvate resistant strains of *C. thermocellum*, lactate dehydrogenase activity was not drastically affected and lactate production was only delayed (Tailliez *et al*., 1989). Further, the resistant strains of *C. thermocellum* became ethanol tolerant and asporogenous. However, due to lack of genetic analysis, it is difficult to conclude whether 3-fluoropyruvate resistance was affecting its own transport across the membrane or else responsible for ethanol tolerance and asporogenous nature of the mutant (Tailliez *et al*., 1989). Marounek *et al*., (1989) reported that *M. elsdenii* is relatively insensitive to many antimicrobial compounds but no reports on 3-fluoropyruvate resistance on *M. elsdenii* were available in literature searched.

The second part of this chapter describes the evaluation of ion-exchange resins for their utility in demonstrating end-product changes under conditions where end-products inhibition is removed. This compares using Amberlite IRA-400 with Amberlite IRA-93 in parent strain ATCC 25940, and was not pursued in the mutant strains due to shortage of time and would not have shown much new information towards the mechanism of adsorption and desorption of end-products to resins studied.

The main objectives of the work reported in this part of the chapter were,

1. to examine the effect of fermentation broth on the adsorption of *n*-hexanoic and *n*-butyric acids by the ion exchange resins.
2. to examine the desorption of *n*-hexanoic acid and *n*-butyric acids from the ion exchange resins by esterification and to recover it as its ester form.

Before ion exchange resin could be applied to actual fermentations, it was important to examine the adsorption and desorption of VFAs from the resin. Since the main product *n*-hexanoic acid, has a negative charge in neutral solution, anion exchange resins were used in this study. Amberlite IRA-400, a strong base anion exchange resin, was chosen and the adsorption of *n*-hexanoic and *n*-butyric acids, the main products of *M. elsdenii*, were investigated under conditions similar to those of the fermentation. Amberlite IRA-93 (weakly basic anion resin) was also tested as an alternative. Further, the esterification of the adsorbed *n*-hexanoic acid was examined using ethanol/sulfuric acid mixtures. Generally three methods for desorption of VFAs from ion exchange resins have been used in previous studies (Deguchi, 1994). One method is to use alkali and acid solutions, the second is to use organic solvents and the third is to use mixtures of organic solvents and alkali or acid solutions. Roddick and Britz (1986) showed that 92% of the *n*-butyric acid and 31% of the *n*-hexanoic acid that had been adsorbed to the strong base anion exchange resin Amberlite IRA-400 was desorbed by both acid and alkali solutions. They suggested that the VFAs that were bound by molecular adsorption were not desorbed by this regime.

4.2 Results

4.2.1 Minimal inhibitory concentrations (MICs) of parent and mutant strains

MICs (the concentration of antimetabolites that significantly inhibited the growth of bacteria) were estimated by spotting undiluted cultures onto THBAG and THBAL (Todd-Hewitt broth solidified with 1.5% agar, supplemented with either 0.8% glucose or lactate, 6% horse blood and 0.05% cysteine-HCl) by using the replica plate technique. MICs for several halogenated analogues of end-products or intermediates of metabolism were tested on the growth sensitivity of three parental strains (ATCC 25940, B159 and T-81) and seven spontaneous mutant strains (ME5, ME7, ME8, ME9, ME12, ME14 and 28.7B). Minimal inhibitory concentrations of the following antimetabolites (abbreviations in parenthesis) and levels used are: pyruvic acid (PA), 0.5-3.0 mg; 3-fluoropyruvate (3-FP), 0.5-2.0 mg; hexanoic acid (HA), 0.05-3.0 mg; 6-bromohexanoyl chloride (6-BHC), 0.1-5.0 mg; acetic acid (AA), 0.5-5.0 mg; bromoacetic acid (BAA), 0.05-0.50 mg; butyric acid (BA), 0.05-5.0 mg; 2-bromobutyric acid (2-BBA), 0.1-5.0 mg; 4-bromobutyric acid (4-BBA), 0.05-5.0 mg; 4-chlorobutyryl chloride (4-CBC), 0.1-5.0 mg; acrylic acid (Acry. A), 0.05-4.0 mg; 2-bromomethyl acrylic acid (2-BMAA), 0.5-2.5 mg; propionic acid (Prop. A), 0.05-5.0 mg; 2-bromoproponyl bromide (2-BPB), 0.5-2.0 mg; 3-chloropropionic acid (3-CPA), 0.05-5.0 mg; and valeric acid (VA), 0.05-5.0 mg. The results are summarized in Table 4.1 and 4.2. The plates of THBAG and THBAL without any antimetabolite were used as a control and the growth profile of all the strains studied are shown in appendix-2 Figure A-2.

4.2.2 MICs on THBAG agar

Differential sensitivity to the end-products and potential antimetabolites was detected on THBAG agar plates. For each group of parent and mutant strains, it was possible to compare resistance to growth inhibition (Table 4.1 and Figures 4.1 – 4.5). Group one mutant strain ME5 was seven times more resistant to 3-fluoropyruvate and showed resistance up to 3 mg/ml of 3-FP compared to its parental strain ATCC 25940 (Figure 4.3). Strain ME5 showed more sensitivity to pyruvic acid, hexanoic acid, 6-BHC, acetic acid, butyric acid, 4-CBC, 2-BMAA, 3-CPA and valeric acid compared to its parental strain ATCC 25940 (Figures 4.1, 4.2, 4.3, 4.4 and 4.5). Strain ME5 showed great sensitivity towards acrylic acid and 2-BPB and showed no growth on the lowest concentration of these compounds used. Strain ME5 was 10-times more sensitive to butyric acid compared to its parental strain ATCC 25940, and growth disappeared when a concentration of 0.3 mg/ml was reached as shown in Figure 4.2. Strain ME5 in the presence of glucose showed great sensitivity to valeric acid and showed MIC at 1 mg/ml, which was approximately four times more sensitive than most other strains studied (Figure 4.1). Parental strain ATCC 25940 growth was sensitive to 3-FP and MIC was 0.4 mg/ml compared to its mutant strain ME5 as shown in Figure 4.3.

Table 4.1: MICs (the concentration of antimetabolites that significantly inhibited the growth of bacteria) were estimated by spotting diluted cultures onto THBAG plates (Todd-Hewitt Broth solidified with 1.5% agar, supplemented with 0.8% glucose, 6% Horse blood and 0.05% cysteine-HCl).

Strain	Parent / Mutant group	MIC (mg/ml)														
		P.A	3-FP	H.A	6-BHC	A.A	BAA	B.A	2-BBA	4-CBC	Acrylic acid	2-BMAA	Propionic acid	2-BPB	3-CPA	V.A
ATCC 25940	Parent 1	3.0	0.4	0.9	3.0	5.0	0.2	3.0	1.0	2.0	0.3	2.0	1.5	2.0	1.0	4.0
ME 5	Mutant 1	2.0	3.0	0.2	2.0	3.0	0.1	0.3	1.0	0.6	–	0.5	2.0	–	0.1	1.0
ME 7	Mutant 1	2.0	1.0	2.0	3.5	4.5	0.25	3.0	1.0	2.0	0.4	1.5	2.0	1.5	1	4.0
B 159	Parent 2	2.0	–	0.8	2.5	4.0	0.4	3.0	1.0	2.0	0.3	2.0	1.5	2.0	0.5	4.0
ME 8	Mutant2	2.0	0.75	1.5	3	4.0	0.4	3.0	1.0	2.0	0.4	2.0	1.5	2.0	0.6	4.0
ME 9	Mutant 2	1.5	0.75	2.5	2.5	5.0	0.4	3.0	1.0	2.0	0.3	2.0	1.5	2.0	0.5	4.0
T-81	Parent 3	0.8	0.75	0.4	2.5	3.0	0.4	2.5	0.6	2.0	0.1	2.0	0.6	2.0	3	3.0
ME 12	Mutant 3	1.5	–	3.0	2.5	2.5	0.3	3	1.0	3.0	0.15	1.5	1.5	1.0	2.5	4.0
ME 14	Mutant 3	1.5	0.5	0.8	2.5	4.5	0.4	2.0	0.1	2.0	0.3	2.0	0.6	2.0	0.5	3.0
28.7B	Parent 4	1.5	0.75	0.8	3.0	4.5	0.25	2.0	1.0	2.0	0.4	1.0	0.6	1.0	0.5	3.5

Acids were neutralized using sodium hydroxide before addition to THBAG medium. P.A (pyruvic acid), 3-FP (3-fluoropyruvate), H.A (hexanoic acid), 6-BHC (6 bromohexanoyl chloride), A.A (acetic acid), BAA (bromoacetic acid), B.A (butyric acid), 2-BBA (2-bromobutyric acid), 4-CBC (4-chlorobutyryl chloride), Acry. Acid (acrylic acid), 2-BMAA (2-bromomethyl acrylic acid), Prop. Acid (propionic acid), 2-BPB (2-bromopropionyl bromide), 3-CPA (3-chloropropionic acid) and V.A (valeric acid), - (no growth shown on minimal concentration tested by these strains). Control plate showed growth of all parental strains and their mutants without additive. Colored shaded areas represent obvious differences between parental strain and their mutants. MICs were determined on two separate occasions.

Table 4.2: MICs (the concentration of antimetabolites that significantly inhibited the growth of bacteria) were estimated by spotting diluted cultures onto THBAL plates (Todd-Hewitt Broth solidified with 1.5% agar, supplemented with 0.8% lactate, 6% Horse blood and 0.05% cysteine-HCl).

Strain	Parent Mutant group	MIC (mg/ml)														
		P.A	3-FP	H.A	6-BHC	A.A	BAA	B.A	2-BBA	4-CBC	Acrylic acid	2-BMAA	Propionic acid	2-BPB	3-CPA	V.A
ATCC 25940	Parent 1	0.5	0.7	0.2	2.5	0.5	–	4.0	1.0	3.5	1.0	0.7	1	–	0.5	1.0
ME 5	Mutant 1	4.5	1.5	1.5	2.5	4.5	0.2	4.5	1.0	3.5	1.0	0.7	3.0	0.6	0.5	2.5
ME 7	Mutant 1	4.5	2.0	2.0	3.5	5.0	0.2	4.5	1.0	3.0	1.5	0.6	1.5	0.5	0.5	2.5
B 159	Parent 2	4	0.5	0.9	3.0	3.0	0.2	4.0	1.0	4.0	0.5	0.6	3.0	0.5	0.5	2.5
ME 8	Mutant2	4.5	2.0	2.0	5.0	5.0	0.2	5.0	1.0	3.5	1.5	0.8	4.0	0.65	0.5	2.5
ME 9	Mutant 2	4.5	2.0	2.0	5.0	4.5	0.2	4.5	1.0	2.0	1.5	0.8	4.0	0.6	0.5	2.5
T-81	Parent 3	2.0	–	–	2.0	2.5	0.15	3.0	–	2.0	0.1	0.6	3.0	–	1.0	0.5
ME 12	Mutant 3	4.0	–	3.5	2.5	3.0	0.25	2.0	1.0	2.0	0.1	0.9	3.0	0.45	1.5	3
ME 14	Mutant 3	4.5	2.0	2.0	2.5	5.0	0.2	2.5	1.0	2.0	1.5	0.9	3.0	–	0.3	2.0
28.7B	Parent 4	4.5	1.0	2.0	2.5	5.0	0.2	2.5	1.0	2.0	1.5	0.9	3.0	–	0.3	2.0

Acids were neutralized using sodium hydroxide before addition to THBAL medium. P.A (pyruvic acid), 3-FP (3-fluoropyruvate), H.A (hexanoic acid), 6-BHC (6 bromohexanoyl chloride), A.A (acetic acid), BAA (bromoacetic acid), B.A (butyric acid), 2-BBA (2-bromobutyric acid), 4-CBC (4-chlorobutyryl chloride), Acry. Acid (acrylic acid), 2-BMAA (2-bromomethyl acrylic acid), Prop. Acid (propionic acid), 2-BPB (2-bromopropionyl bromide), 3-CPA (3-chloropropionic acid) and V.A (valeric acid), - (no growth shown on minimal concentration tested by these strains). Control plate showed growth of all parental strains and their mutants without additive. Colored shaded areas represent obvious differences between parental strain and their mutants. MICs were determined on two separate occasions.

Figure 4.1: Minimal Inhibitory Concentrations (MICs) of a) pyruvic acid b) acetic acid and c) valeric acid (mg/ml) on the growth of *M. elsdenii* strains on THBAG and THBAL medium.

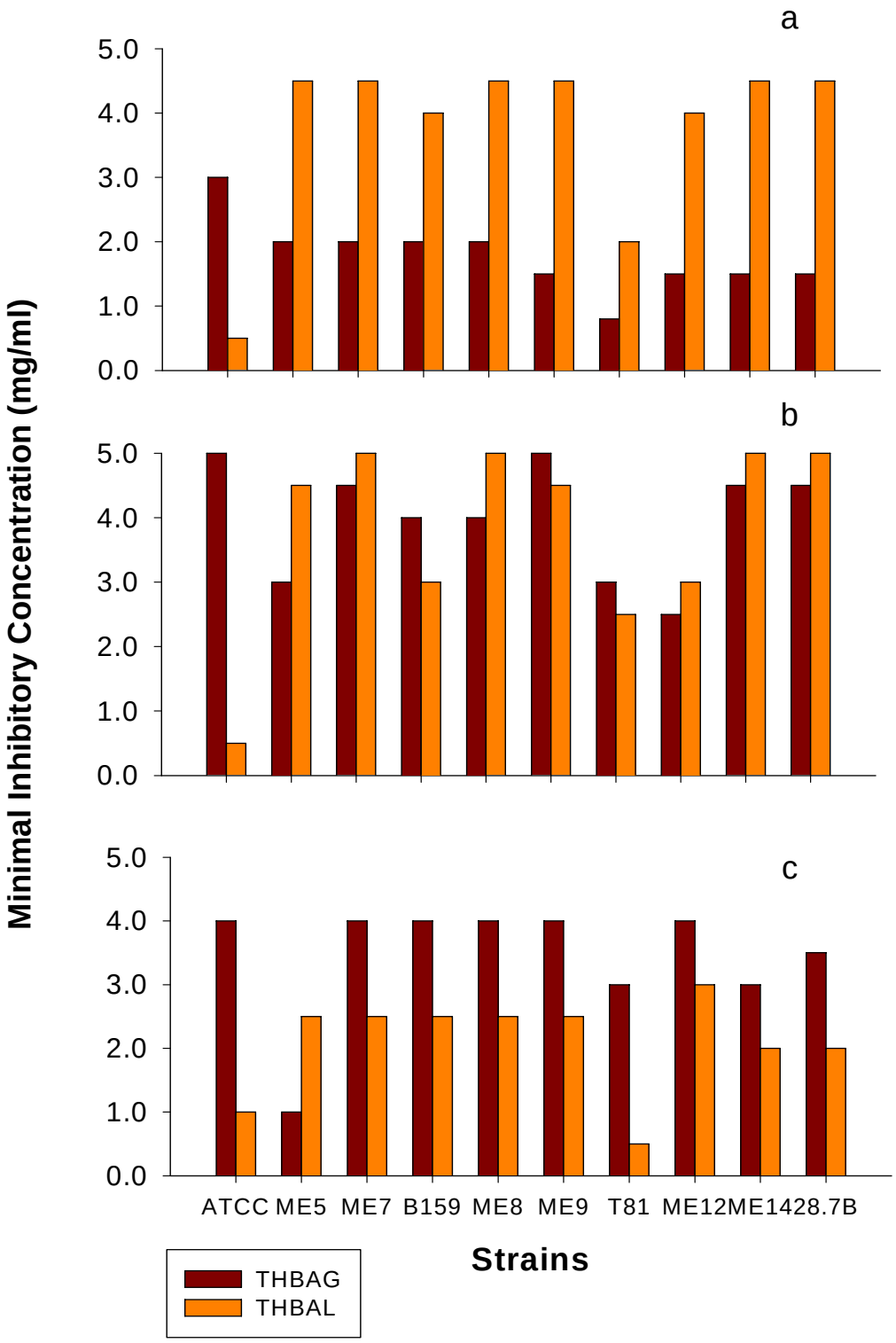


Figure 4.2: Minimal Inhibitory Concentrations (MICs) of a) butyric acid b) hexanoic acid and c) propionic acid (mg/ml) on the growth of *M. elsdenii* strains on THBAG and THBAL medium.

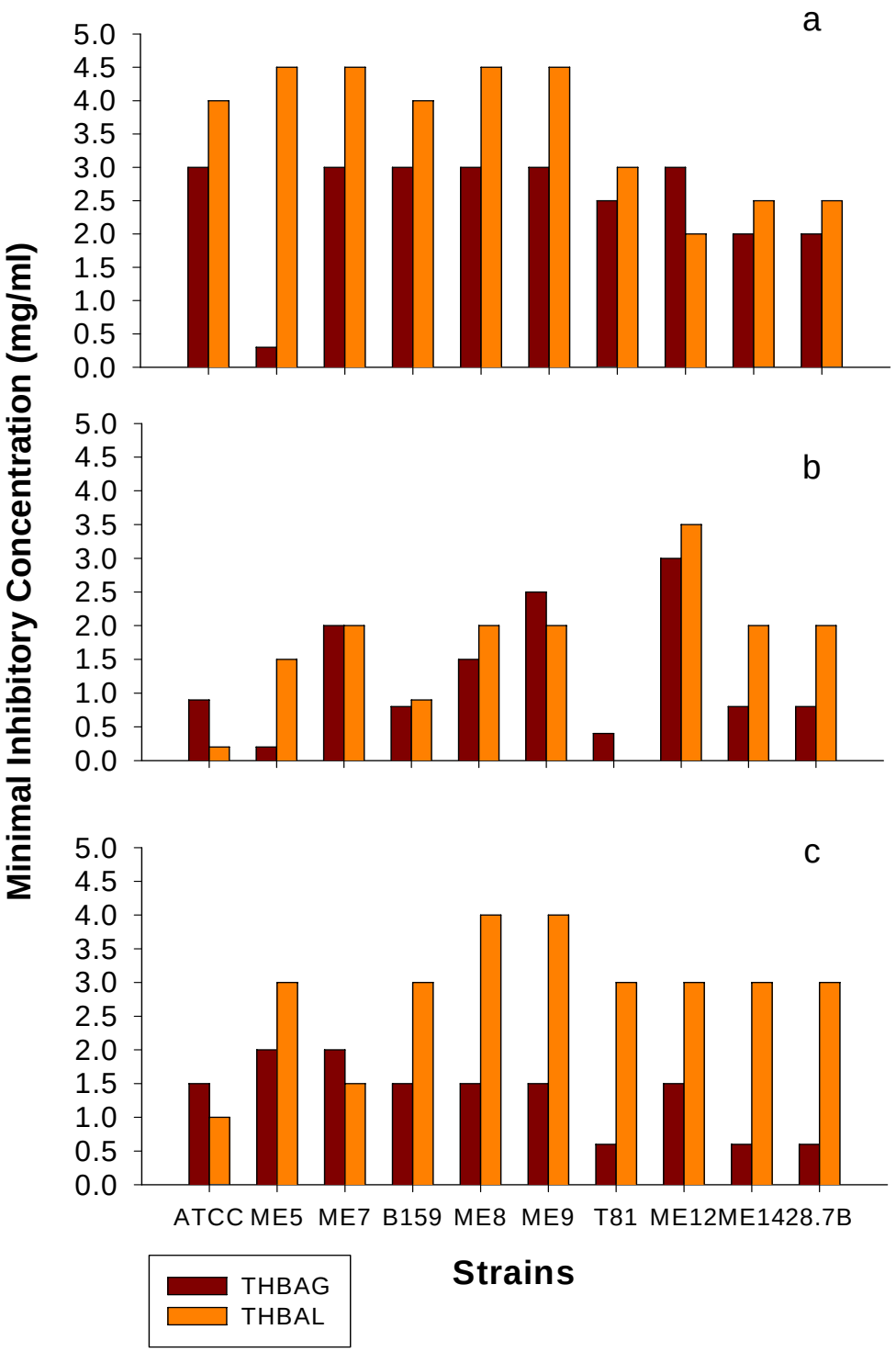


Figure 4.3: Minimal Inhibitory Concentrations (MICs) of a) 3-fluoropyruvate b) 3-chloropropionic acid and c) 2-bromopropionyl bromide (mg/ml) on the growth of *M. elsdenii* strains on THBAG and THBAL medium.

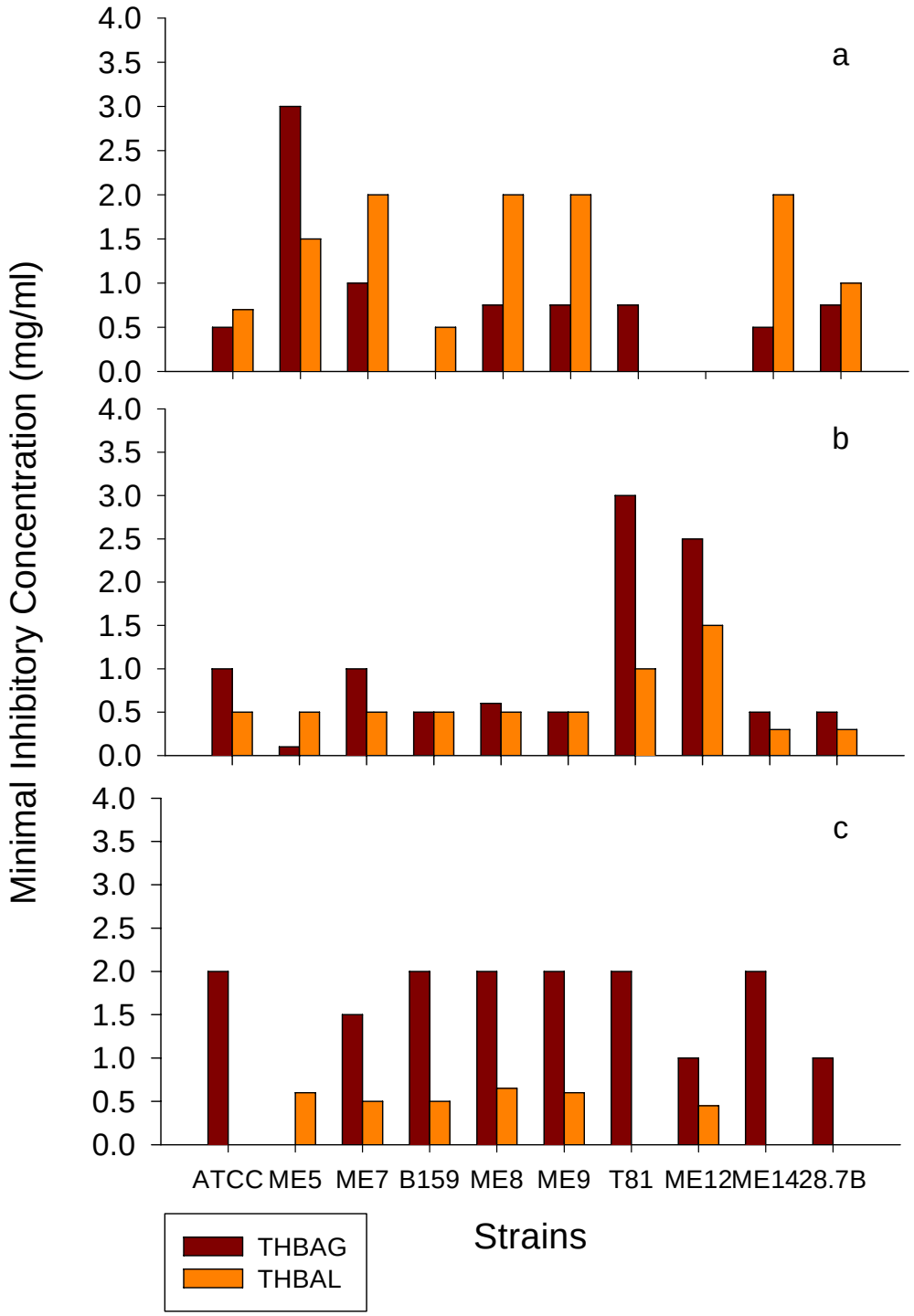


Figure 4.4: Minimal Inhibitory Concentrations (MICs) of a) 2-bromomethyl acrylic acid b) acrylic acid and c) bromoacetic acid (mg/ml) on the growth of *M. elsdenii* strains on THBAG and THBAL medium.

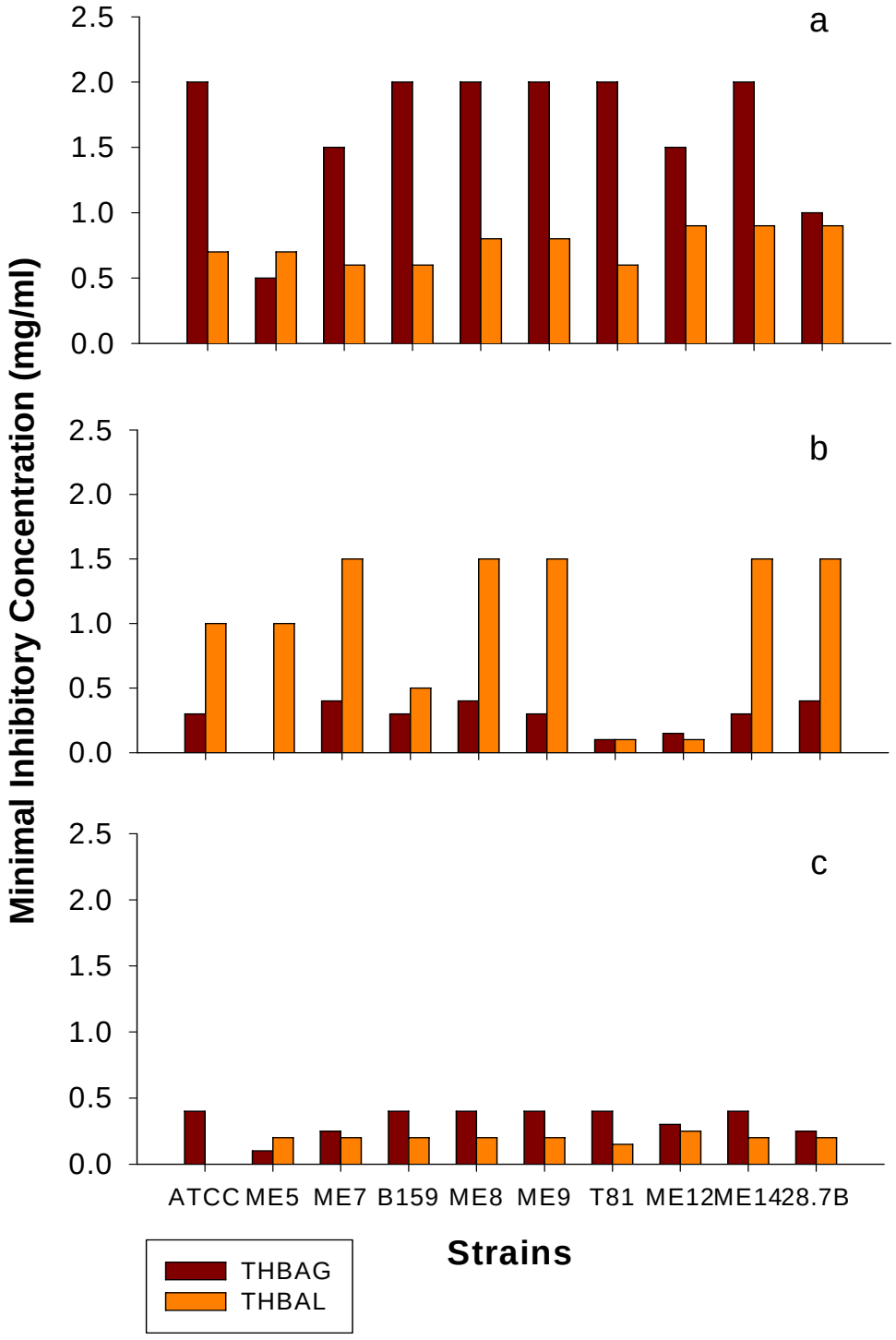
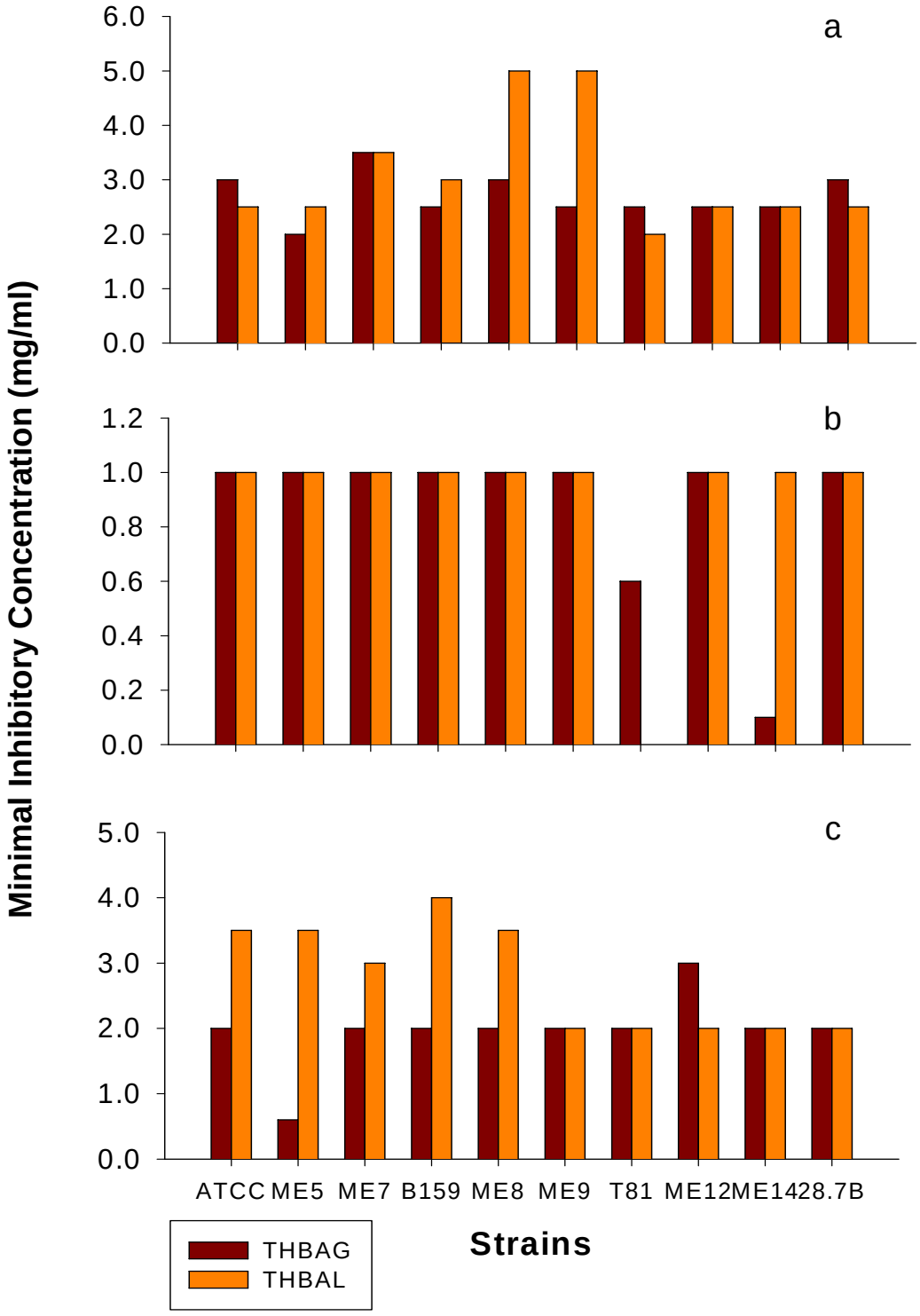


Figure 4.5: Minimal Inhibitory Concentrations (MICs) of a) 6-bromohexanoyl chloride b) 2-bromobutyric acid and c) 4-chlorobutyryl chloride (mg/ml) on the growth of *M. elsdenii* strains on THBAG and THBAL medium.



Group two parental strain B159 and group three mutant strain ME12 did not show any growth when tested on lowest concentration of 3-fuoropyruvate. Group three parent strain T-81 was sensitive to pyruvic acid, 2-BBA and propionic acids compared to its mutant strain ME12 (Table 4.1), but was, however, resistant to 3-fluoropyruvate compared to its mutant strain ME12 (Figure 4.3). Strain ME12 was resistant to pyruvic acid, hexanoic acid, 2-BBA and propionic acid compared to its parental strain T-81 (Table 4.1). Group two parental strain B159 was sensitive to 3-fluoropyruvate and showed no growth when tested on the lowest concentration compared to its mutant strains ME8 and ME9. Strains ME8 and ME9 were resistant to 3-FP and hexanoic acid compared to its parental strain B159 (Figure 4.3). Group three mutant strains ME14 was resistant to pyruvic acid, hexanoic acid, acetic acid and acrylic acid, however, sensitive to 3-FP, 2-BBA and 3-CPA compared to its parental strain T-81 (Table 4.1). All other results of MICs are presented in appendix-2 Figures A-14 – A-23.

4.2.3 MICs on THBAL agar

Differential sensitivity to the end-products and potential antimetabolites was detected on THBAL agar plates. For each group of parent and mutant strains, it was possible to compare relative sensitivity to growth inhibition (Table 4.2 and Figures 4.1 – 4.5). Group one mutant strain ME5 showed greater resistant to pyruvic acid, 3-fluoropyruvic acid, hexanoic acid, acetic acid, bromoacetic acid, propionic acid, 2-BPB and valeric acid compared to its parental strain ATCC 25940 as shown in Table 4.2. Mutant strain ME5 was approximately eight times more resistant to pyruvic acid, hexanoic acid and acetic acid compared to its parent strain ATCC 25940. Parental strain ATCC 25940 was very sensitive to bromoacetic acid and 2-BPB and did not show any growth when tested on the lowest concentration of these compounds (Table 4.2). Strain ME5 was two times more resistant to 3-fluoropyruvate compared to its parental strain ATCC 25940, while approximately three times more resistant to propionic acid and valeric acid compared to its parental strain ATCC 25940. Group two parental strains B159 was sensitive to 3-fluoropyruvate, hexanoic acid, 6-BHC, acetic acid and acrylic acid compared to its mutant strains ME8 and its parental strain ATCC 25940. Group two parental strains B159 was sensitive to 3-fluoropyruvate, hexanoic acid, 6-BHC, acetic acid and acrylic acid compared to its mutant strains ME8 and ME9 (Table 4.2). Group two mutant strains ME8 and ME9 were very resistant to 3-FP, hexanoic acid, 6-BHC, acetic acid and acrylic acid compared to their parental strain B159 (Table 4.2). Group three parental strains T-81 was sensitive to 3-FP, hexanoic acid, 2-BBA and 2-BPB and did not show any growth when tested on the lowest concentration of these compounds (Table 4.2). Group three mutant strains ME12 showed the same pattern of sensitivity to 3-FP as its parental strain T-81. Strain ME12 and ME14 were two times more resistant to pyruvic acid compared to its parental strain T-81 (Figure 4.1). Strain ME12 was very resistant to hexanoic acid (3.5 mg/ml) compared to all the strains studied. Strain ME14 was resistant to 3-FP, hexanoic acid, 2-BBA, acrylic acid and valeric acid compared to its parental strain T-81 (Table 4.2). All other results of MICs are presented in appendix-2 Figures A-14 – A-23.

The MIC studies confirmed that the mutants were distinct from the parents and therefore further studies were conducted for their metabolic end-products and their recovery from medium in small and large-volumes culture using different physiological conditions.

4.2.4 Effects of substrates on the production of VFAs in small-volume cultures of parent and 3FP^r mutants of *M. elsdenii*

4.2.4.1 Effects of glucose on volatile fatty acids

M. elsdenii strains were grown on 50 ml peptone-yeast extract plus 0.8% glucose (PYG) medium under strictly anaerobic uncontrolled pH conditions without stirring during 144 h to obtain the end-products profile, as shown in Figures 4.6a, 4.6.1a, and 4.6.2a. Ten different strains (ATCC 25940, ME5, ME7, T-81, B159, ME9, ME10, ME12, ME14 and 28.7B) were used in this study to select the appropriate parental and mutant strains for further studies at fermentation level. In the presence of glucose, the hexanoic and butyric acids were the major end-products in all above strains studied (Figure 4.6a, 4.6.1a and 4.6.2a). Strain ME5 produced 2 mM, the highest amount of valeric acid compared to all other strains studied. Strain ME14 produced 1.5 mM of iso-hexanoic acid as shown in Figure 4.6a. Most of the strains produced hexanoic acid in the range of 3 mM to 10 mM. The lowest concentration of hexanoic acid, that is 3 mM, was observed by the strain 28.7B and the highest concentration 10 mM was observed by the strain ME12. The concentration of hexanoic acid by the strain ATCC 25940 and ME5 was approximately 9 mM. The concentration of butyric acid been produced by both ATCC 25940 and ME5 was approximately 4 mM respectively. Total acids produced by ME5 and ATCC 25940 were approximately 14 mM and was the highest amongst all other strains studied. Comparison of the main acids such as butyric, valeric and hexanoic acids along with their total acids production by the parental strains of *M. elsdenii* are given in Figures 4.6.1a and 4.6.2a. An example gas chromatograms for fatty acids detected in *M. elsdenii* strains grown on peptone-yeast-glucose (PYG) medium are shown in appendix-2 Figures A-24 – A-26.

4.2.4.2 Effects of lactate on volatile fatty acids

All the *M. elsdenii* strains described above were grown on 50 ml peptone-yeast plus 0.8% lactate (PYL) medium under strictly anaerobic uncontrolled pH conditions without stirring during 144 h to obtain the end-products profile as shown in Figures 4.6b, 4.6.1b, 4.6.2b and 4.6.3a.

In the presence of lactate, all strains produced acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids. Strain ATCC 25940, ME5, B159 and ME9 did not produce any iso-hexanoic acid while strain ME9 and ME14 did not produce *n*-hexanoic acid. Hexanoic acid was the minor product in the presence of lactate medium. Most of the strains produced hexanoic acid in the range of 0.4 mM to 1.5 mM. Strain ME5 produced 8 mM, the highest amount of butyric acid compared to all other strains studied.

Figure 4.6: End-products detected in peptone–yeast plus a) glucose b) lactate c) glucose plus lactate medium following growth of *M. elsdenii* strains during 144 h in 50 ml culture without stirring and no pH controlled. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.

Subordinate figures 4.6.1, 4.6.2 show the data presented here in derivatise between butyric, valeric and hexanoic acids and 4.6.3 shows the enlargement for acetate and propionate.

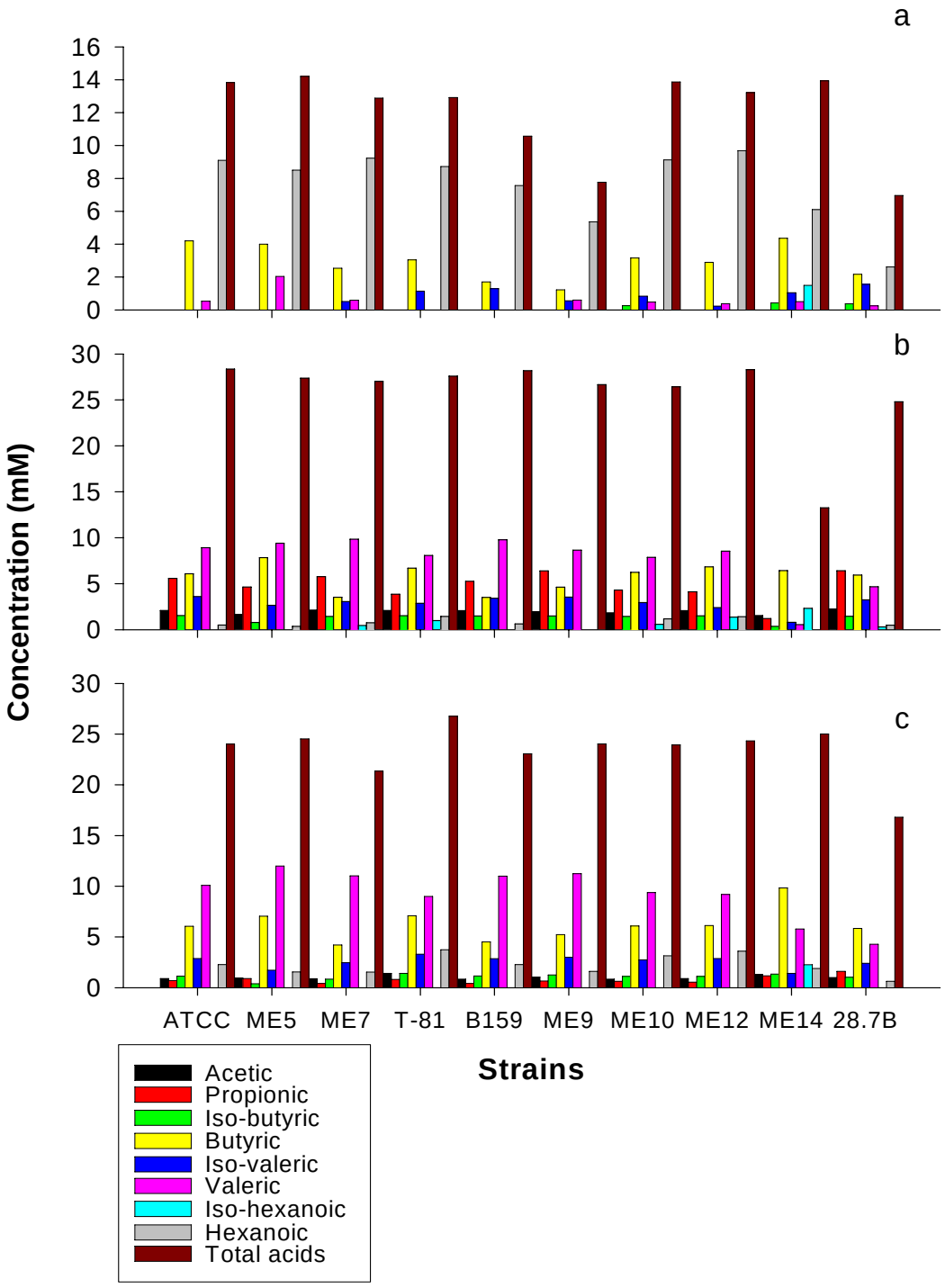


Figure 4.6.1: Comparison of butyric, valeric and hexanoic acids in peptone-yeast medium following growth of *M. elsdenii* and their mutant strains on a) glucose b) lactate c) glucose plus lactate medium during 144 h in 50 ml culture without stirring and uncontrolled pH conditions. Figures represent the average and standard deviation of triplicate experiments.

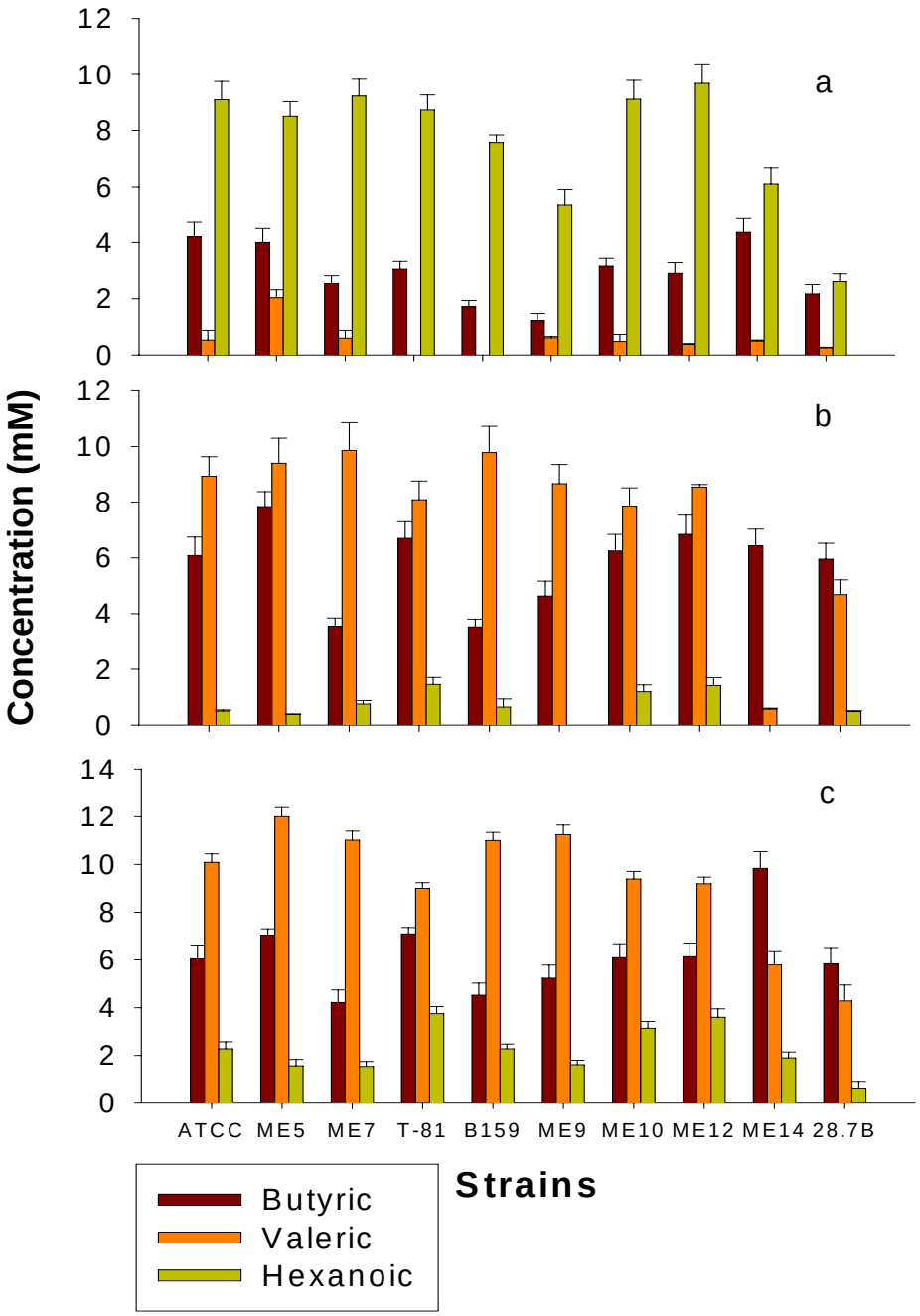


Figure 4.6.2: Comparison of butyric, valeric, hexanoic and total acids produced in peptone-yeast medium following growth of *M. elsdenii* parental strains (ATCC 25940, T-81 and B159) on a) glucose b) lactate c) glucose plus lactate during 144 h in 50 ml culture without stirring under uncontrolled pH conditions. Figures represent the average and standard deviation of triplicate experiments.

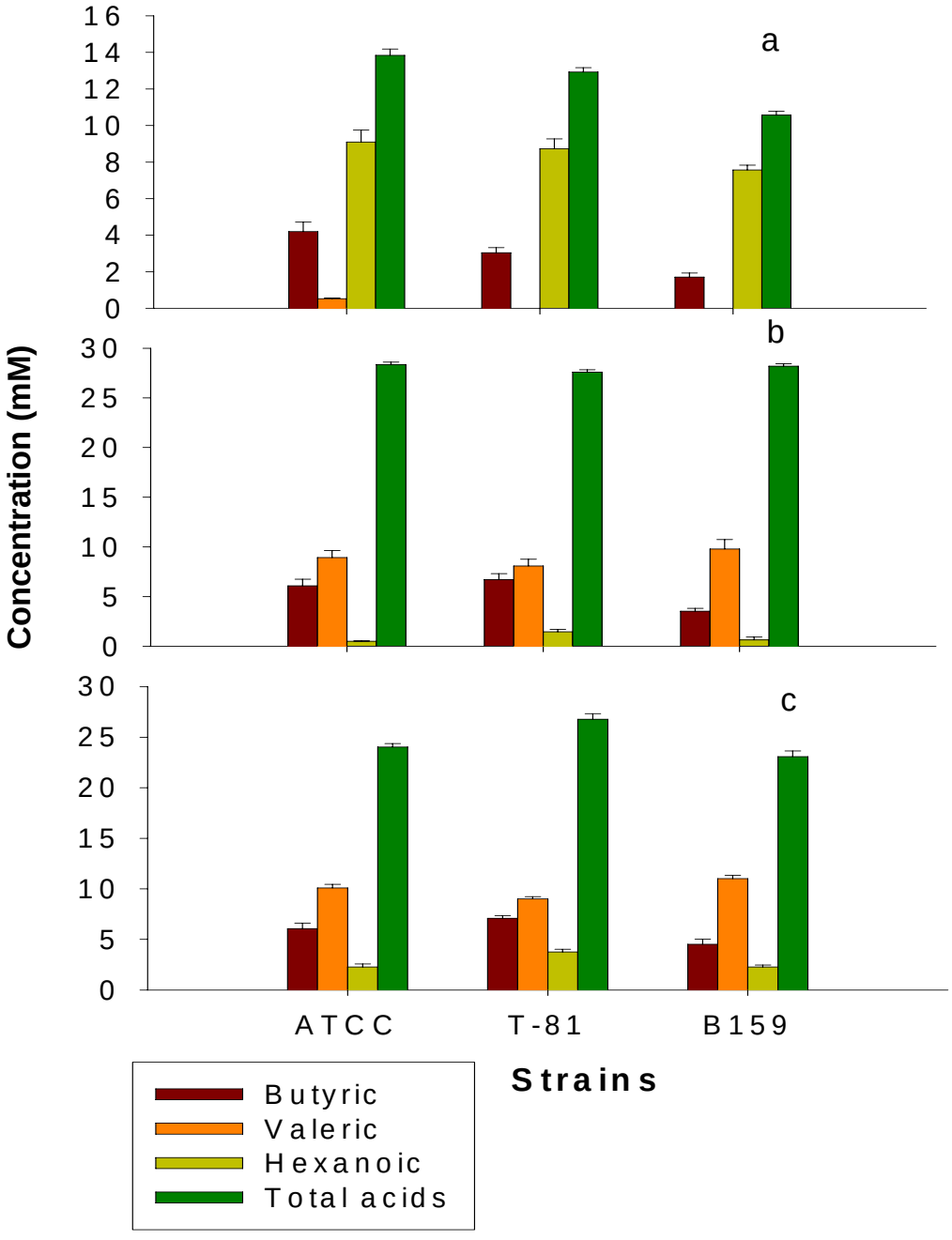
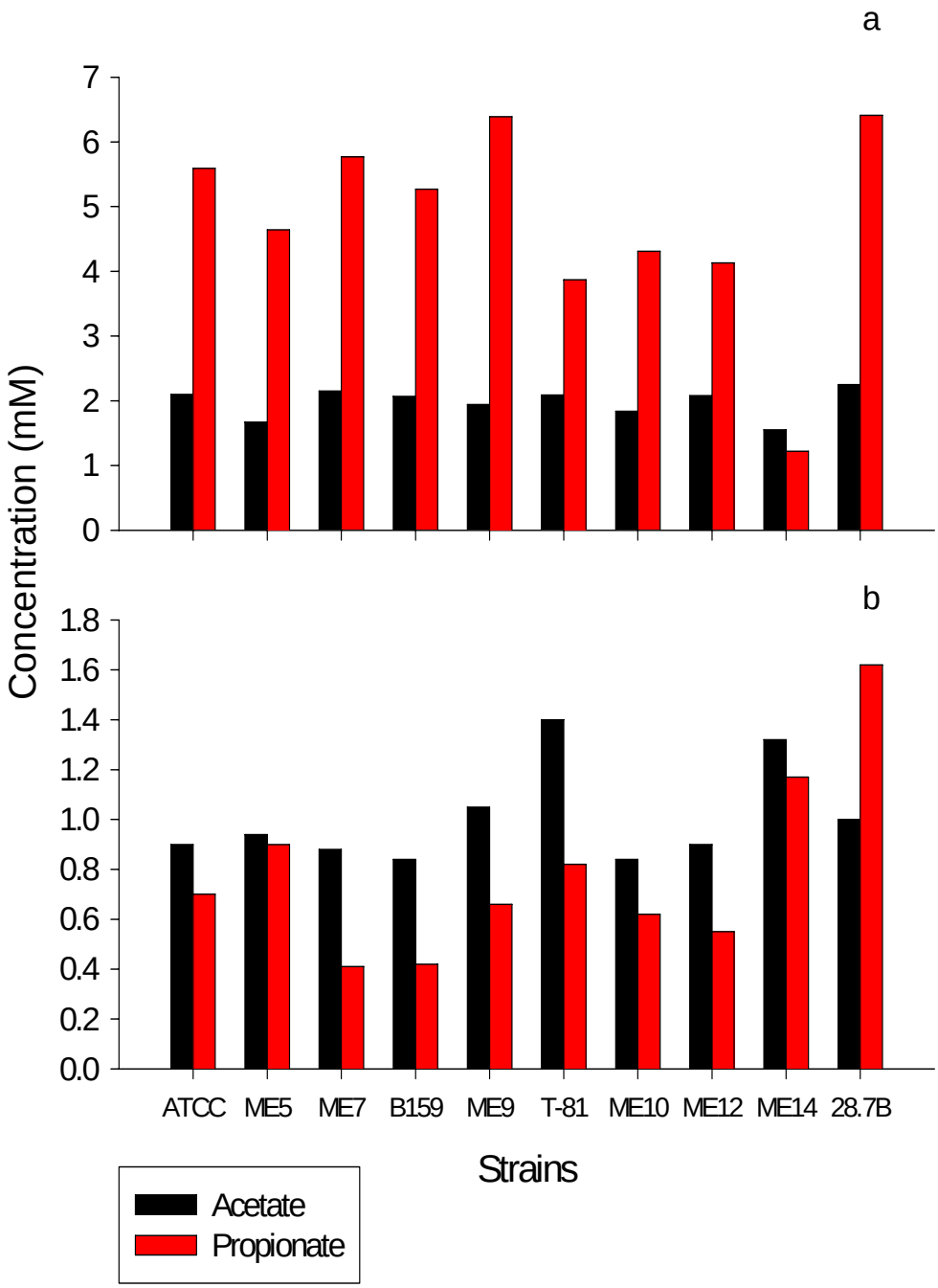


Figure 4.6.3: Enlarged for acetate and propionate detected in peptone-yeast medium following growth of *M. elsdenii* parental and their mutant strains on a) lactate b) glucose plus lactate during 144 h in 50 ml culture without stirring under uncontrolled pH conditions. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.



The concentration of valeric acid produced by all the strains was in the range of 0.6 mM to 10 mM. The concentration of valeric acid by the strain ATCC 25940 and ME5 was 9 mM and 10 mM respectively as shown in Figure 4.6.1b. Total acids produced by ATCC 25940 and ME5 were 28 mM and 27 mM respectively as shown in Figure 4.6b. Comparison of main acids such as butyric, valeric and hexanoic acids along with their total acids production by the parental strains of *M. elsdenii* are given in Figures 4.6.1b and 4.6.2b. The propionate productions are the one of the major acids and were in the range of 4 - 6 mM in all the above strains studied, while acetate was the minor product in the presence of PYL medium as shown in Figure 4.6.3a. An example gas chromatograms for fatty acids detected in *M. elsdenii* grown on peptone-yeast-lactate (PYL) medium are shown in appendix-2 Figures A-24 to A-26.

4.2.4.3 Effects of mixed substrates on volatile fatty acids

M. elsdenii strains were grown on 50 ml of peptone-yeast with 0.8% glucose plus 0.8% lactate (PYG+L) medium under strictly anaerobic uncontrolled pH conditions without stirring during 144 h time duration to obtain the end-products profile as shown in Figures 4.6c, 4.6.1c, 4.6.2c and 4.6.3b. Ten different strains (ATCC 25940, ME5, ME7, T-81, B159, ME9, ME10, ME12, ME14 and 28.7B) were grown in the presence of glucose and lactate. All these strains produced acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and hexanoic acids. Strain ME14 produced the highest amount of butyric acid (9 mM) compared to all other strains studied. Strain ME5 produced the highest concentration of valeric acid (12 mM) compared to all the strains studied as shown in Figure 4.6c. Total acids produced by ATCC 25940 and ME5 were 24 mM and 25 mM as shown in Figure 4.6c. Comparison of main acids such as butyric, valeric and hexanoic acids along with their total acids production by the parental strains of *M. elsdenii* are given in Figures 4.6.1c and 4.6.2c. The acetate and propionate are the minor acids (Figure 4.6.3b) produced in the presence of glucose plus lactate medium. Example gas chromatograms for fatty acids detected in *M. elsdenii* grown on peptone-yeast-glucose plus lactate (PYG + L) medium are shown in appendix-2 Figures A-24 – A-26.

4.2.4.4 Optimisation of pH for the production of VFAs

For microorganisms, the pH of the broth is one of the most important parameters affecting growth and product formation. Initially, the optimal pH for growth and acid production by *M. elsdenii* strains (ATCC 25940, ME5, and ME7) were determined by culturing in peptone-yeast plus 4% glucose (PYG) medium by using 1L Applicon automated pH controlled fermenters at different fixed pHs between 5.5 to 7.5 as shown in Figure 4.7, 4.7.1 and 4.7.2. Two days old grown 10% inoculum was used for each fermenter and was sampled at regular intervals and was terminated at 72 h. The optimal pH in terms of growth rate in terms of protein concentrations and hexanoic plus butyric acid production was at pH 6.5 (Figure 4.7.1 and 4.7.2). The production of hexanoic and butyric acid were low at acidic value pH 5.5 compared to pH 7.5.

Figure 4.7: Effect of different set pH values on the end-products detected in peptone-yeast-glucose medium following growth of *M. elsdenii* strains a) ATCC 25940 b) ME5 and c) ME7 during 72 h culture in one litre volume Applicon fermenters with constant stirring. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.

Subordinate figure 4.7.1 shows the enlargement for hexanoic and butyric acids and 4.7.2 shows the effect of pH values on protein concentrations.

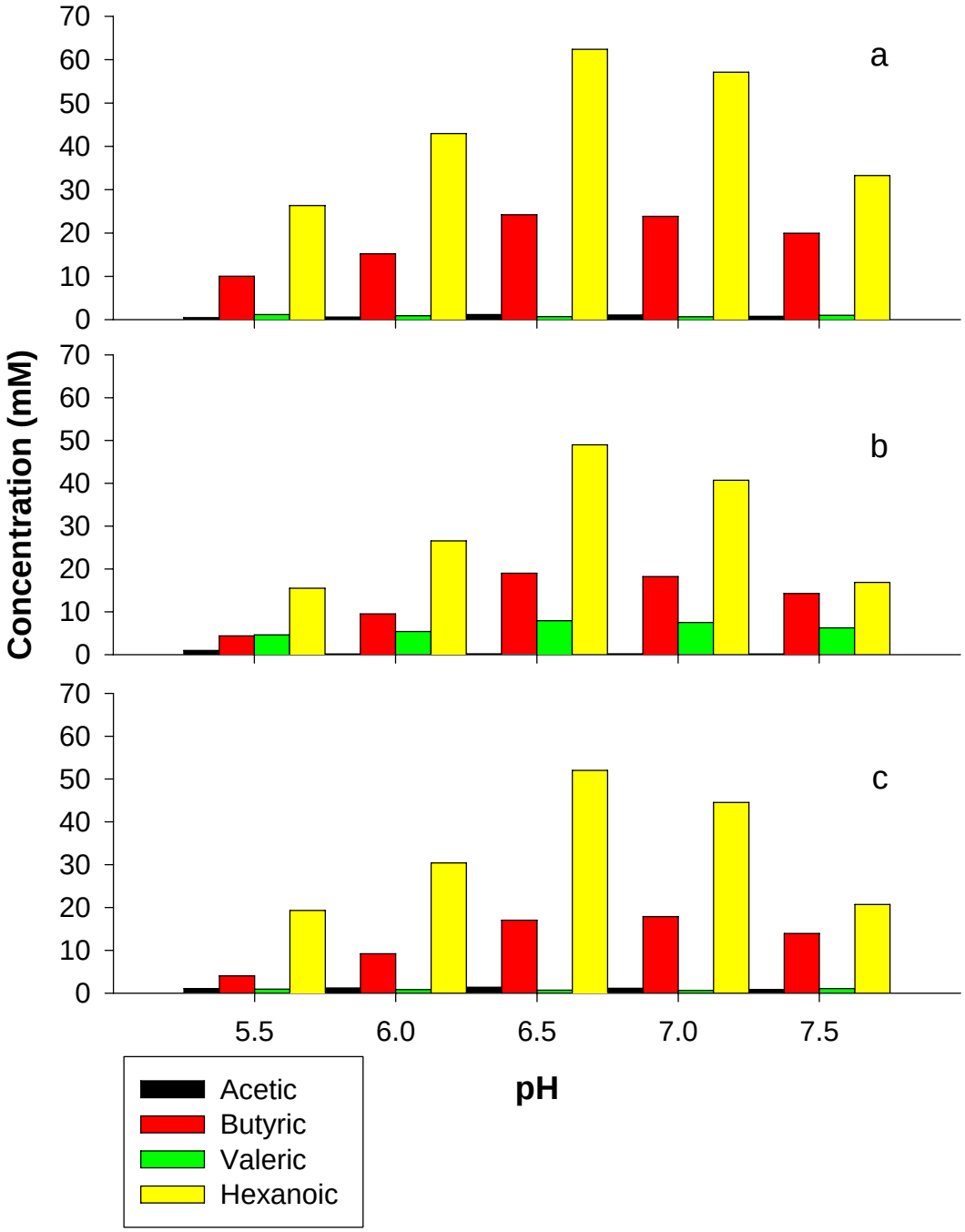


Figure 4.7.1: Effect of different set pH values on the production of a) hexanoic acid and b) butyric acid in peptone-yeast-glucose medium following growth of *M. elsdenii* parental (ATCC 25940) and mutant (ME5 and ME7) strains during 72 h culture in one litre volume Applicon fermenters with constant stirring. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.

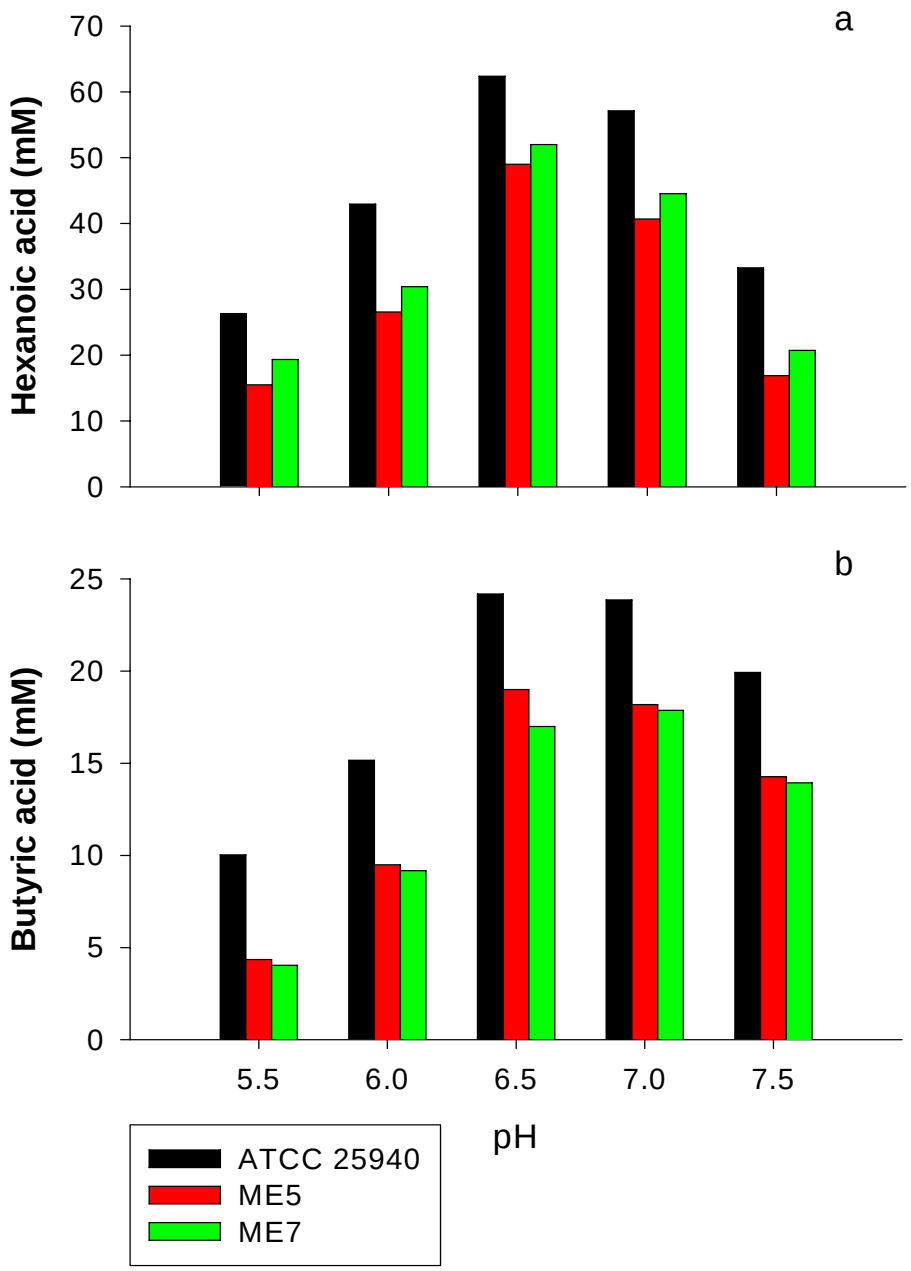
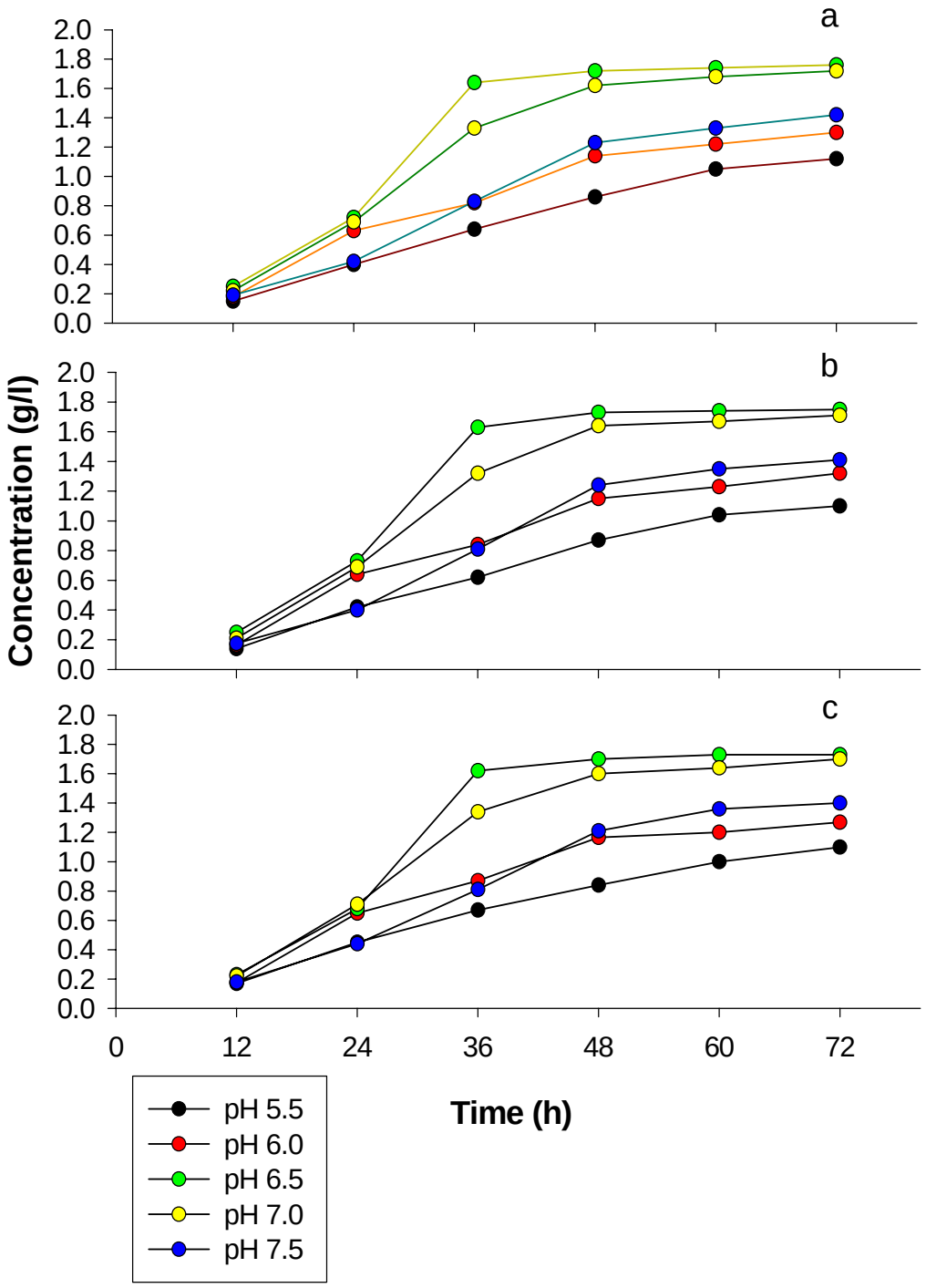


Figure 4.7.2: Effect of different set pH values on protein concentration in peptone-yeast-glucose medium following growth of *M. elsdenii* strains a) ATCC 25940 b) ME5 and c) ME7 during 72 h culture in one litre volume Applicon fermenters with constant stirring. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.



However, the production of both acids was slightly lower at pH 7.0 compared to pH 6.5 (Figure 4.7.1). The pH values above 7.0 and below 6.0 showed significant reduction in the end-products as shown in Figure 4.7. By comparison of the values at these times, it can be seen that at pH 6.5 there was greater production of *n*-hexanoic and *n*-butyric acid. When the pH of broth was above 7.0, *M. elsdenii* tends to produced relatively shorter chain acids (i.e., *n*-butyric acid), where as when the pH was lower, more of the longer chain acids (i.e., *n*-hexanoic acid) produced. The strain ATCC 25940 produced 62 mM of hexanoic and 24 mM of butyric acid at pH 6.5. Strain ME5 produced hexanoic acid (49 mM) and butyric acid (19 mM) at pH 6.5. Strain ME7 produced 52 mM of hexanoic acid and 17 mM of butyric acid at pH 6.5 as shown in Figure 4.7.1a and b. Strain ME5 produced the lowest amount of hexanoic acid at pH 5.5, which was the half of the amount produced by strain ATCC 25940 (Figure 4.7.1a) shows that this mutant is more sensitive towards lower acidic pH values compared to parent strain ATCC 25940.

4.2.5 Effects of substrates in batch fermentations

4.2.5.1 Effects of glucose on volatile fatty acids

A comparative study was carried out to optimise the growth and total acids production from *M. elsdenii* using different glucose concentrations (3%, 4%, 5% and 6%) in peptone-yeast media. Total acids production was optimal at 4% glucose level in automated controlled pH 6.5 (data not shown) Applicon fermenters. On the basis of these findings the *M. elsdenii* strains were grown on peptone-yeast plus 4% glucose (PYG) media in 1 litre Applicon fermenters during 144 h at automated controlled pH 6.5 to obtain the end-products profile as shown in Figure 4.8a, 4.8.1a and 4.8.2a. Strain ATCC 25940 and ME7 produced *n*-hexanoic acid in the presence of glucose at the concentration of 58 mM and 53 mM respectively. This is slightly higher compared to strain ME5 (49 mM) as shown in Figure 4.8.2a. The production of butyric acid for the strain ATCC 25940 and ME7 was 29 mM and 23 mM respectively. While strain ME5 showed the least production of both these acids compared to ATCC 25940 and ME7 as shown in Figure 4.8.2a. Strain ME5 has shown five times more production of valeric acid compared to other two strains studied as shown in Figure 4.8.2a.

Total acids produced in the media by ATCC 25940, ME5 and ME7 are shown in Figures 4.8a and 4.8.1a. Strain ATCC 25940 produced the highest amount of acids compared to other strains. Comparison of main acids such as butyric, valeric and hexanoic acids along with their total acids production by the parental strains of *M. elsdenii* are given in Figures 4.8a and 4.8.1a. Samples were collected at 24, 48, 72, 96, 120 and 144 h according to the method given in the materials and methods and the time course for the end-products detected in peptone-yeast-glucose medium as shown in appendix-2 Figures A-27 – A-30.

Figure 4.8: End-products detected in peptone-yeast plus a) glucose b) lactate c) glucose plus lactate medium following growth of *M. elsdenii* strains during 144 h in one litre volume Applicon fermenters at automated controlled pH 6.5. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.

Subordinate figures 4.8.1, 4.8.2 show the data presented here in derivatise forms to allow comparison between butyric, valeric, and hexanoic acids and 4.8.3 shows the enlargement for acetate and propionate.

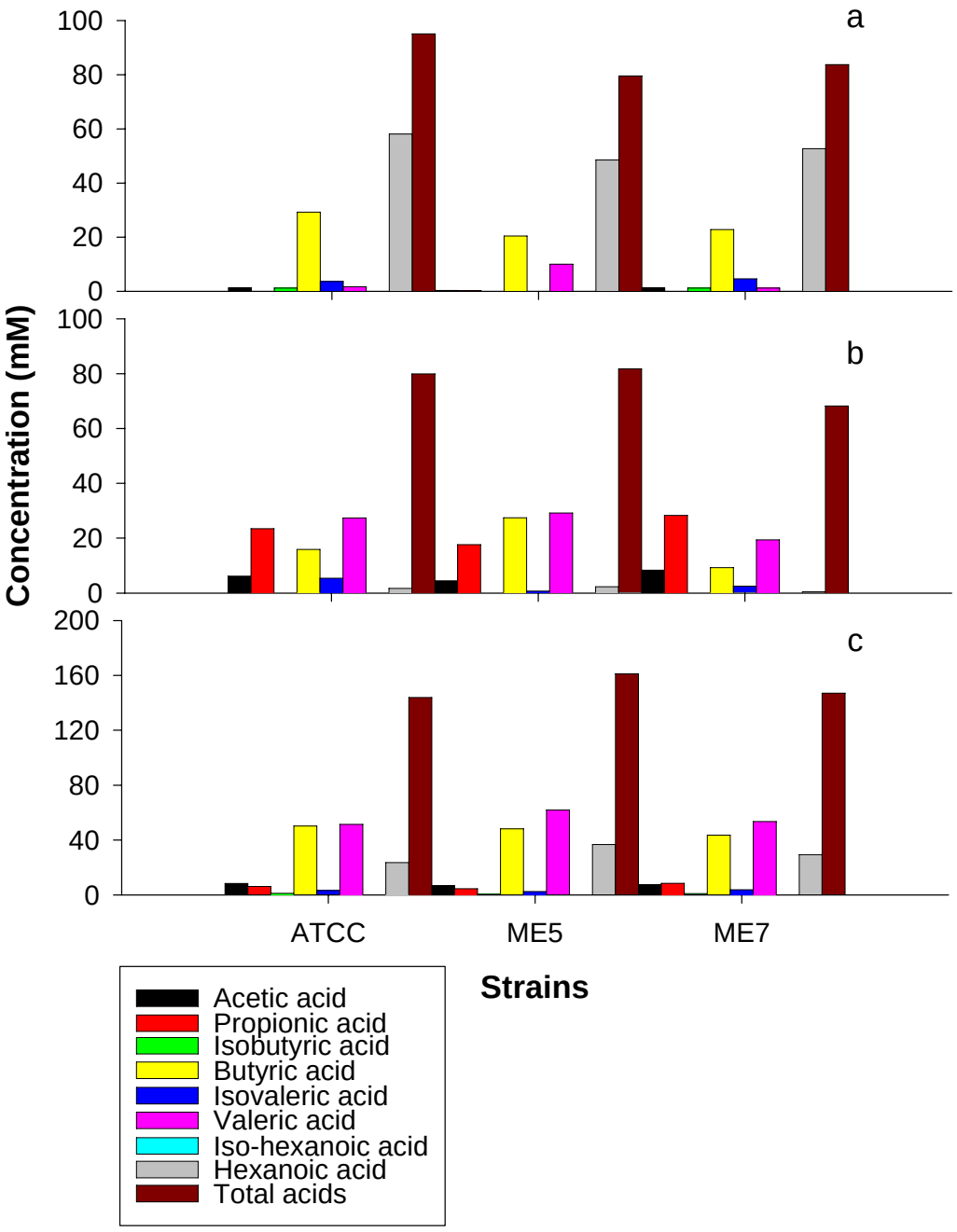


Figure 4.8.1: End-products detected in peptone-yeast medium following growth of *M. elsdenii* strains (ATCC 25940, ME5 and ME7) on a) glucose b) lactate c) glucose plus lactate during 144 h in one litre volume Applicon fermenters with stirring at automated controlled pH 6.5. Y-scale on figure c is twice of figure a and b. Figures represent the average and standard deviation of triplicate experiments.

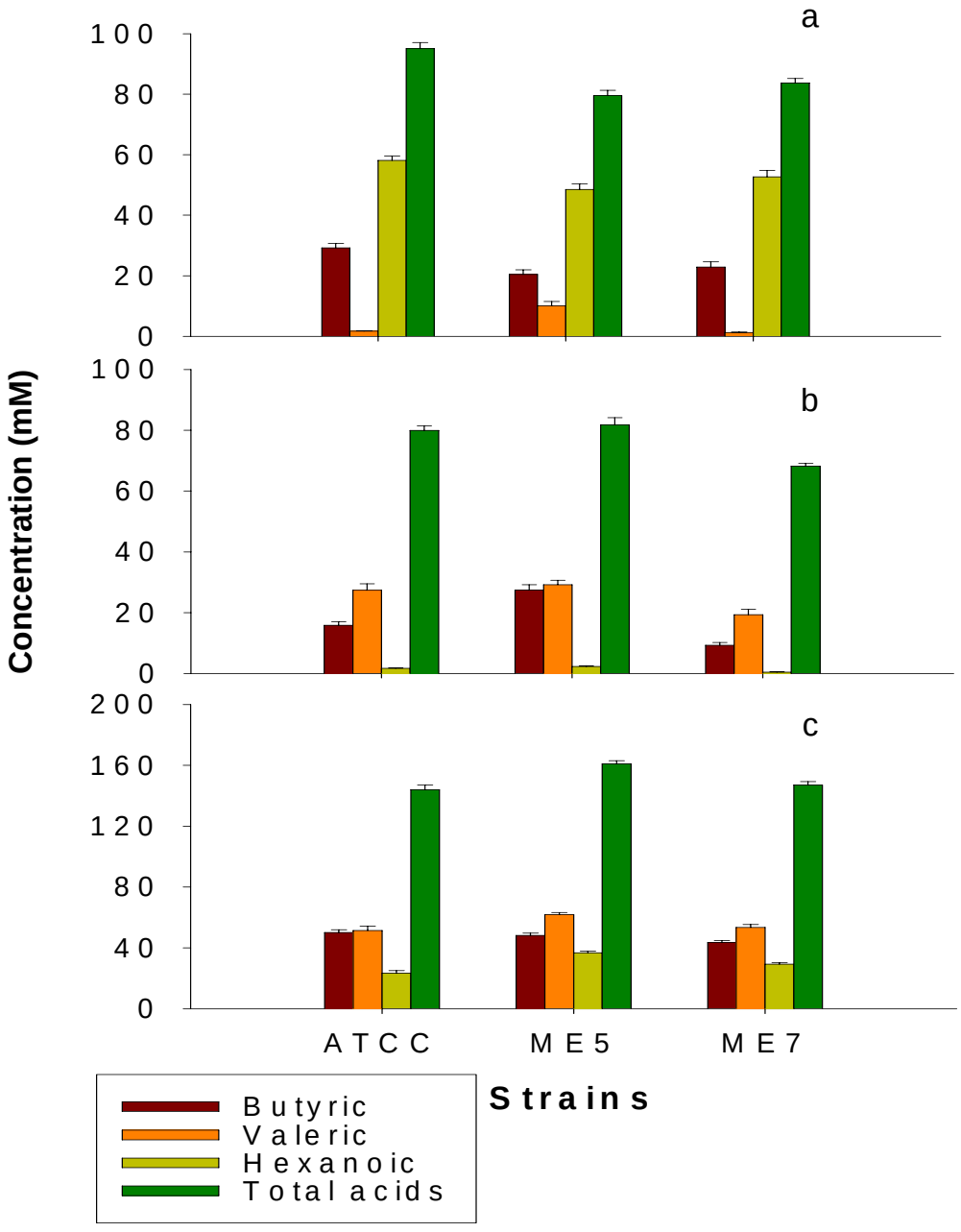
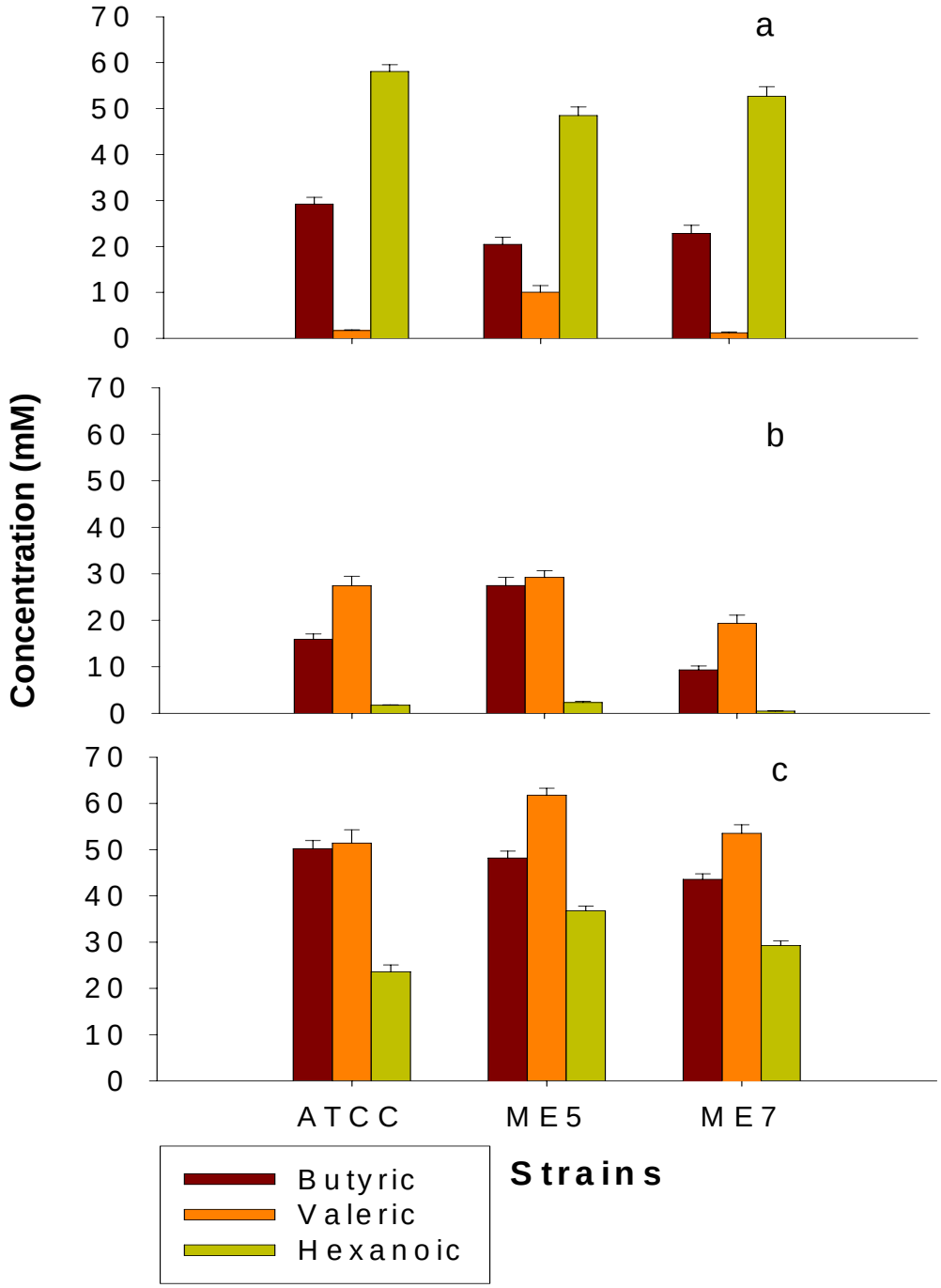


Figure 4.8.2: Comparison of butyric, valeric and hexanoic acids detected in peptone-yeast medium following growth of *M. elsdenii* strains (ATCC 25940, ME5 and ME7) on a) glucose b) lactate c) glucose plus lactate during 144 h in one litre volume Applicon fermenters with stirring at automated controlled pH 6.5. Figures represent the average and standard deviation of triplicate experiments.



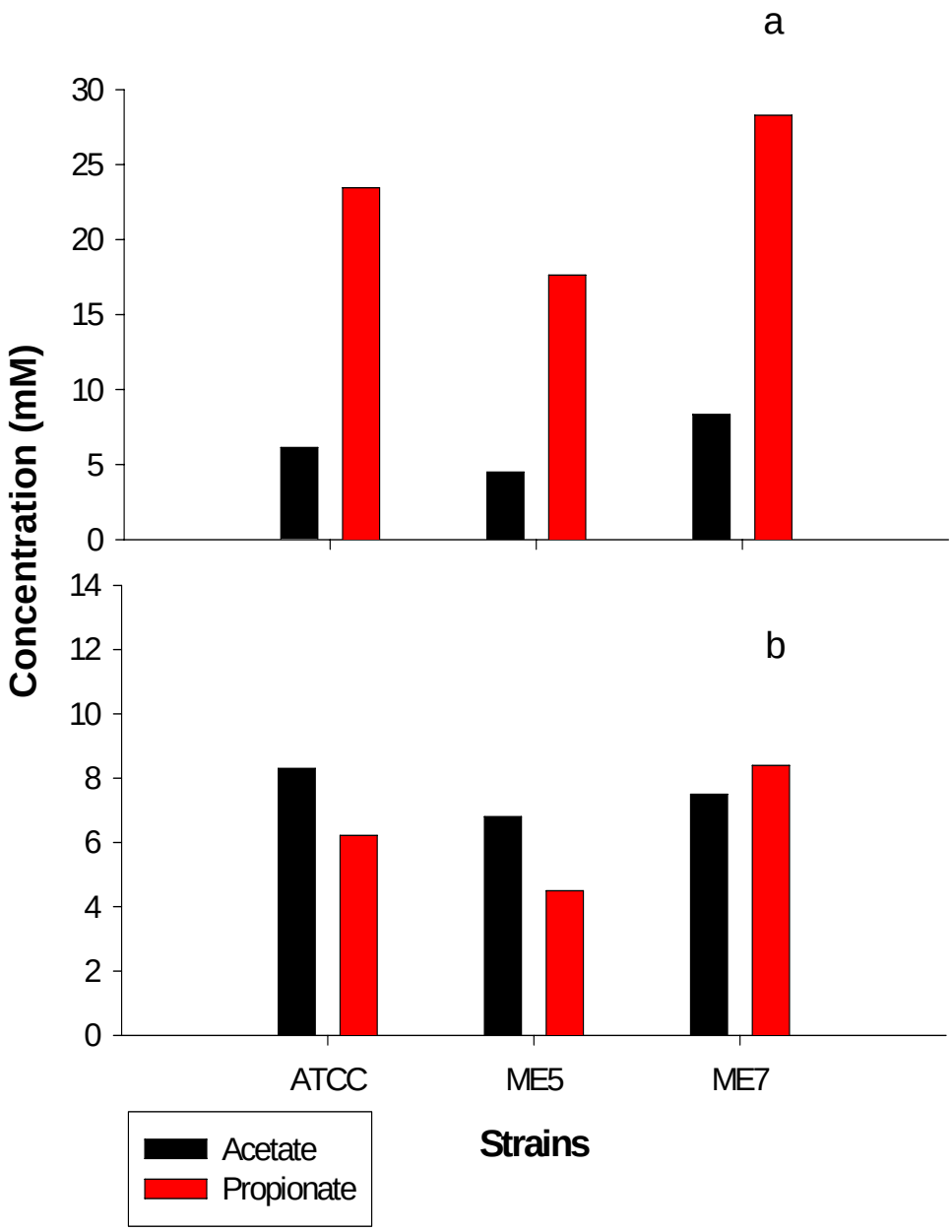
4.2.5.2 Effects of lactate on volatile fatty acids

M. elsdenii were grown on peptone-yeast plus 4% lactate (PYL) media in 1L volume Applicon fermenters during 144 h at automated controlled pH 6.5 to obtain the end-products profile as shown in Figure 4.8b, 4.8.1b and 4.8.2b. Samples were collected according to the method given in the Materials and Methods. *M. elsdenii* grown on lactate as a substrate showed altered fatty acids profile compared to the cells grown on glucose. Strains ATCC 25940, ME5, and ME7 produced valeric acid at the highest concentration, whilst hexanoic acid was the lowest. These strains also produced acetic, propionic and butyric acids in the presence of lactate as a substrate. Valeric acid produced by the strains ME5, ATCC 25940, and ME7 was 29 mM, 27 mM and 19 mM respectively. ME5 produced 28 mM of butyric acid which was significantly higher compared to strain ATCC 25940 and ME7. However, strain ME7 shows the highest concentration of propionic acid that is 28 mM compared to ATCC 25940 and ME5 as shown in Figure 4.8.3a. Total acids produced by ATCC 25940 and ME5 were higher compared to strain ME7. Figure 4.8.1b shows strain ME5 produced more butyric and valeric acid compared to ATCC 25940 and ME7.

4.2.5.3 Effects of mixed substrates (glucose plus lactate) on volatile fatty acids

M. elsdenii were grown on peptone-yeast plus 4% glucose plus 4% lactate (PYG+L) media in 1L volume Applicon fermenters during 144 h at automated controlled pH 6.5 to obtain the end-products profile as shown in Figure 4.8c, 4.8.1c and 4.8.2c. Samples were collected according to the method given in the materials and methods. In the presence of mixed substrates, the major end-products by strains ATCC 25940 and ME5 were valeric, butyric, and hexanoic acids, and the minor products were acetic and propionic acids as shown in Figure 4.8c. However, strain ME7 showed propionic, valeric, and butyric acids as a major end-products and hexanoic acid as a minor product as in Figure 4.8c. It seems that in the presence of mixed substrates, the bacteria utilises lactate in preference to glucose as a substrate. The concentration of valeric acid produced by strain ATCC 25940, ME5, and ME7 were 51 mM, 62 mM, and 54 mM respectively as shown in Figure 4.8.1c. The amount of butyric acid produced by the strain ATCC 25940, ME5, and ME7 were 50 mM, 48 mM and 44 mM respectively as shown in Figure 4.8.1c. The highest concentrations of valeric and *n*-hexanoic acids produced by the strain ME5 are 62 mM and 37 mM as in Figure 4.8.1c. Total acids produced by the strain ME5 were 161 mM compared to the strain ATCC 25940 and ME7 as shown in Figure 4.8c. Strain ATCC 25940 and ME7 produced more acetic acid than ME5 strain, however, ME7 produced highest amount of propionic acid compared to ATCC 25940 and ME5 strains as shown in Figure 4.8.3b.

Figure 4.8.3: Effect of a) lactate and b) glucose plus lactate on the end-products acetate and propionate detected in peptone-yeast medium following growth of *M. elsdenii* strains (ATCC 25940, ME5 and ME7) during 144 h in one litre volume Applicon fermenters with stirring at automated controlled pH 6.5. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.



4.2.6 Binding and desorption capacity of resins for hexanoic and butyric acids from water and PYG

4.2.6.1 Binding or adsorption of *n*-hexanoic and *n*-butyric acids by Amberlite IRA-93 and Amberlite IRA-400 resins

Binding capacity was tested for both Amberlite IRA-93 and Amberlite IRA-400 resins using hexanoic and butyric acids at concentrations of 60 mM and 20 mM respectively (the proportions normally seen in fermentation broths) in water as a neutral solution or in PYG (Table 4.3), using equivalent wet weights (100 g) of resin for 2 h with constant stirring at 100 rpm.

Table 4.3: Binding capacity of resins Amberlite IRA-93 and Amberlite IRA-400 for hexanoic and butyric acids from water and PYG media. HA, BA represents hexanoic and butyric acid respectively.

Resin	Sorption				Desorption			
	Water		PYG Medium		Water		PYG Medium	
	HA (%)	BA (%)	HA (%)	BA (%)	HA (%)	BA (%)	HA (%)	BA (%)
IRA–93	98	97	56	24	95	87	99	93
IRA–400	63	36	72	51	85	70	87	73

A further purpose of this experiment was to determine the approximate amounts of *n*-hexanoic and *n*-butyric acids adsorbed and the effect of fermentation broth during fermentation experiments.

For this experiment, 100 g (wet) Amberlite IRA-93 (free base form) or Amberlite IRA-400 (chloride form) was added to 400 ml water or PYG broth at pH 6.5 in a separate flask as described earlier. The flasks placed in a shaking water bath at 37^{o}C for 2 h, and the solution sampled at different time intervals. The solutions were assayed for *n*-butyric and *n*-hexanoic acids (as described in Materials and Methods section 2.4.3.4) and the amount of acids that had been adsorbed to the resin were calculated from the amount remaining in solution. It was observed that equilibrium was reached within 20-40 min depending on the system (data not shown). In this experiment Amberlite IRA-93 adsorbed 98% of *n*-hexanoic acid and 97% of *n*-butyric acid from water solution while the same resin adsorbed 56% and 24% of *n*-hexanoic and butyric acids respectively in PYG medium (Table 4.3). In case of Amberlite IRA-400, the resin adsorbed 63% and 36% hexanoic and butyric acids in water solution and 72% and 51% of the corresponding acids in PYG medium (Table 4.3). It can be seen that both resins adsorbed more *n*-hexanoic than *n*-butyric acid. In the presence of Amberlite IRA-93, there was greater adsorption of the acids from the water solution than from the fermentation broth, whilst in the case of Amberlite IRA-400 there was considerable adsorption of acids from the PYG medium. The fermentation broth contains several salts and other ionic materials, some of which occupy the functional groups of the ion exchange resin. The greater adsorption of *n*-hexanoic acid compared with *n*-butyric acid suggests that the VFA chain length may affect the degree of adsorption, i.e., that molecular adsorption may be involved. Since the

dissociation of *n*-hexanoic acid depends on pH, the pH of the solution will have a major effect on the degree of molecular adsorption. Amberlite IRA-93 adsorbed a larger proportion of both acids compared to Amberlite IRA-400 when the acids were in water but less when the acids were in PYG, indicating the larger binding capacity of Amberlite IRA-93 on a wet weight basis. Both acids were desorbed more effectively by esterification from Amberlite IRA-93 resin. This indicated that Amberlite IRA-93 might have some attractive features over Amberlite IRA-400 in terms of product recovery in the form of esters. The process of desorption of acids depends on the hydrodynamic conditions provided between the resin beads and solution. It is possible to reuse the resins for the VFAs adsorption and desorption after regeneration process of the resins.

4.2.6.2 Esterification (desorption) of the resin-adsorbed *n*-hexanoic and *n*-butyric acids

After investigating the adsorption of *n*-hexanoic and *n*-butyric acids to the resins, the next objective was to study desorption of both acids from Amberlite IRA-93 and Amberlite IRA-400 resins. In earlier studies, the recovery of *n*-hexanoic acid adsorbed to Amberlite IRA-400 by alkali and acid (HCl) wash had been about 31% (Roddick and Britz, 1987). Deguchi (1994) reported desorption of resin-adsorbed *n*-hexanoic acid by dilute sulfuric acid and recovery was approximately 50% for broth (PYG) media from Amberlite IRA-400 (chloride form). This author (Deguchi 1994) also reported that the recovery of *n*-hexanoic acid by sulfuric acid and ethanol mixture from the resin Amberlite IRA-400 (chloride form) by esterification was 98%. In the present study, the desorption of hexanoic and butyric acid from Amberlite IRA-93 by using sulfuric acid and ethanol mixture was 95% and 87% respectively in water solution and 99% and 93% from PYG broth as shown in Table 4.3. In case of Amberlite IRA-400, the desorption of hexanoic and butyric acids was 85% and 70% respectively in water solution and 87% and 73% from PYG broth mentioned in Table 4.3. Desorption of both acids were greater from PYG medium in Amberlite IRA-93 and Amberlite IRA-400 resins as given in Table 4.3. The esterification reaction mixture (solution only) was sampled periodically and the concentration was measured by Gas Chromatography (see section 2.4.2. in Materials and Methods).

4.2.6.3 Effect of fermentation broth on the adsorption and esterification of *n*-hexanoic and *n*-butyric acids with Amberlite IRA-93 and Amberlite IRA-400 resins

It has been shown that Amberlite IRA-400 and Amberlite IRA-93 can adsorb *n*-hexanoic acid from PYG broth, and it can be desorbed from those resins by esterification (Table 4.3). It was possible that the presence of the organism *M. elsdenii*, which secretes a slimy surface coating, and its metabolites other than *n*-butyric and *n*-hexanoic acids, may affect the processes of adsorption and esterification. To investigate the adsorption and esterification of fermentatively produced *n*-hexanoic and *n*-butyric acid using Amberlite IRA-93 and Amberlite IRA-400 resins as adsorbents such study was undertaken.

Batch fermentation in the presence of *M. elsdenii* was conducted for 84 h under pH controlled and uncontrolled conditions and samples were collected at different time intervals as described in Materials and Methods. At the end of fermentation, resin samples were collected and subjected to the esterification. The concentration of VFAs in the fermentation broth initially and after absorption, and the concentration of esters produced were determined as described in Materials and Methods section 2.4.3.4, according to the formulas mentioned in appendix-3.

4.2.6.4 Effects of resins on pH and glucose in batch fermentations

Initially, the optimal pH for growth and acid production by *M. elsdenii* was determined by culturing in fermenters at different fixed pHs between 5.5 and 7.5 over 72 h, without resins. The optimal pH in terms of growth rate and hexanoic plus butyric acids production was at pH 6.5 (Figure 4.7.2 and 4.7.1). On the basis of findings, all the pH-controlled experiments were carried out close to pH 6.5. In controls which had no resins added, pH control increased glucose utilisation relative to pH uncontrolled fermentations, as shown previously. When the pH was not controlled and Amberlite IRA-400 (chloride form) was added at 24 h, the pH dramatically decreased (Figure 4.9d). In contrast, addition of Amberlite IRA-93 (free base form) the pH was not affected and this remained around neutral (Figure 4.9d). This meant that using the Amberlite IRA-93 had an advantage in that it did not release a counter ion on binding of anions so that it exerts a buffering effect on the fermentation broth, thus reducing the degree of pH control needed and the amount of alkali used to maintain the optimum pH. In pH uncontrolled fermentations with Amberlite IRA-400 added, the rate and extent of glucose utilisation was 26% less when compared to Amberlite IRA-93 under the same conditions (Figure 4.9a) and residual glucose was detected at the end of the fermentation period of 84 h. In fermentation, which was pH controlled and had, either Amberlite IRA-93 or Amberlite IRA-400 added, the utilisation of glucose was almost complete (98% used in 84 h), indicating the positive effect of adding the resins (Figure 4.9a).

4.2.6.5 Effects of Amberlite IRA-93 (free base form) resin on *n*-hexanoic and *n*-butyric acids in batch fermentations

When the pH was controlled at 6.5 with no resins added, hexanoic and butyric acids increased from levels of 23 mM and 10 mM for pH uncontrolled cultures to 70 mM and 23 mM respectively (Figure 4.9b and 4.9c). Addition of Amberlite IRA-93 resin to *M. elsdenii* fermentations resulted significant increases in the production of hexanoic and butyric acids compared to fermentations without resins, for both pH uncontrolled (pHnc) or controlled (pHc) cultures (Figure 4.9b and 4.9c). For pH uncontrolled fermentations, in the presence of Amberlite IRA-93 (free base form) resin, the amount of hexanoic and butyric acids was 78 mM and 34 mM respectively. Two fermenters (volume of PYG broth = 800 ml) were set up as described in Materials and Methods section and each was inoculated with 10% of *M. elsdenii* two days old inoculum. The pH of one fermenter was controlled at 6.5 with sterile 10 M sodium hydroxide while other fermenter was run under pH uncontrolled condition. Amberlite IRA-93 (weak base anion exchange) resin was added in both fermenters at 24 h time point.

Figure 4.9 (a-d): Comparison of end-products and glucose utilization in peptone-yeast-glucose medium following growth of *M. elsdenii* strain ATCC 25940 in the presence of Amberlite IRA-93, Amberlite IRA-400, no resin (pH controlled and uncontrolled) for a) glucose consumption b) production of hexanoic acid c) production of butyric acid and d) pH values during 84 h in one litre volume Applicon fermenters. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.

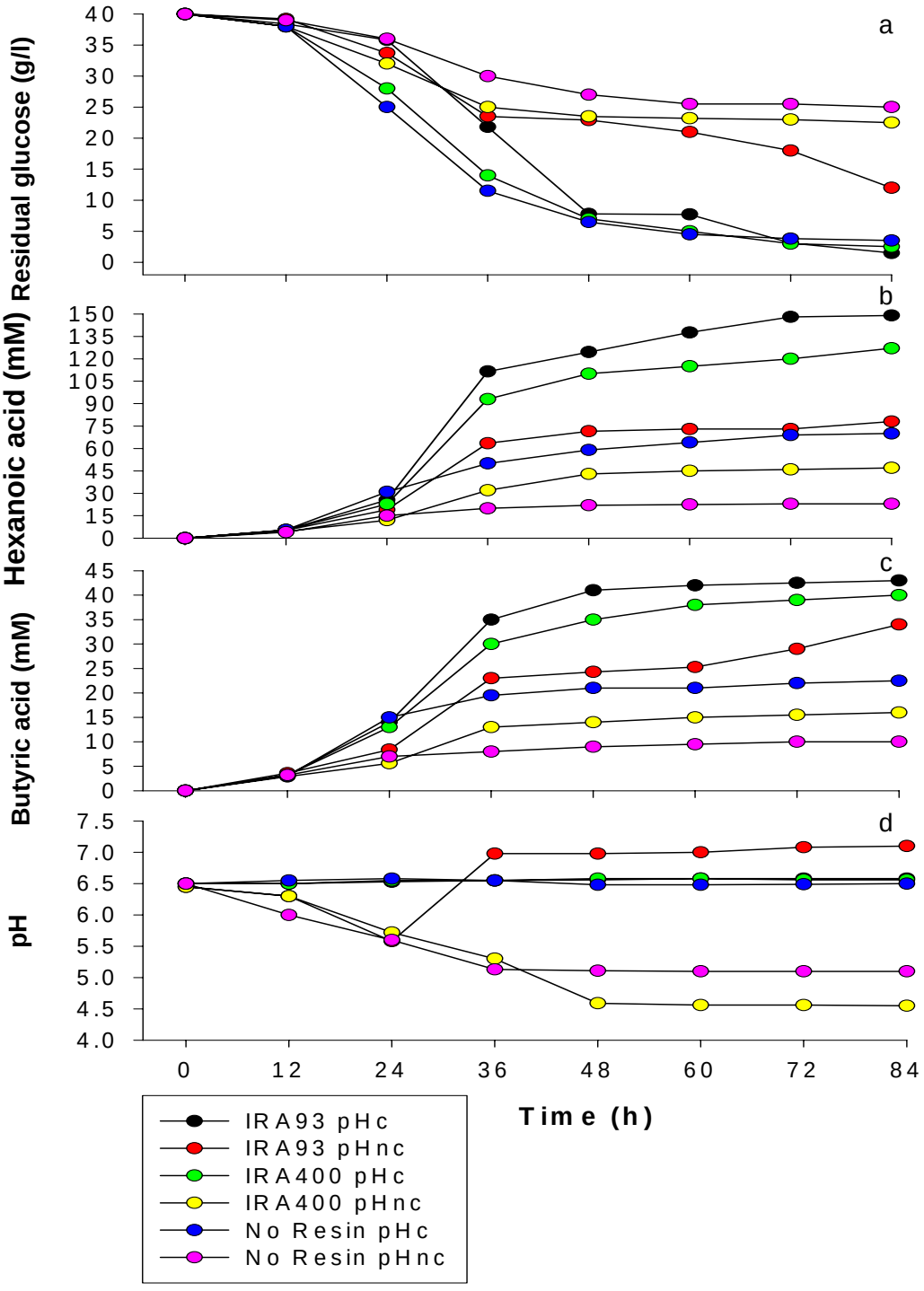
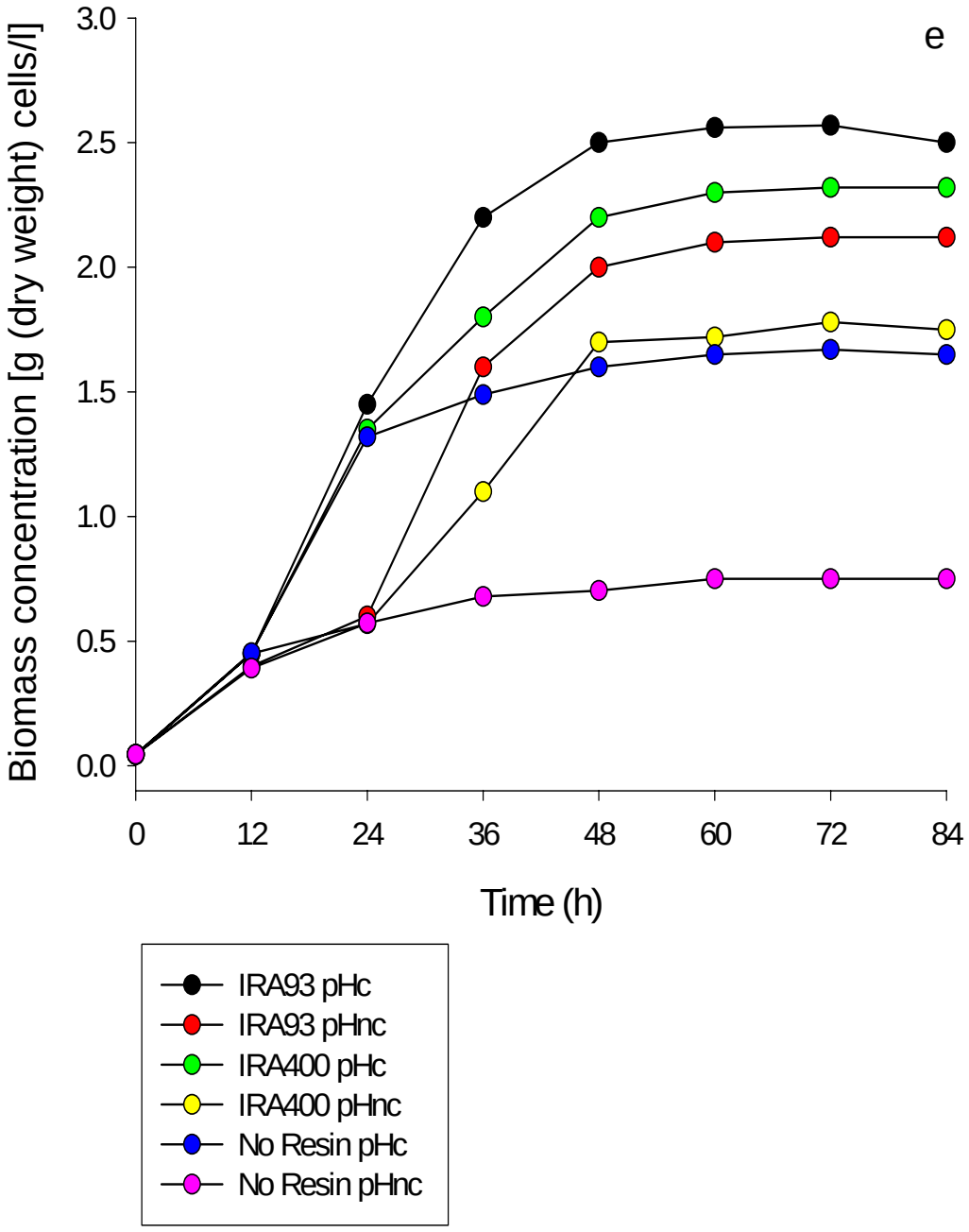


Figure 4.9 (e): Effect of pH and resin on the biomass concentration of *M. elsdenii* strain ATCC 25940 in the presence of Amberlite IRA-93, Amberlite IRA-400, no resin (pH controlled and uncontrolled) during 84 h culture in one litre volume Applicon fermenters. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.



On termination of the fermentation, 21 ml of wet resin was taken, dried and placed in screw-capped Erlenmeyer flask. Ethanol (100 ml, 80.02 g) and sulfuric acid (4.01 g, i.e., 5% weight of ethanol) was added to the dried resin and the flask was incubated with shaking at 55^{o}C. Solution samples were removed periodically up to 84 h and the concentration of VFAs and their esters were determined as described in materials and methods. The time course for the fermentations is shown in Figure 4.9 (a) – (e) and the final results of the esterification of the resin-adsorbed acids appears in Table 4.4, the values being corrected to give the amounts of acids and esters recoverable from the total amount of resin used in the fermentation and the results are summarized in Table 4.4.

Table 4.4: Summary of the effect of the addition of Amberlite IRA-93 (free base form) and Amberlite IRA-400 (chloride form) resins and no resins on VFA production during 84 h fermentation under controlled and uncontrolled pH conditions by *M. elsdenii*.

	No Resin pHc	No Resin pHnc	IRA-93 pHc	IRA-93 pHnc	IRA-400 pHc	IRA-400 pHnc
V_b (l)	0.80	0.80	1.066	1.066	0.933	0.933
C^b_{HEX}	70	23	24	5	16.5	3.2
C^b_{BUTY}	22.5	10	21.4	3.3	13	3.8
$R^b_{HEX/BUTY}$	3.1	2.3	1.12	1.5	2.3	0.8
C^e_{HEX}	70	23	95	46	88	32
C^e_{BUTY}	22.5	10	34	21	31	12
$R^e_{HEX/BUTY}$	3.1	2.3	2.8	2.2	2.8	2.7
S (g/l)	36.5	15	38.5	28	37.5	17.5
$Y_{p/s}$ (HEX)(g/g)	0.22	0.18	0.29	0.20	0.27	0.21
$Y_{p/s}$ (BUT)(g/g)	0.05	0.06	0.08	0.07	0.07	0.06
P_{HEX}	0.10	0.03	0.13	0.06	0.12	0.04
P_{BUTY}	0.02	0.01	0.04	0.02	0.03	0.03
T_h	84 h	84 h	84 h	84 h	84 h	84 h

V_b = broth volume, C^b_{HEX} = concentration of *n*-hexanoic acid in broth, C^b_{BUTY} = concentration of *n*-butyric acid in broth, $R^b_{HEX/BUTY}$ = C^b_{HEX}/C^b_{BUTY}; ratio of *n*-hexanoic acid concentration to *n*-butyric acid concentration in broth, C^e_{HEX} = effective concentration of *n*-hexanoic acid, C^e_{BUTY} = effective concentration of *n*-butyric acid, S = glucose consumption, $R^e_{HEX/BUTY}$ = C^e_{HEX}/C^e_{BUTY}, ratio of effective concentration of *n*-hexanoic acid to *n*-butyric acid. $Y_{p/s}$ (HEX) = Yield of *n*-hexanoic acid, $Y_{p/s}$ (BUTY) = Yield of *n*-butyric acid, P_{HEX} = volumetric productivity of *n*-hexanoic acid, P_{BUTY} = volumetric productivity of *n*-butyric acid, T_h = 84 h (duration of fermentation).

For pH-controlled fermentations, the amount of total acids increased in the both resins, the concentration of hexanoic and butyric acids in the presence of IRA-93 pH controlled (pHc) fermentations were 149 mM and 43 mM respectively. In the presence of resin, the overall glucose consumption increased by 26% (Table 4.4). The glucose consumption increased markedly after the addition of the resin,

however, after 36 h, the consumption of glucose in the presence of the resin under pH controlled samples was only slightly higher than the pH uncontrolled samples (Figure 4.9a).

The yield was increased in the presence of the resin. This would be due to the change in the product composition since the pH for the control and the resin-containing system was the same. The value of $R^{e}_{HEX/BUTY}$ was decreased in the presence of the resin (Table 4.4). Therefore, due to the higher affinity of the resin for *n*-hexanoic acid than for *n*-butyric acid, there was greater adsorption of *n*-hexanoic acid by the resin, greater relief of inhibition of its production by the cells and hence greater production of the acid leading to an increased yield. Comparison of C^{b}_{BUTY} and C^{e}_{BUTY} values show that the resin adsorbed n-butyric acid and that the production of this VFA was enhanced in the presence of the resin (Table 4.4). These results provide further evidence that Amberlite IRA-93 has a lower affinity for *n*-butyric acid than does Amberlite IRA-400. The volumetric productivity for *n*-hexanoic acid and *n*-butyric acid was increased for the resin containing system (Table 4.4). The calculations of the fermentation results are given in the appendix A-3.

In conclusion, in the presence of Amberlite IRA-93 (free base) resin under controlled pH, it is found that:

1. The effective concentration of *n*-hexanoic acid increased by 26%.
2. The effective concentration of *n*-butyric acid increased by 34%.
3. The glucose consumption and the yield of *n*-hexanoic acid increased.
4. The ratio of the effective concentration of *n*-hexanoic acid to *n*-butyric acid ($R^{e}_{HEX/BUTY}$) decreased.
5. The volumetric productivity for both acids was increased.

Comparison of the pH uncontrolled Amberlite IRA-93 and Amberlite IRA-400 resin experiments, show higher effective concentration, high volumetric productivity and high glucose consumption for both acids for Amberlite IRA-93 pH uncontrolled resin because of the buffering effect of the Amberlite IRA-93 resin as shown in Table 4.4.

4.2.6.6 Effects of Amberlite IRA-400 (chloride form) resins on *n*-hexanoic and *n*-butyric acids in batch fermentations

When the pH was controlled at 6.5 with no resins added, hexanoic and butyric acids increased from levels of 23 mM and 10 mM for pH uncontrolled cultures to 70 mM and 23 mM respectively (Figure. 4.9b and 4.9c). Addition of Amberlite IRA-400 resin to *M. elsdenii* fermentations resulted significant increases in the production of hexanoic and butyric acids compared to fermentations without resins, for both pH uncontrolled (pHnc) or controlled (pHc) cultures (Figure 4.9b and 4.9c). For pH uncontrolled fermentations, in the presence of Amberlite IRA-400 (chloride form) resin, the concentration of hexanoic and butyric acids was 47 mM and 16 mM respectively. Two fermenters (volume of PYG broth = 800 ml) were set up as described in Materials and Methods section and each was inoculated with 10% of *M.*

elsdenii two days old inoculum. The pH of one fermenter was controlled at 6.5 with sterile 10 M sodium hydroxide while other fermenter was run under pH uncontrolled condition. Amberlite IRA-400 (chloride form) resin was added in both fermenters at 24 h time point.

On termination of the fermentation, 21 ml of wet resin was taken, dried and placed in screw-capped Erlenmeyer flask. Ethanol (100 ml, 80.02 g) and sulfuric acid (4.01 g, i.e., 5% weight of ethanol) was added to the dried resin and the flask was incubated with shaking at 55^{o}C. Solution samples were removed periodically up to 84 h and the concentration of VFAs and their esters were determined as described in Materials and Methods. The time course for the fermentations is shown in Figure 4.9 (a) – (e) and the final results of the esterification of the resin-adsorbed acids appears in Table 4.4, the values being corrected to give the amounts of acids and esters recoverable from the total amount of resin used in the fermentation and the results are summarized in Table 4.4. For pH-controlled fermentations, the amount of total acids increased in the both resins, the concentration of hexanoic and butyric acids in the presence of Amberlite IRA-400 pH controlled (pHc) fermentations were 127 mM and 40 mM respectively as shown in Figure 4.9b and 4.9c. The glucose consumption increased markedly after the addition of the resin, however, after 36 h, the consumption of glucose in the presence of the resin under pH controlled samples was only slightly higher than the pH uncontrolled samples (Figure 4.9a).

It was also observed that the yield was increased in the presence of the resin. This seems to be due to the change in the product composition since the pH for the control and the resin-containing system was the same. The value of $R^{e}_{HEX/BUTY}$ was decreased in the presence of the resin (Table 4.4). Therefore, due to the higher affinity of the resin for *n*-hexanoic acid than for *n*-butyric acid, there was greater adsorption of *n*-hexanoic acid by the resin, greater relief of inhibition of its production by the cells and hence greater production of the acid leading to an increased yield. Comparison of C^{b}_{BUTY} and C^{e}_{BUTY} values show that the resin adsorbed *n*-butyric acid and that the production of this VFA was enhanced in the presence of the resin (Table 4.4). These results provide further evidence that Amberlite IRA-400 has a higher affinity for *n*-butyric acid than does Amberlite IRA-93. The volumetric productivity for *n*-hexanoic and *n*-butyric acids was increased for the resin containing system (Table 4.4).

In conclusion, in the presence of IRA-400 (chloride form) resin under controlled pH, it is found that:

1. The effective concentration of *n*-hexanoic acid increased by 20%.
2. The effective concentration of *n*-butyric acid increased by 27%.
3. The glucose consumption and the yield of *n*-hexanoic acid increased.
4. The ratio of the effective concentration of *n*-hexanoic acid to *n*-butyric acid ($R^{e}_{HEX/BUTY}$) decreased.
5. The volumetric productivity for both acids was increased.

Comparison of the pH uncontrolled IRA-400 and IRA-93 resin experiments, show lower effective concentration, low volumetric productivity and low glucose consumption for both acids for IRA-400 pH uncontrolled resin as shown in Table 4.4.

Addition of IRA-400 resin to *M. elsdenii* fermentations resulted in significant increases in the production of hexanoic and butyric acids compared to fermentations without resins, for both pH uncontrolled (pHnc) or controlled (pHc) cultures (Figure 4.9b and 4.9c). For pH uncontrolled fermentations, in the presence of IRA-400 (chloride form) resin, the concentration of hexanoic and butyric acids was 47 mM and 16 mM respectively. For pH-controlled fermentations, the concentration of hexanoic and butyric acids in the presence of IRA-400 were 127 mM and 40 mM respectively.

The presence of Amberlite IRA-93 with controlled pH fermentations shows the biomass concentration of 2.5 g dry weight cells/l (Figure 4.9e) and this increase was observed up to 48 h, whereas in the presence of Amberlite IRA-400 with controlled pH fermenters, the biomass concentration of 2.2 g dry weight cells/l, was slightly lower than IRA-93. However, in uncontrolled pH fermenters, the presence of IRA-93 resin shows significant increase of biomass concentration, 2.0 g dry weight cells/l compared to IRA-400, which was 1.7 g dry weight cells/l under the same conditions. Both the resins with either controlled or uncontrolled pH conditions show higher amount of biomass compared to the fermenters without resin (Figure 4.9e). However, the controlled pH without resin showed significantly high biomass concentration, 1.6 g dry weight cells/l compared to uncontrolled pH fermenters in the absence of resin (0.7 g dry weight cells/l).

The time course for the fermentations is shown in Figure 4.9 (a) – (e). The final results of the esterification of the resin-adsorbed acids appears in Table 4.4, the values being corrected to give the amounts of acids and esters recoverable from the total amount of resin used in the fermentation and the results are summarized in Table 4.4. In the presence of the resin pH controlled experiments, the effective concentration of *n*-hexanoic and *n*-butyric acids was increased by 20% and 27% respectively.

4.3 Discussion

A series of mutants spontaneously resistant to 3-FP had been isolated and preliminary characterization showed that these mutants display different sensitivity to end-products. Furthermore different patterns of end products made were seen, particularly when the parent and mutant strains were cultured in a mixture of substrates.

MICs were determined for growth on glucose and lactate to determine whether the major carbon source influenced their growth. The growth was observed on both major carbon sources for all the parents and their mutant strains. The mutants showed different patterns of sensitivity to potential antimetabolites. For example, ME5 when grown on glucose was outstanding in that it was more sensitive to pyruvic, hexanoic, acetic, butyric, 4-CBC, acrylic, 2-BMAA, 2-BPB, 3-CPA and valeric acids, as well as being $3FP^r$ compared to the ATCC 25940 parent strain (Table 4.1),

confirming its original phenotype. Though mutant strain ME5 was seven times more resistant to 3-FP and showed complete inhibition towards 2-BPB at the least concentration tested than the parental strain ATCC 25940 when grown on glucose that might lead to high activity of lactate dehydrogenase. All the *M. elsdenii* strains on THBAG plates showed almost four times more resistance to acrylic acid compared to its halogen analogue 2-bromomethyl acrylic acid (Figure 4.4). Strain ME12 was most sensitive to acetic acid (Figure 4.1) when grown on glucose compared to other parental and mutant strains studied. Strain ME12 showed no growth on glucose or lactate and were highly sensitive to 3-fluoropyruvate (Figure 4.3). Most of the strains studied on THBAG and THBAL plates showed eight times more sensitivity to bromoacetic acid (Figure 4.4) compared with acetic acid (Figure 4.1), with the exception of ATCC 25940 that showed the same pattern of growth on both. The parent strains ATCC 25940 and B159 showed similar MICs for end-products on THBAG plates but strain T-81 was more sensitive to pyruvate, hexanoic acid, acetic acid, acrylic acid and propionic acid than other parents and the wild-type strain 28.7B.

When grown on THBAL plates, strain ME5 showed resistance to pyruvic, 3-FP, hexanoic, acetic, bromoacetic, propionic acid, 2-BPB and valeric acid (Table 4.2). All the mutants grown on THBAL medium were almost two to four times more resistant to 3-FP compared to their parent strains except strain T-81, which showed no growth even at the minimum concentration tested. The phenotype of mutant strain ME5 was verified in that it was consistent with previous unpublished data and it implies that ME5 strain may be a metabolic variant.

Parental strain T-81 and its mutant ME14 was very sensitive to 2-bromopropionyl bromide in lactate media and showed no growth at the lowest concentration tested (Figure 4.3). Strain T-81 also showed no growth on 3-FP, hexanoic, 2-BBA and 2-BPB in the presence of lactate (Table 4.2). All the strains grown on THBAL plates showed almost six times more sensitivity to 3-chloropropionic acid (Figure 4.3) compared with propionic acid (Figure 4.2). The growth of most strains studied on THBAL medium showed almost three to four times more sensitive to 2-bromobutyric acid (Figure 4.5) compared with butyric acid (Figure 4.2) but strain T-81 showed complete growth inhibition on the lowest concentration tested. Parental strain B159 and its mutant strains ME8 and ME9 in the presence of glucose showed relatively more sensitivity to pyruvate, 3-FP, butyric acid, acrylic acid and propionic acid compared with lactate containing media. However, B159 had shown no growth on glucose medium with 3-FP but showed growth in the presence of lactate (Figure 4.3). Initially it was important to investigate end-products formation in terms of volatile fatty acids in small volume cultures (50 ml) containing different substrates and with the combination of substrates for the each parent and their mutant strains using uncontrolled pH conditions without stirring for 144 h growth periods. In the presence of glucose, the hexanoic acid was the major end-product produced by all the strains studied. Wild-type strain 28.7B produced the lowest concentration of hexanoic acid, whilst mutant ME12 produced the highest concentration of hexanoic acid (Figure 4.6a, 4.6.1a and 4.6.2a). Mutant strain ME5 produced the highest amount of valeric acid and the total acids produced by ME5 was also the highest amongst all the

strains studied (Figure 4.6a, 4.6.1a and 4.6.2a). However, when *M. elsdenii* parental and mutant strains were grown on lactate medium under uncontrolled pH condition, all the strains studied produced acetic, propionic, butyric, and valeric acids (Figure 4.6b, 4.6.1b, 4.6.2b and 4.6.3a). The major end-product on lactate medium was valeric, butyric and propionic acid produced by all the strains studied. However, in the presence of lactate medium, almost eight times more propionic acid (Figure 4.6.3a) was produced compared to the mixed substrates by most of the strains studied. In the presence of lactate, strain ME5 produced the highest amount of butyric acid compared to all other strains studied. Valeric and butyric acids were the major end-products while acetate and propionate was the minor end-products in the presence of mixed substrates (Figure 4.6c, 4.6.1c, 4.6.2c and 4.6.3b). Strain ME5 produced the highest concentration of valeric acid compared to all other strains studied (Figure 4.6c). These results showed that same mutants resistant to 3-FP showed different end-product formation to their parent strains.

After these preliminary studies on end-product profiles under uncontrolled pH, studies were conducted to confirm the metabolic capacity of the mutants in fermenters. The first step was to optimise the pH for growth, the most important parameter affecting growth and end-product formation during fermentation for the parental strain ATCC 25940 and mutants ME5 and ME7 when grown in the presence of peptone-yeast-glucose medium (Figure 4.7). The optimal pH in terms of growth rate and hexanoic and butyric acid production was at pH 6.5 (Figure 4.7a and 4.7b). An interesting observation was the significant reduction in the end-products when the pH values of the fermenters were below 6 and above 7. When the pH of broth was above 7, the *M. elsdenii* strains tend to produce relatively shorter chain acids, whereas when the pH was lower, more of the longer chain acids were produced on the basis of butyrate/hexanoate ratios. Mutant strain ME5 produced the less amount of hexanoic acid at pH 5.5 compared to its parent strain ATCC 25940 (Figure 4.7a), showing that this mutant is more sensitive towards lower pH values.

Parental strain ATCC 25940 and its mutant ME5 and ME7 were further tested for the end-products under controlled pH fermenters with continuous stirring in the presence of glucose, lactate and glucose plus lactate medium at pH 6.5. Mutant strain ME5 produced the highest concentration of valeric acid compared to ATCC 25940 and ME7 in the presence of glucose (Figure 4.8.2a). Other major acids produced by these strains were hexanoic and butyric acids. In the presence of lactate, the parental strain ATCC 25940, mutants ME5 and ME7 produced acetic, propionic, butyric and valeric acids. Valeric acid was the highest concentration produced by these three strains while hexanoic acid was least concentration detected (Figure 4.8b, 4.8.1b and 4.8.2b). In the mixed substrates (glucose plus lactate), all these strains produced acetic, propionic, butyric, valeric and hexanoic acids (Figure 4.8c, 4.8.1c and 4.8.2c). However, the major products were butyric, valeric and hexanoic acids. However, the major products were butyric, valeric and hexanoic acids by the strain ATCC 25940, ME5 and ME7. Total acids produced were greater in the presence of mixed substrates compared to single substrate used. Changes in metabolism seen for these mutants (indicated by altered production of certain short

chain organic acids) can be attributed to changes in levels of enzymes involved in pyruvate or lactate metabolism.

M. elsdenii is a ruminal soluble-substrate fermenting species. The composition of its fermentation end-products was not the same in media with different energy sources, evidently due to the predominance of preferential fluxes and/or higher activities of individual enzymes within the overall metabolic space of the bacterium depending on the nature of the substrate (lactate or glucose or their mixture) used. Furthermore, single-and mixed-substrate fermentation differed as mentioned above in the experiments that are in conjunction with previous studies. Uncontrolled pH cultures of *M. elsdenii* strains with or without stirring produced lower amount of acids than the comparable controlled pH and stirred cultures.

M. elsdenii strains had previously been shown to be sensitive to growth inhibition by the end-products of metabolism (Margaret Britz personal communication). In particular, as the chain length of the acid increased, the severity of inhibition increased so that hexanoic acid was most toxic. However, use of ion-exchange resins had been shown to alleviate product inhibition so that metabolism and growth continued if acidic end products were continually stripped (Roddick and Britz, 1997).

Initially the Amberlite IRA-93 (weak base anion-exchange resin) and Amberlite IRA-400 (strong base anion-exchange resin) resins were tested for their ability to adsorb and desorb butyric and hexanoic acids from water and peptone-yeast-glucose media (Table 4.3). It was observed that both resins adsorbed more *n*-hexanoic acid than *n*-butyric acid, which suggested that the VFA chain length might affect the degree of adsorption. Both acids were desorbed more effectively by esterification from Amberlite IRA-93 resin. This indicated that Amberlite IRA-93 might have some attractive features above Amberlite IRA-400 in terms of product recovery in the form of esters. Roddick and Britz (1997) had shown that culturing *M. elsdenii* under pH controlled conditions significantly increased the amount of acid end-products and including Amberlite IRA-400, a strong anion exchange resin, significantly increased total acids produced, presumably due to removal of end-product inhibition. Roddick and Britz (1987) reported an increase in production of *n*-hexanoic acid by *M. elsdenii* from 7.4 g/l to 13.1 g/l using Amberlite IRA-400 added to the fermenter. However, hexanoic acid bound strongly to Amberlite IRA-400, necessitating its removal using *in situ* esterification (Roddick and Britz, 1997). There were several problems associated with adsorption methods. One was the possible toxicity of the adsorbent to the microorganisms (Wang, 1981), and the other was the non-specific adsorption of the by-products and broth components (Lencki *et al*., 1983). This may lead to removal of nutrients from the broth causing reduction of microbial activity, reduction of adsorption capacity for the product (Roffler *et al*., 1984) and interference with product purification and /or removal due to the presence of substances other than the product on the adsorbent. When ion exchange resin is used, the accumulation of the ions released from the resin as materials are adsorbed may inhibit product synthesis by the microorganisms, e.g., by altering the pH.

Further studies were carried out on the adsorption and desorption of both acids on the fermentation level in peptone-yeast-glucose media under pH controlled and pH

uncontrolled conditions (Table 4.4) for the parental strain ATCC 25940. Results indicated that using the Amberlite IRA-93 had an advantage in that it did not release a counter ion on binding of anions so that it exerts a buffering effect on the fermentation broth, thus reducing the degree of pH control needed and the amount of alkali used to maintain the optimum pH. When the pH was controlled at 6.5 with no resins added, butyric and hexanoic acid production increased 2-3 times respectively compared to uncontrolled pH conditions. After the introduction of Amberlite IRA-93 resin in controlled pH fermenters, the amount of butyric and hexanoic acids were doubled compared to the samples without resin. In the presence of Amberlite IRA-93 resin under controlled pH, the effective concentration of *n*-hexanoic acid increased by 26%, the effective concentration of *n*-butyric acid increased by 34%, the glucose consumption and the yield of *n*-hexanoic acid increased. The ratio of the effective concentration of *n*-hexanoic acid to *n*-butyric acid decreased, and the volumetric productivity for both acids was increased. Comparison with pH uncontrolled Amberlite IRA-93 and Amberlite IRA-400 resin experiments showed that the effective concentration, volumetric productivity for both acids and glucose consumption were higher for Amberlite IRA-93 pH uncontrolled resin compared with Amberlite IRA-400 due to buffering effect of this resin (Table 4.4). The presence of Amberlite IRA-93 with controlled pH fermentations showed the high biomass concentration (Figure 4.9e) and this increase was observed up to 48 h, whereas in the presence of Amberlite IRA-400 with controlled pH fermenters, the biomass concentration was slightly lower than Amberlite IRA-93. However, in uncontrolled pH fermenters, the presence of Amberlite IRA-93 resin showed significant increase of biomass concentration compared to Amberlite IRA-400 under the same conditions. Both the resins with controlled or uncontrolled pH conditions showed higher amount of biomass compared to the fermenters without resin (Figure 4.9e), although fermenters run with controlled pH and without resin showed significantly high biomass concentration compared to pH uncontrolled fermenters in the absence of the resin. This suggests that the adsorption of VFAs, particularly of *n*-hexanoic acid, by the resins reduces the growth inhibition by acidic end-products, but for Amberlite IRA-400 the release of the resultant counter ion causes some inhibition of growth as shown previously by Deguchi 1994.

The proportion of butyric and hexanoic acids seen with or without resins was similar therefore it was decided not to pursue similar studies with the mutants. These studies enabled us to characterise and verify the mutants and their parental strains. Their fermentation products were analysed on single and mixed-substrates and compared for the recovery from the fermentation broth by using ion-exchange resins. Different proportions of the end-products in mutants compared with their parental strains suggested carrying out studies on enzymes and genes involved in metabolism. Parental strain ATCC 25940 and its mutants were selected to study the enzymes activity involved in the production of major end-products along with their metabolic routes. The amount of any particular enzyme is regulated at the level of RNA and protein synthesis and controlled by regulatory mechanism; however, constitutive enzymes produced at moderate levels all the time and are involved in metabolic activities. Some of these enzymes activities are measured and their genetic sequence analyses are described in the following chapter.

CHAPTER 5

5 STUDIES ON ENZYMES INVOLVED IN THE FERMENTATION OF GLUCOSE AND LACTATE AND INVESTIGATION OF SELECTED GENES IN 3FP^r MUTANTS AND PARENT STRAIN ATCC 25940

5.1 Introduction

A major aspect of this chapter was to investigate the biochemical and genetic systems involved in short and long chain volatile fatty acid production by *M. elsdenii*. *M. elsdenii* strains were grown on glucose, lactate and mixed substrates (glucose plus lactate) as described in the previous chapter and characterized in terms of sensitivity to end-products and anti-metabolites plus products made in batch and pH controlled fermenters.

When the parent and their mutant strains were grown in the presence of glucose under uncontrolled pH, hexanoic acid was the major end-product by all the strains studied. Under similar conditions, only mutant ME5 produced significant amounts of valeric acid, this was in contrast to all other strains, which evidenced diversity amongst the strains in terms of their metabolic activities.

It was also noted that *M. elsdenii* strains produced acetic, propionic, butyric and valeric acids in the presence of lactate as a carbon source in peptone-yeast medium, and, furthermore, under these conditions it was shown that the mutant strain ME5 produced higher concentrations of valeric and butyric acids among all the strains studied. In the presence of lactate medium, it appears that propionates and acetate are the minor end-products in all the strains. Similarly, acetates and propionates were also the minor end-products in the presence of mixed substrates by the entire parent and their mutant strains. Also, compared to all other strains, in the presence of mixed carbon substrates, the mutant strain ME5 produced higher concentrations of valeric acid while ME14 showed the highest amount of butyric acid.

These results indicated that these mutants display different degrees of sensitivity to their end-products and anti-metabolites made in batch and pH-controlled fermenters, using either single or mixed carbon sources. This observation is supported by the fact that it has been reported that *M. elsdenii* can utilize several sugars and lactate at the same time and the substrate preferences are, in order: glucose, maltose, lactate and sucrose (Russell and Baldwin, 1978; Russell *et al*., 1979). *M. elsdenii* has a mixed acid metabolism, as the bacterium converts its preferred substrates, glucose, maltose, lactate and sucrose, into acetic, butyric and finally *n*-hexanoic acid *via* a series of serial condensations of C_2 subunits of acetyl CoA derived from the metabolism of C_3 acid pyruvate. Pyruvate is derived most probably from the glycolysis of carbohydrate to generate the extra reducing equivalents needed to regulate its metabolism (Pacaud *et al*., 1985; Roddick and Britz, 1986).

Mutants that are 3-FP resistant had altered fermentation profiles, which suggested that the flow of carbon *via* pyruvate as a key intermediate in the intermediary metabolism had changed. Production of *n*-valeric acid was enhanced when the same mutants grew on lactate or glucose plus lactate under controlled and uncontrolled pH conditions. This led to a study of the enzymes involved in pyruvate metabolism that may be sensitive to inhibition by 3-FP and may have been altered in $3FP^r$ mutants either at the level of enzyme structure or level of its synthesis.

The general aim of this chapter was to investigate the biochemical and genetic systems involved in short and long chain carbon fatty acids production to elucidate changes in the metabolic pathways in anti-metabolite-resistant mutants of *M. elsdenii*.

The approach used was:

a) to detect and measure the lactate dehydrogenase and pyruvate dehydrogenase enzyme activities in crude cell free extracts of parent ATCC 25940 and its mutant ME5 and ME7 strains, to determine if changes in specific activity might be responsible for varied product formation;

b) to study the organism on a molecular genetic level by sequencing the genes involved in pyruvate metabolism such as short-chain acyl-CoA dehydrogenase (SCAD), electron-transferring flavoprotein subunit α (*etfA*) and subunit β (*etfB*) gene and lactate dehydrogenase (LDH) genes; and

c) to detect and quantify the production of lactic and pyruvic acids by parental strain ATCC 25940 and its mutant ME5 and ME7 strains during fermentation studies in the presence of glucose, to determine whether this was consistent with measured enzyme activities.

5.2 Results

5.2.1 Pyruvate dehydrogenase (PDH) activity in cell-free extracts of parental and mutant strains

M. elsdenii strains were grown on peptone-yeast extract plus 4% glucose (PYG) media in 1 litre Applicon fermenters for 48 h with automated pH control at 6.5 with constant stirring. Cells were collected and cell free extract prepared as described in section 2.5.3.1. Pyruvate dehydrogenase activity was measured under anaerobic conditions in cell-free extracts of the parental strain ATCC 25940 and mutants ME5 and ME7 for cells collected from cultures at intervals of 24, 36, and 48 h. Enzyme activity was measured under anaerobic conditions by the reduction of methyl viologen, detected by increases in absorbance at 600 nm. Cell-free extracts were prepared under anaerobic conditions to avoid loss in activity due to O_2 exposure, since similar enzymes from other anaerobes were sensitive to O_2 inactivation (Britz and Wilkinson, 1978). Table 5.1 shows the pyruvate dehydrogenase specific activity in milli-International Units (M.I.U.) per milligram of protein. High activity of the enzyme was observed in the logarithmic phase of growth (24 h) and then became very low during stationary phase of growth, but was still detectable. Strain ATCC

25940 showed the highest activity (172 M.I.U /mg protein) after culture for 24 h then this gradually decreased to 60 M.I.U. at 36 h to 4 M.I.U. at 48 h of culture. The lowest enzyme activity was observed in the samples collected at 48 h as shown in Table 5.1. Strain ME7 showed similar maximum activity (164 M.I.U.) at 24 h that also gradually decreased to 66 M.I.U. at 36 h to 6 M.I.U. at 48 h of culture. Strain ME5 showed much less enzyme activity for pyruvate dehydrogenase, ranging from 0.06-1.7 M.I.U which was 100 times lower than activity detected in the parental strain ATCC 25940, as shown in Table 5.1.

Table 5.1: Specific activity of pyruvate dehydrogenase and lactate dehydrogenase at different growth phases.

Enzyme Activity	Specific activity (M.I.U./mg protein)								
	ATCC 25940			ME 5			ME 7		
	Time (h)								
	24[a]	36	48	24	36	48	24	36	48
Pyruvate Dehydrogenase (PDH)	172	60	4.0	1.7	0.24	0.06	164	66	6.0
Lactate Dehydrogenase (LDH)	0.42	0.26	0.24	161	77	12	0.75	0.32	0.2

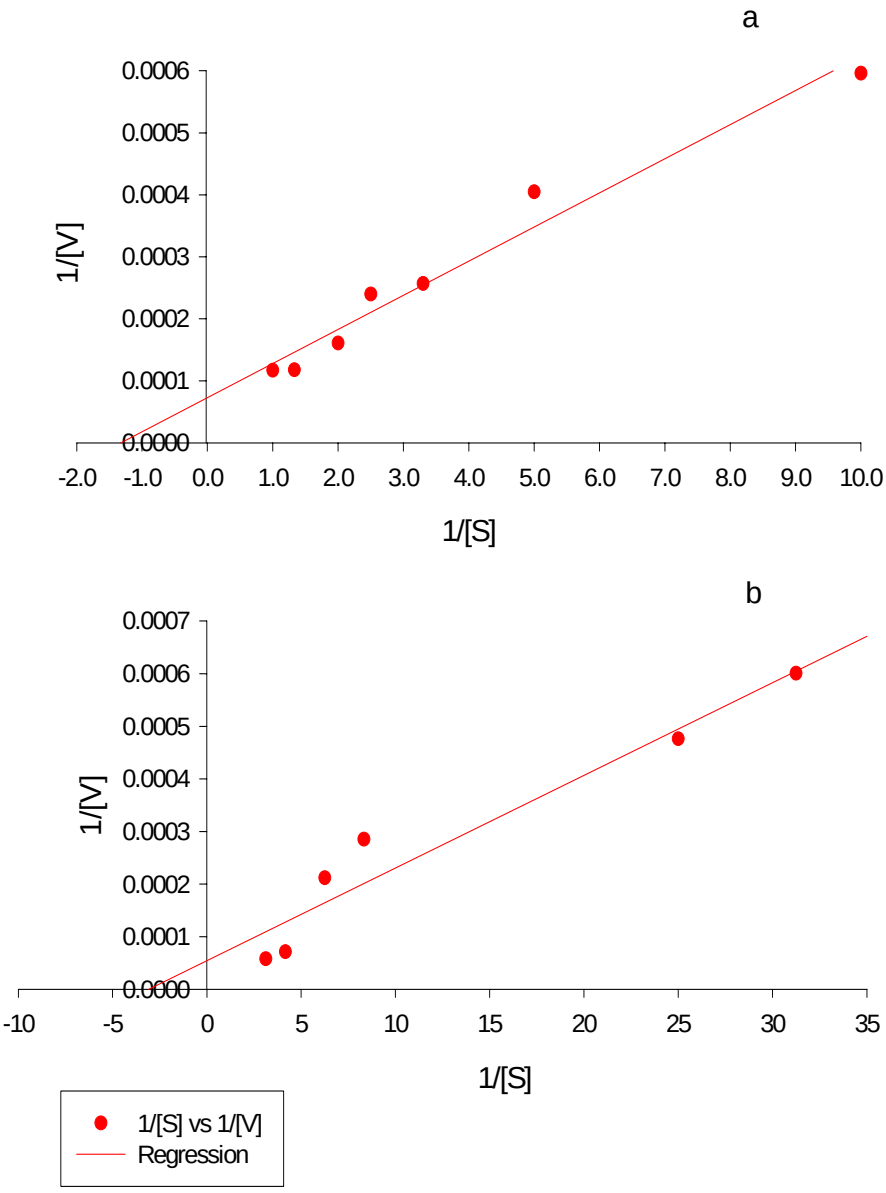
[a] Cells were harvested within 24 to 48 h grown in peptone-yeast-glucose medium.

M.I.U. is milli-International units.

5.2.2 Lactate dehydrogenase (LDH) activity in cell-free extracts of parental and mutant strains

M. elsdenii strains were grown on peptone-yeast plus 4% glucose (PYG) media in 1 litre Applicon fermenters for 48 h with automated pH control at 6.5 with constant stirring. Cells were collected and cell-free extracts prepared as described in section 2.5.3.1. Lactate dehydrogenase activity was measured under anaerobic conditions in cell-free extracts of the parental strain ATCC 25940 and mutants ME5 and ME7 for cells collected at intervals of 24, 36, and 48 h. Table 5.1 shows the lactate dehydrogenase specific activity in M.I.U. per milligram of protein. Strain ME5 showed highest activity (161 M.I.U.) at 24 h and this gradually decreased to 77 M.I.U. at 36 h and to 12 M.I.U. for cells harvested at 48 h. The lowest enzyme activity was observed in samples collected at 48 h cultures. Strain ATCC 25940 showed maximum specific activity (0.42 M.I.U.) at 24 h, and then this gradually decreased to 0.26 M.I.U. at 36 h to 0.24 M.I.U. at 48 h of culture. Strain ME7 also showed much less enzyme activity than ME5, ranging from 0.20-0.75 M.I.U., with the highest activity observed at 24 h. Extracts of ATCC 25940 and mutant ME7 showed 100 times less enzyme activity compared to mutant ME5, as shown in Table 5.1. For all these strains, the specific activity of the enzyme was far greater in exponential phase and then this decreased to very low levels by stationary phase. Kinetic studies of LDH were undertaken by measuring K_m and V_{max} values for pyruvate and NADH and are shown in Figure 5.1a, b. The K_m values for pyruvate and NADH were 2.46 mM and 0.23 mM respectively. The V_{max} was calculated to be 33.33 mM for pyruvate and 16.7 mM for NADH.

Figure 5.1: Lineweaver-Burke plot for a) pyruvate as substrate and b) NADH as substrate for lactate dehydrogenase. The cell free extracts were prepared as described in Materials and Methods (section 2.5.3.1) for *M. elsdenii* mutant strain ME5 and K_m and V_{max} were determined using extracts of cells harvested from 24 h cultures.



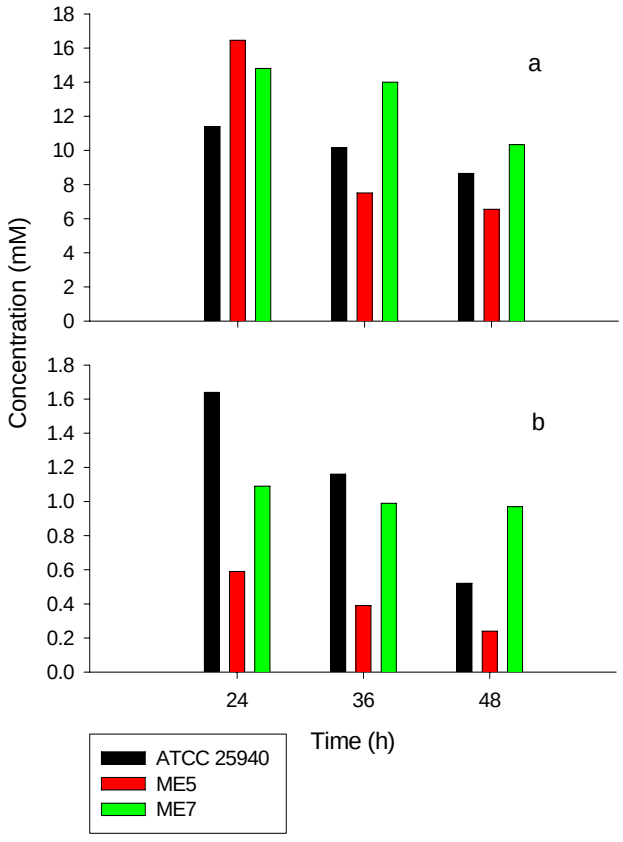
Because the specific activity in strain ATCC 25940 and ME7 were significantly lower, similar kinetic studies were not performed. A control experiment for LDH activity was run in the absence of cell-free extract to allow for background reading and the decrease in the absorbance was observed in the presence of pyruvate or NADH as a substrate or cofactor respectively. See appendix-2 Figures A-10 and A-11 for an example of enzyme activity assays.

5.2.3 Detection of lactic acid and pyruvic acid in fermentation broths of *M. elsdenii* strains (ATCC 25940, ME5 and ME7) by HPLC

M. elsdenii strains were grown on peptone-yeast plus 4% glucose (PYG) media in 1 litre Applicon fermenters for 48 h with automated pH control at 6.5. Samples were collected according to the method given in the section 2.4.1. Lactic and pyruvic acids were detected by HPLC (section 2.4.4.4) in the *M. elsdenii* cultures at 24, 36, and 48 h, as shown in Figure 5.2. The highest concentration of lactic acid (16.5 mM) for the strain ME5 was detected in the samples collected at an incubation time of 24 h. The highest concentration of lactic acid in the ATCC 25940 and ME7 strains was 11.4 mM and 15 mM respectively at 24 h incubation.

The pyruvic acid concentration was very low in all the strains ATCC 25940, ME5 and ME7, relative to lactic acid that ranged from 0.24-1.64 mM. The highest concentration of pyruvic acid (1.64 mM) for strain ATCC 25940 was detected in samples collected at 24 h. The highest concentration of pyruvic acid (0.59 mM) for the strain ME5 was detected in the samples collected at 24 h incubation.

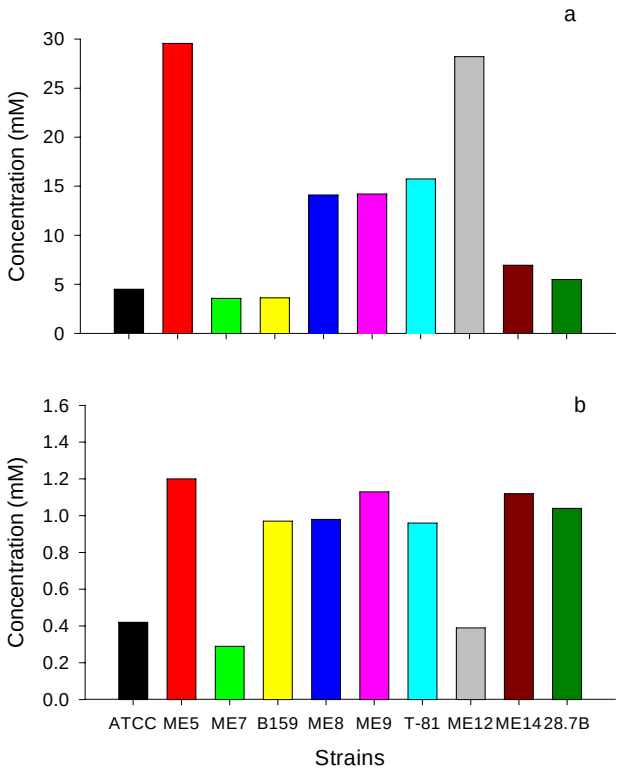
Figure 5.2: Measurement of a) lactic acid and b) pyruvic acid by HPLC in peptone-yeast-glucose medium following growth of *M. elsdenii* parental and mutant strains during 48 h culture in 1L Applicon fermenter at pH 6.5 with constant stirring. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.



5.2.4 Detection of lactic acid and pyruvic acid in small-volume culture of *M. elsdenii* strains by HPLC

After detecting lactic and pyruvic acids in strains ATCC 25940, ME5 and ME7, using pH controlled fermenters, all strains used in this thesis were screened for lactic and pyruvic acids using 50 ml peptone-yeast extract plus 0.8% glucose (PYG) medium cultures-conditions used for routine detection of VFAs. The 144 h duration samples were chosen, because at this time the maximum concentrations of VFAs were observed. Lactic acid was detected by HPLC (section 2.4.4.4) in the *M. elsdenii* cultures as shown in Figure 5.3a. The parental strain ATCC 25940 produced 5 mM of lactic acid compared to its mutants ME7 (4 mM) and ME5 (30 mM). The parental strain T-81 produced 16 mM of lactic acid compared to its mutants ME12 (28 mM) and ME14 (7 mM). The parental strain B159 produced 4 mM of lactic acid compared to its mutants ME8 and ME9 produced 14 mM of lactic acid respectively. The highest concentration of lactic acid was produced by the strain ME5 followed by ME12 strain as shown in Figure 5.3a.

Figure 5.3: Measurement of a) lactic acid and b) pyruvic acid by HPLC in peptone-yeast-glucose medium following growth of parental and mutant strains of *M. elsdenii* during 144 h incubation in 50 ml culture without stirring and no pH control. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.



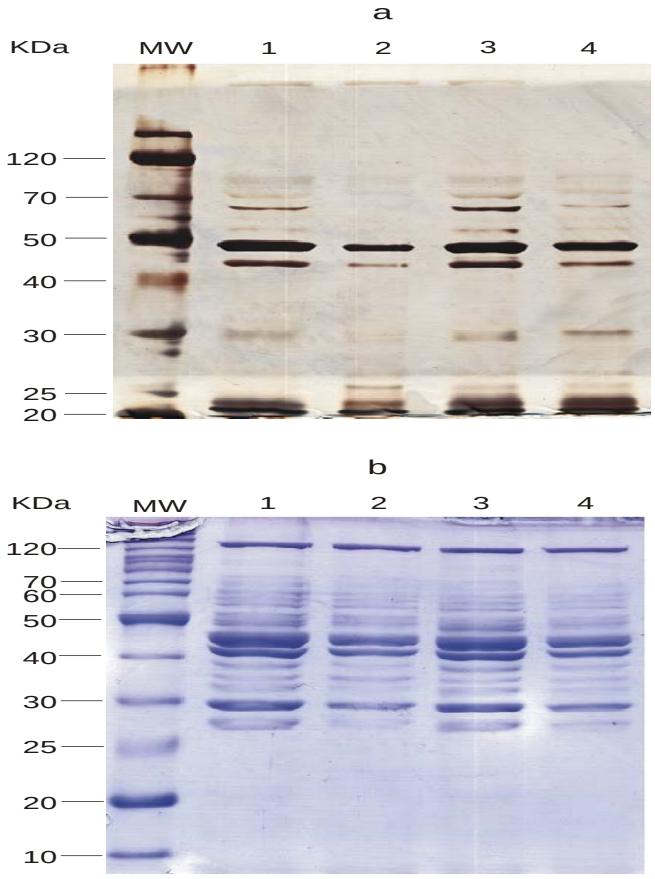
Pyruvic acid was detected by HPLC (section 2.4.4.4) in the *M. elsdenii* cultures as shown in Figure 5.3b. The parental strain ATCC 25940 produced 0.4 mM of pyruvic acid compared to its mutants ME5 (1.2 mM) and ME7 (0.3 mM). The parental strain T-81 produced 1 mM of pyruvic acid compared to its mutants ME12 (0.4 mM) and ME14 (1.12 mM).

The parental strain B159 produced 1 mM of pyruvic acid compared to its mutants ME8 and ME9 1 mM and 1.13 mM respectively. The accumulation of pyruvic acid ranged between 0.3 mM to 1.2 mM in all the strains studied as shown in Figure 5.3b. These data show that ME5 could be distinguished from its parent ATCC 25940 on the basis of accumulating high concentrations of both pyruvic and lactic acids in stationary phase cultures without pH control.

5.2.5 Protein profiles of parent and mutant strains of *M. elsdenii* on SDS-PAGE

Cell-free extracts of parental (ATCC 25940) and mutant (ME5) strains of *M. elsdenii* collected at 24 and 48 h intervals were subjected to SDS-PAGE (section 2.5.4) under reducing conditions to separate the proteins. The gels were stained with Coomassie blue or silver staining (section 2.5.4.3 and 2.5.4.4 respectively) to see the differences among the strains to relate with the enzyme activities, as shown in Figure 5.4a and b. The banding patterns of mutant strain ME5 are not distinct from the parent ATCC 25940 strain and cannot explain the changes observed during assays of enzymes, suggesting that the changes were in minor proteins or in their structure rather than at the synthesis level. Two-dimentional protein gels are the choice in future to see the differences among the strains.

Figure 5.4: SDS-PAGE of cell extracts of parent and mutant strains of *M. elsdenii* stained for total protein with a) Silver and b) Coomassie blue.

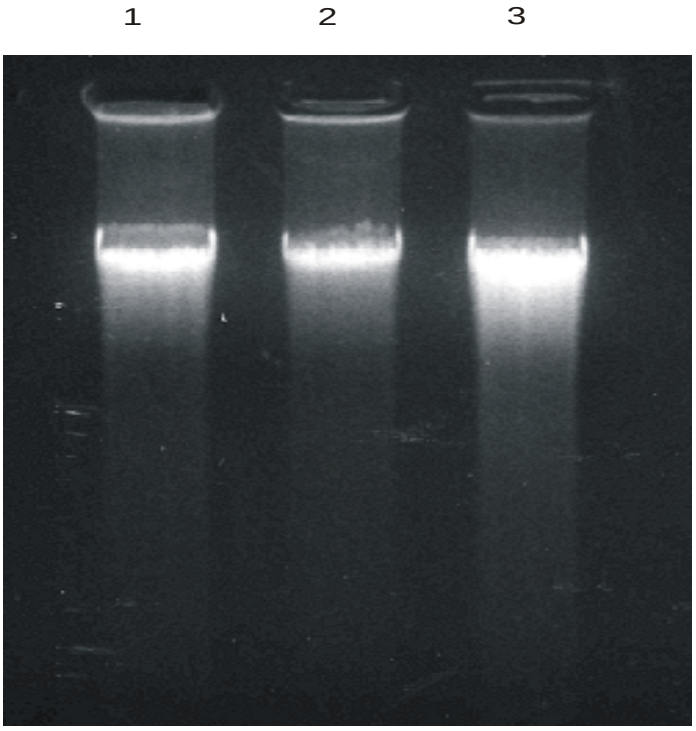


Lane 1: ATCC 25940 (24 h) Lane 2: ME5 (24 h)
Lane 3: ATCC 25940 (48 h) Lane 4: ME5 (48 h)
MW: Molecular Weight Standards ranging from 10 to 220 kDa (Kilodaltons)

5.3 Isolation of Genomic DNA from the strains of *M. elsdenii*

Genomic DNA of three strains of *M. elsdenii* (ATCC 25940, ME5 and ME7) was isolated from overnight grown culture in 50 ml peptone-yeast extract plus 0.8% glucose (PYG) medium as described in the section 2.6.1.2 and shown in Figure 5.5. The concentration of DNA was measured by the method given in section 2.6.1.5 and used in all the following genetic studies on these strains.

Figure 5.5: Isolation and purification of genomic DNA from *M. elsdenii* strains.



Lane 1: Strain ATCC 25940 Lane 2: Strain ME5 Lane 3: Strain ME7

5.3.1 PCR cloning and sequencing of gene coding for Short-Chain Acyl-CoA Dehydrogenase (SCAD) from *M. elsdenii* parental (ATCC 25940) and mutant (ME5 and ME7) strains

5.3.1.1 Construction of primers

Primers for the amplification of Short-Chain Acyl-CoA Dehydrogenase (SCAD) genes from total genomic DNA isolated from *M. elsdenii* parental (ATCC 25940) and mutant strains ME5 and ME7 were designed in this study as listed in Table 5.2 from the published sequence of SCAD by Becker *et al*., 1993. The primers were designed from the published *M. elsdenii* strain ATCC 25940 SCAD sequences (Accession No: L04528) for full-length (1.55 kb) PCR product to compare any change in SCAD gene sequences in the parental and mutant strains in relation to their metabolic routes.

Table 5.2: Synthetic oligonucleotide primers employed in the PCR amplification of genomic DNA of *M. elsdenii* strain for Short Chain Acyl CoA dehydrogenase (SCAD).

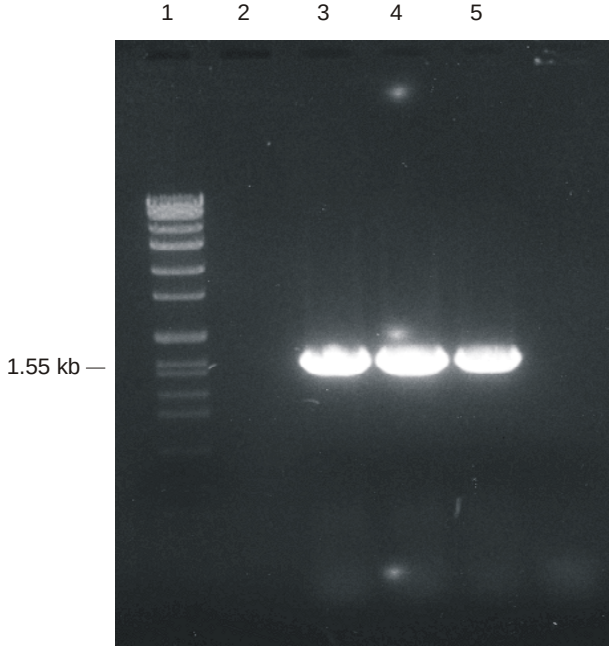
Primer	Sequence 5' to 3'	Position (nucleotide number)	Length (nucleotide)	Hybridize to
MEF–4	AGAGACGTACGTGATCAAGAAGAT	67–90	24	SCAD
MER–6	CGCATGTGACGAGCAACCGGATAT	1405–1428	24	SCAD
MER–7	CTGCAGTGTCCGGAACCTGTTTGA	1599–1622	24	SCAD

F and R indicating forward and reverse primers respectively and were designed in this study from the published sequence of SCAD by Becker *et al*., 1993.

5.3.1.2 PCR amplification products

These oligonucleotides then served as primers (MEF–4 and MER–7) in a polymerase chain reaction (PCR) using genomic DNA from *M. elsdenii* strains ATCC 25940, ME5 and ME7. PCR amplification products (1.55 Kb) of the SCAD gene were obtained by using Expand™ Long Template PCR system. The conditions for the PCR were as follows: initial denaturation for 2 min at 94°C, followed by 10 s at 94°C; annealing, 30 s at 55°C; elongation, 90 s at 68°C for 9 cycles, followed by further denaturation at 94°C for 10 s; annealing, 30 s at 55°C; elongation, 90 s at 68°C with extension of 20/s/cycle for 19 cycles followed by final elongation at 68°C for 7 min. The PCR mixture was analysed by 1% agarose gel electrophoresis and a single band was obtained corresponding to the expected 1.55 kb fragment as shown in Figure 5.6.

Figure 5.6: PCR amplification for SCAD gene from the *M. elsdenii* strains using genomic DNA.



Lane 1: SPP molecular weight markers
Lane 2: Negative control (no DNA)
Lane 3: Strain ATCC 25940 (1.55 kb PCR product using primers MEF–4 and MER–7)
Lane 4: Strain ME5 (1.55 kb PCR product using primers MEF–4 and MER–7)
Lane 5: Strain ME7 (1.55 kb PCR product using primers MEF–4 and MER–7)

5.3.1.3 SCAD gene sequence analysis

Purified PCR amplification products of the SCAD gene for the strain ATCC 25940, ME5 and ME7 of *M. elsdenii* are shown in Figure 5.6 were used as a template for cyclo-sequencing reactions. The primers used for sequencing were MEF–4 (forward) and MER–7 (reverse) for 5'–3' and 3'–5' direction respectively. The nucleotide sequence of 1.55 kb fragment of three strains was determined using the ABI-PRISM Dye Terminator Cycle Sequencing Kit in an automated DNA sequencer as described in section 2.6.12. After sequence assembly (Figure 5.7), the completed sequence of the SCAD gene (1.55 kb) of the three *M. elsdenii* strains was found to represent the whole gene. A sequence similarity search based on the NCBI database was performed using ANGIS and BLASTN analysis, which showed that the SCAD gene was identical to the published sequence for *M. elsdenii* Accession number L04528. Figure 5.7 shows the multiple sequences alignment of the SCAD gene sequences of strains ATCC 25940, ME5, ME7 and Accession no. L04528, which was performed using a multiple comparison program Clustal.

Figure 5.7: Multiple sequence alignment of the Short-chain Acyl-CoA dehydrogenase (SCAD) gene sequence of *M. elsdenii* strains ATCC 25940, ME5, ME7 and database sequence L04528 using a multiple comparison program (Clustal).

	10	**20**	**30**	**40**	
1	ATGGATTTTA	ACTTAACAGA	TATTCAACAG	GACTTCTTAA	L04528
1	ATGGATTTTA	ACTTAACAGA	TATTCAACAG	GACTTCTTAA	ME7
1	ATGGATTTTA	ACTTAACAGA	TATTCAACAG	GACTTCTTAA	ATCC 25940
1	ATGGATTTTA	ACTTAACAGA	TATTCAACAG	GACTTCTTAA	ME5
	50	**60**	**70**	**80**	
41	AACTCGCTCA	TGATTTCGGC	GAAAAGAAAT	TAGCACCGAC	L04528
41	AACTCGCTCA	TGATTTCGGC	GAAAAGAAAT	TAGCACCGAC	ME7
41	AACTCGCTCA	TGATTTCGGC	GAAAAGAAAT	TAGCACCGAC	ATCC 25940
41	AACTCGCTCA	TGATTTCGGC	GAAAAGAAAT	TAGCACCGAC	ME5
	90	**100**	**110**	**120**	
81	CGTTACGGAA	CGCGACCACA	AAGGTATTTA	TGACAAAGAA	L04528
81	CGTTACGGAA	CGCGACCACA	AAGGTATTTA	TGACAAAGAA	ME7
81	CGTTACGGAA	CGCGACCACA	AAGGTATTTA	TGACAAAGAA	ATCC 25940
81	CGTTACGGAA	CGCGACCACA	AAGGTATTTA	TGACAAAGAA	ME5
	130	**140**	**150**	**160**	
121	CTCATCGACG	AATTGCTCAG	CCTCGGTATT	ACCGGCGCTT	L04528
121	CTCATCGACG	AATTGCTCAG	CCTCGGTATT	ACCGGCGCTT	ME7
121	CTCATCGACG	AATTGCTCAG	CCTCGGTATT	ACCGGCGCTT	ATCC 25940
121	CTCATCGACG	AATTGCTCAG	CCTCGGTATT	ACCGGCGCTT	ME5
	170	**180**	**190**	**200**	
161	ACTTCGAAGA	AAAATACGGC	GGTTCCGGCG	ATGACGGCGG	L04528
161	ACTTCGAAGA	AAAATACGGC	GGTTCCGGCG	ATGACGGCGG	ME7
161	ACTTCGAAGA	AAAATACGGC	GGTTCCGGCG	ATGACGGCGG	ATCC 25940
161	ACTTCGAAGA	AAAATACGGC	GGTTCCGGCG	ATGACGGCGG	ME5
	210	**220**	**230**	**240**	
201	CGACGTTTTG	AGCTACATCC	TCGCTGTTGA	AGAATTGGCT	L04528
201	CGACGTTTTG	AGCTACATCC	TCGCTGTTGA	AGAATTGGCT	ME7
201	CGACGTTTTG	AGCTACATCC	TCGCTGTTGA	AGAATTGGCT	ATCC 25940
201	CGACGTTTTG	AGCTACATCC	TCGCTGTTGA	AGAATTGGCT	ME5

	250	260	270	280	
241	AAATACGACG	CTGGTGTTGC	TATCACCTTG	TCGGCAACGG	L04528
241	AAATACGACG	CTGGTGTTGC	TATCACCTTG	TCGGCAACGG	ME7
241	AAATACGACG	CTGGTGTTGC	TATCACCTTG	TCGGCAACGG	ATCC 25940
241	AAATACGACG	CTGGTGTTGC	TATCACCTTG	TCGGCAACGG	ME5
	290	**300**	**310**	**320**	
281	TTTCCCTTTG	CGCTAACCCG	ATTTGGCAGT	TCGGTACAGA	L04528
281	TTTCCCTTTG	CGCTAACCCG	ATTTGGCAGT	TCGGTACAGA	ME7
281	TTTCCCTTTG	CGCTAACCCG	ATTTGGCAGT	TCGGTACAGA	ATCC 25940
281	TTTCCCTTTG	CGCTAACCCG	ATTTGGCAGT	TCGGTACAGA	ME5
	330	**340**	**350**	**360**	
321	AGCTCAGAAA	GAAAAATTCC	TCGTTCCTTT	GGTTGAAGGC	L04528
321	AGCTCAGAAA	GAAAAATTCC	TCGTTCCTTT	GGTTGAAGGC	ME7
321	AGCTCAGAAA	GAAAAATTCC	TCGTTCCTTT	GGTTGAAGGC	ATCC 25940
321	AGCTCAGAAA	GAAAAATTCC	TCGTTCCTTT	GGTTGAAGGC	ME5
	370	**380**	**390**	**400**	
361	ACTAAACTCG	GCGCTTTCGG	CTTGACCGAA	CCGAACGCAG	L04528
361	ACTAAACTCG	GCGCTTTCGG	CTTGACCGAA	CCGAACGCAG	ME7
361	ACTAAACTCG	GCGCTTTCGG	CTTGACCGAA	CCGAACGCAG	ATCC 25940
361	ACTAAACTCG	GCGCTTTCGG	CTTGACCGAA	CCGAACGCAG	ME5
	410	**420**	**430**	**440**	
401	GTACTGATGC	TTCCGGCCAG	CAGACCATTG	CTACGAAGAA	L04528
401	GTACTGATGC	TTCCGGCCAG	CAGACCATTG	CTACGAAGAA	ME7
401	GTACTGATGC	TTCCGGCCAG	CAGACCATTG	CTACGAAGAA	ATCC 25940
401	GTACTGATGC	TTCCGGCCAG	CAGACCATTG	CTACGAAGAA	ME5
	450	**460**	**470**	**480**	
441	CGATGACGGC	ACTTACACGT	TGAACGGCTC	CAAGATCTTC	L04528
441	CGATGACGGC	ACTTACACGT	TGAACGGCTC	CAAGATCTTC	ME7
441	CGATGACGGC	ACTTACACGT	TGAACGGCTC	CAAGATCTTC	ATCC 25940
441	CGATGACGGC	ACTTACACGT	TGAACGGCTC	CAAGATCTTC	ME5
	490	**500**	**510**	**520**	
481	ATCACCAACG	GCGGCGCTGC	TGACATCGAC	ATTGTCTTCG	L04528
481	ATCACCAACG	GCGGCGCTGC	TGACATCGAC	ATTGTCTTCG	ME7
481	ATCACCAACG	GCGGCGCTGC	TGACATCGAC	ATTGTCTTCG	ATCC 25940
481	ATCACCAACG	GCGGCGCTGC	TGACATCGAC	ATTGTCTTCG	ME5
	530	**540**	**550**	**560**	
521	CTATGACCGA	TAAGAGCAAA	GGCAACCACG	GCATTACAGC	L04528
521	CTATGACCGA	TAAGAGCAAA	GGCAACCACG	GCATTACAGC	ME7
521	CTATGACCGA	TAAGAGCAAA	GGCAACCACG	GCATTACAGC	ATCC 25940
521	CTATGACCGA	TAAGAGCAAA	GGCAACCACG	GCATTACAGC	ME5
	570	**580**	**590**	**600**	
561	CTTCATCCTC	GAAGACGGTA	CTCCGGGCTT	TACTTACGGC	L04528
561	CTTCATCCTC	GAAGACGGTA	CTCCGGGCTT	TACTTACGGC	ME7
561	CTTCATCCTC	GAAGACGGTA	CTCCGGGCTT	TACTTACGGC	ATCC 25940
561	CTTCATCCTC	GAAGACGGTA	CTCCGGGCTT	TACTTACGGC	ME5

	610	620	630	640	
601	AAGAAAGAAG	ACAAGATGGG	CATCCATACT	TCGCAGACCA	L04528
601	AAGAAAGAAG	ACAAGATGGG	CATCCATACT	TCGCAGACCA	ME7
601	AAGAAAGAAG	ACAAGATGGG	CATCCATACT	TCGCAGACCA	ATCC 25940
601	AAGAAAGAAG	ACAAGATGGG	CATCCATACT	TCGCAGACCA	ME5
	650	**660**	**670**	**680**	
641	TGGAACTCGT	ATTCCAGGAC	GTCAAAGTTC	CGGCTGAAAA	L04528
641	TGGAACTCGT	ATTCCAGGAC	GTCAAAGTTC	CGGCTGAAAA	ME7
641	TGGAACTCGT	ATTCCAGGAC	GTCAAAGTTC	CGGCTGAAAA	ATCC 25940
641	TGGAACTCGT	ATTCCAGGAC	GTCAAAGTTC	CGGCTGAAAA	ME5
	690	**700**	**710**	**720**	
681	CATGCTCGGC	GAAAGAAGGCA	AAGGCTTCAA	GATTGCTATG	L04528
681	CATGCTCGGC	GAAAGAAGGCA	AAGGCTTCAA	GATTGCTATG	ME7
681	CATGCTCGGC	GAAAGAAGGCA	AAGGCTTCAA	GATTGCTATG	ATCC 25940
681	CATGCTCGGC	GAAAGAAGGCA	AAGGCTTCAA	GATTGCTATG	ME5
	730	**740**	**750**	**760**	
721	ATGACCTTGG	ACGGCGGCCG	TATCGGCGTT	GCTGCTCAGG	L04528
721	ATGACCTTGG	ACGGCGGCCG	TATCGGCGTT	GCTGCTCAGG	ME7
721	ATGACCTTGG	ACGGCGGCCG	TATCGGCGTT	GCTGCTCAGG	ATCC 25940
721	ATGACCTTGG	ACGGCGGCCG	TATCGGCGTT	GCTGCTCAGG	ME5
	770	**780**	**790**	**800**	
761	CTCTCGGCAT	TGCAGAAGCT	GCTTTGGCAG	ATGCTGTTGA	L04528
761	CTCTCGGCAT	TGCAGAAGCT	GCTTTGGCAG	ATGCTGTTGA	ME7
761	CTCTCGGCAT	TGCAGAAGCT	GCTTTGGCAG	ATGCTGTTGA	ATCC 25940
761	CTCTCGGCAT	TGCAGAAGCT	GCTTTGGCAG	ATGCTGTTGA	ME5
	810	**820**	**830**	**840**	
801	ATACTCCAAA	CAGCGTGTAC	AGTTCGGCAA	ACCGCTCTGC	L04528
801	ATACTCCAAA	CAGCGTGTAC	AGTTCGGCAA	ACCGCTCTGC	ME7
801	ATACTCCAAA	CAGCGTGTAC	AGTTCGGCAA	ACCGCTCTGC	ATCC 25940
801	ATACTCCAAA	CAGCGTGTAC	AGTTCGGCAA	ACCGCTCTGC	ME5
	850	**860**	**870**	**880**	
841	AAATTCCAGT	CCATTTCCTT	CAAACTGGCT	GACATGAAGA	L04528
841	AAATTCCAGT	CCATTTCCTT	CAAACTGGCT	GACATGAAGA	ME7
841	AAATTCCAGT	CCATTTCCTT	CAAACTGGCT	GACATGAAGA	ATCC 25940
841	AAATTCCAGT	CCATTTCCTT	CAAACTGGCT	GACATGAAGA	ME5
	890	**900**	**910**	**920**	
881	TGCAGATCGA	AGCTGCTCGT	AACCTCGTTT	ACAAAGCTGC	L04528
881	TGCAGATCGA	AGCTGCTCGT	AACCTCGTTT	ACAAAGCTGC	ME7
881	TGCAGATCGA	AGCTGCTCGT	AACCTCGTTT	ACAAAGCTGC	ATCC 25940
881	TGCAGATCGA	AGCTGCTCGT	AACCTCGTTT	ACAAAGCTGC	ME5
	930	**940**	**950**	**960**	
921	TTGCAAGAAA	CAGGAAGGCA	AACCCTTCAC	CGTTGACGCT	L04528
921	TTGCAAGAAA	CAGGAAGGCA	AACCCTTCAC	CGTTGACGCT	ME7
921	TTGCAAGAAA	CAGGAAGGCA	AACCCTTCAC	CGTTGACGCT	ATCC 25940
921	TTGCAAGAAA	CAGGAAGGCA	AACCCTTCAC	CGTTGACGCT	ME5

	970	980	990	1000	
961	GCTATCGCAA	AACGCGTTGC	TTCCGACGTC	GCTATGCGCG	L04528
961	GCTATCGCAA	AACGCGTTGC	TTCCGACGTC	GCTATGCGCG	ME7
961	GCTATCGCAA	AACGCGTTGC	TTCCGACGTC	GCTATGCGCG	ATCC 25940
961	GCTATCGCAA	AACGCGTTGC	TTCCGACGTC	GCTATGCGCG	ME5
	1010	**1020**	**1030**	**1040**	
1001	TAACGACCGA	AGCTGTCCAG	ATCTTCGGCG	GCTATGGCTA	L04528
1001	TAACGACCGA	AGCTGTCCAG	ATCTTCGGCG	GCTATGGCTA	ME7
1001	TAACGACCGA	AGCTGTCCAG	ATCTTCGGCG	GCTATGGCTA	ATCC 25940
1001	TAACGACCGA	AGCTGTCCAG	ATCTTCGGCG	GCTATGGCTA	ME5
	1050	**1060**	**1070**	**1080**	
1041	CAGCGAAGAA	TATCCGGTTG	CTCGTCACAT	GCGCGATGCT	L04528
1041	CAGCGAAGAA	TATCCGGTTG	CTCGTCACAT	GCGCGATGCT	ME7
1041	CAGCGAAGAA	TATCCGGTTG	CTCGTCACAT	GCGCGATGCT	ATCC 25940
1041	CAGCGAAGAA	TATCCGGTTG	CTCGTCACAT	GCGCGATGCT	ME5
	1090	**1100**	**1110**	**1120**	
1081	AAGATTACTC	AGATCTACGA	AGGCACGAAC	GAAGTTCAGC	L04528
1081	AAGATTACTC	AGATCTACGA	AGGCACGAAC	GAAGTTCAGC	ME7
1081	AAGATTACTC	AGATCTACGA	AGGCACGAAC	GAAGTTCAGC	ATCC 25940
1081	AAGATTACTC	AGATCTACGA	AGGCACGAAC	GAAGTTCAGC	ME5
	1130	**1140**	**1150**	**1160**	
1121	TCATGGTTAC	AGGCGGTGCT	CTGTTAAGAT	AA	L04528
1121	TCATGGTTAC	AGGCGGTGCT	CTGTTAAGAT	AATTGAAGTT	ME7
1121	TCATGGTTAC	AGGCGGTGCT	CTGTTAAGAT	AATTGAAGTT	ATCC 25940
1121	TCATGGTTAC	AGGCGGTGCT	CTGTTAAGAT	AATTGAAGTT	ME5
	1170	**1180**	**1190**	**1200**	
1161					L04528
1161	TATGCTCGGG	CCTGGCCCTT	TGCTGGGCCC	GTTACATAAA	ME7
1161	TATGCTCGGG	CCTGGCCCTT	TGCTGGGCCC	GTTACATAAA	ATCC 25940
1161	TATGCTCGGG	CCTGGCCCTT	TGCTGGGCCC	GTTACATAAA	ME5
	1210	**1220**			
1201					L04528
1201	AAAAGATTTT	AGGAGGCAAAA			ME7
1201	AAAAGATTTT	AGGAGGCAAAA			ATCC 25940
1201	AAAAGATTTT	AGGAGGCAAAA			ME5

5.3.1.4 SCAD amino acid sequence analysis

Compared to the amino acid sequence of SCAD Accession No: L04528 with the parental (ATCC 25940), and mutant strains (ME5 and ME7), no change could be observed in the amino acid sequence of SCAD gene when aligned with the database sequence Accession No: L04528 indicating that they showed 100% homology as shown in Figure 5.8.

Figure 5.8: Amino acid alignments of Short-chain Acyl-CoA dehydrogenase (SCAD) from *M. elsdenii* strains ATCC 25940, ME5, ME7 and database sequence L04528. The alignments were performed with Clustal.

	10	20	30	40	
1	MDFNLTDIQQ	DFLKLAHDFG	EKKLAPTVTE	RDHKGIYDKE	L04528
1	MDFNLTDIQQ	DFLKLAHDFG	EKKLAPTVTE	RDHKGIYDKE	ME7
1	MDFNLTDIQQ	DFLKLAHDFG	EKKLAPTVTE	RDHKGIYDKE	ATCC 25940
1	MDFNLTDIQQ	DFLKLAHDFG	EKKLAPTVTE	RDHKGIYDKE	ME5
	50	60	70	80	
41	LIDELLSLGI	TGAYFEEKYG	GSGDDGGDVL	SYILAVEELA	L04528
41	LIDELLSLGI	TGAYFEEKYG	GSGDDGGDVL	SYILAVEELA	ME7
41	LIDELLSLGI	TGAYFEEKYG	GSGDDGGDVL	SYILAVEELA	ATCC 25940
41	LIDELLSLGI	TGAYFEEKYG	GSGDDGGDVL	SYILAVEELA	ME5
	90	100	110	120	
81	KYDAGVAITL	SATVSLCANP	IWQFGTEAQK	EKFLVPLVEG	L04528
81	KYDAGVAITL	SATVSLCANP	IWQFGTEAQK	EKFLVPLVEG	ME7
81	KYDAGVAITL	SATVSLCANP	IWQFGTEAQK	EKFLVPLVEG	ATCC 25940
81	KYDAGVAITL	SATVSLCANP	IWQFGTEAQK	EKFLVPLVEG	ME5
	130	140	150	160	
121	TKLGAFGLTE	PNAGTDASGQ	QTIATKNDDG	TYTLNGSKIF	L04528
121	TKLGAFGLTE	PNAGTDASGQ	QTIATKNDDG	TYTLNGSKIF	ME7
121	TKLGAFGLTE	PNAGTDASGQ	QTIATKNDDG	TYTLNGSKIF	ATCC 25940
121	TKLGAFGLTE	PNAGTDASGQ	QTIATKNDDG	TYTLNGSKIF	ME5
	170	180	190	200	
161	ITNGGAADIY	IVFAMTDKSK	GNHGITAFIL	EDGTPGFTYG	L04528
161	ITNGGAADIY	IVFAMTDKSK	GNHGITAFIL	EDGTPGFTYG	ME7
161	ITNGGAADIY	IVFAMTDKSK	GNHGITAFIL	EDGTPGFTYG	ATCC 25940
161	ITNGGAADIY	IVFAMTDKSK	GNHGITAFIL	EDGTPGFTYG	ME5
	210	220	230	240	
201	KKEDKMGIHT	SQTMELVFQD	VKVPAENMLG	EEGKGFKIAM	L04528
201	KKEDKMGIHT	SQTMELVFQD	VKVPAENMLG	EEGKGFKIAM	ME7
201	KKEDKMGIHT	SQTMELVFQD	VKVPAENMLG	EEGKGFKIAM	ATCC 25940
201	KKEDKMGIHT	SQTMELVFQD	VKVPAENMLG	EEGKGFKIAM	ME5
	250	260	270	280	
241	MTLDGGRIGV	AAQALGIAEA	ALADAVEYSK	QRVQFGKPLC	L04528
241	MTLDGGRIGV	AAQALGIAEA	ALADAVEYSK	QRVQFGKPLC	ME7
241	MTLDGGRIGV	AAQALGIAEA	ALADAVEYSK	QRVQFGKPLC	ATCC 25940
241	MTLDGGRIGV	AAQALGIAEA	ALADAVEYSK	QRVQFGKPLC	ME5
	290	300	310	320	
281	KFQSISFKLA	DMKMQIEAAR	NLVYKAACKK	QEGKPFTVDA	L04528
281	KFQSISFKLA	DMKMQIEAAR	NLVYKAACKK	QEGKPFTVDA	ME7
281	KFQSISFKLA	DMKMQIEAAR	NLVYKAACKK	QEGKPFTVDA	ATCC 25940
281	KFQSISFKLA	DMKMQIEAAR	NLVYKAACKK	QEGKPFTVDA	ME5
	330	340	350	360	
321	AIAKRVASDV	AMRVTTEAVQ	IFGGYGYSEE	YPVARHMRDA	L04528
321	AIAKRVASDV	AMRVTTEAVQ	IFGGYGYSEE	YPVARHMRDA	ME7
321	AIAKRVASDV	AMRVTTEAVQ	IFGGYGYSEE	YPVARHMRDA	ATCC 25940
321	AIAKRVASDV	AMRVTTEAVQ	IFGGYGYSEE	YPVARHMRDA	ME5
	370	380			
361	KITQIVEGTN	EVQLMVTGGA	LLR		L04528
361	KITQIVEGTN	EVQLMVTGGA	LLR		ME7
361	KITQIVEGTN	EVQLMVTGGA	LLR		ATCC 25940
361	KITQIVEGTN	EVQLMVTGGA	LLR		ME5

5.4 PCR Amplification and sequencing of gene coding for Electron-transferring Flavoprotein (ETF) subunit α (*etfA*) and subunit β (*etfB*) from *M. elsdenii* parental (ATCC 25940) and mutant (ME5) strain

5.4.1 Construction of primers

Primers for the amplification of *etfA* and *etfB* genes from total genomic DNA isolated from *M. elsdenii* parental ATCC 25940 and mutant ME5 strains were designed, as listed in Table 5.3.

Table 5.3: Synthetic oligonucleotide primers employed in the PCR amplification of genomic DNA of *M. elsdenii* strain for electron-transferring flavoprotein β subunit (*etfB*) and electron-transferring flavoprotein α subunit (*etfA*) genes.

Primer	Sequence 5' to 3'	Position (nucleotide number)	Length (nucleotide)	Hybridize to
ETFF1	TAGGAGGCAAAACGTAAATGG	6–26	21	*etfB*
ETFR1	GATTAAATGATTTTCTGGGCAACC	814–837	24	*etfB*
ETFF2	ATGGAAATATTGGTATGTGTC	23–43	21	*etfB*
ETFR2	GATGAGTTCGACGAATTTGGTCAT	1438–1461	24	*etfA*
ETFF4	AGTTGAAGATGACAACGAACAGGC	763–786	24	*etfB*
ETFF5	CTGCACGCAGCTCGGCGTTGA	1227–1247	21	*etfA*
ETFR3	AGCTTCTTACATATCTCTATG	2081–2101	21	*etfA*

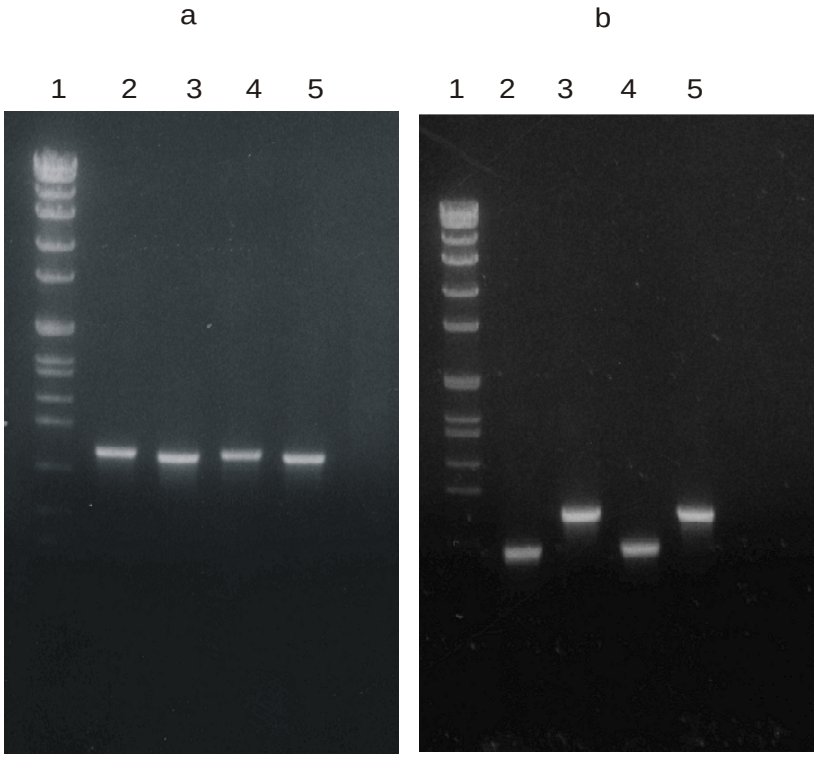
F and R indicating forward and reverse primers and were designed in this study from the published sequence of ETF genes by (O'Neill *et al*., 1998).

The primers were designed from the published ETF sequences (Accession No: AF072475) for full-length (2.1 kb) PCR product to compare any change in the parental and its mutant strain in relation to their metabolic pathways.

5.4.2 PCR amplification products

These oligonucleotides then served as primers (ETFF1, ETFR1, ETFF2, ETFR2 ETFF4, ETFF5 and ETFR3) in a polymerase chain reaction (PCR) using genomic DNA from *M. elsdenii* strains ATCC 25940 and ME5. PCR amplification products (698, 814, 831, and 874 bp) of the ETF subunit α (*etfA)* and subunit β (*etfB*) gene were obtained by using AmpliTaq DNA polymerase. The conditions for the PCR were as follows: Initial denaturation for 5 min at 95^{o}C; annealing, 90 s at 50^{o}C; elongation, 3 min at 72^{o}C for one cycle, followed by denaturation for 90 s 95^{o}C; annealing, 90 s at 50^{o}C; elongation, 3 min at 72^{o}C for 24 cycles followed by the final elongation cycle for 10 min at 72^{o}C. The PCR mixture was analysed by 1% agarose gel electrophoresis as shown in Figure 5.9a and b. Parallel experiment was run with negative control (no DNA) and shown no reaction.

Figure 5.9: PCR amplification products of different sizes for a) subunit β (*etfB*) and b) subunit α (*etfA*) genes from *M. elsdenii* strains using genomic DNA.



Panel a: Subunit β (*etfB*) gene
Lane 1: SPP molecular weight markers
Lane 2: ATCC 25940 (831 bp product using primer ETFF1 and ETFR1)
Lane 3: ATCC 25940 (814 bp product using primer ETFF2 and ETFR1)
Lane 4: ME5 (831 bp product using primer ETFF1 and ETFR1)
Lane 5: ME5 (814 bp product using primer ETFF2 and ETFR1)
Lane 6: Negative control (no DNA)

Panel b: subunit α (*etfA*) gene
Lane 1: SPP molecular weight markers
Lane 2: ATCC 25940 (698 bp product using primer ETFF4 and ETFR2)
Lane 3: ATCC 25940 (874 bp product using primer ETFF5 and ETFR3)
Lane 4: ME5 (698 bp product using primer ETFF4 and ETFR2)
Lane 5: ME5 (874 bp product using primer ETFF5 and ETFR3)
Lane 6: Negative control (no DNA)

5.4.3 Electron-transferring Flavoprotein gene (*etfA* and *etfB*) sequence analysis

Purified PCR amplification products of the subunit β (*etfB*) and subunit α (*etfA)* gene for the strain ATCC 25940 and ME5 of *M. elsdenii* that are shown in Figure 5.9a and b were used as a template for cyclo-sequencing reactions. The forward primers used for sequencing were ETFF1, ETFF2, ETFF4 and ETFF5 and reverse primer were ETFR1, ETFR2 and ETFR3 for 5'–3' and 3'–5' direction respectively. The anticipated PCR products (698, 814, 831, and 874 bp) were detected. The nucleotide sequence of these purified PCR fragments of both strains was determined using the ABI-PRISM Dye Terminator Cycle Sequencing Kit in an automated DNA sequencer as

described in section 2.6.12. After sequence assembly (Figure 5.10), the completed sequence of the subunits *etfA* and *etfB* gene (1.85 kb) of ATCC 25940, and ME5 was found to represent the whole gene. A sequence similarity search based on the NCBI database was performed using ANGIS using BLASTN analysis, which showed that the *etfA* and *etfB* gene was identical to the published sequence for *M. elsdenii* Accession number AF072475 except on positions 1693 and 1694 (shown in blue) nucleotide bases. Figure 5.10 shows the multiple sequence alignment of the *etfB* and *etfA* gene sequences of strain ATCC 25940, ME5 and AF072475, which was performed using a multiple comparison program (Clustal).

Figure 5.10: Multiple sequence alignment of the Electron-transferring Flavoprotein subunit β (*etfB*) and subunit α (*etfA*) gene sequence of *M. elsdenii* strains ATCC 25940, ME5 and database sequence AF072475 using a multiple comparison program (Clustal).

Start etfB

	10	20	30	40	
1	ATGGAAATAT	TGGTATGTGT	CAAACAGGTT	CCGGACACTG	ATCC 25940
1	ATGGAAATAT	TGGTATGTGT	CAAACAGGTT	CCGGACACTG	WT (AF072475)
1	ATGGAAATAT	TGGTATGTGT	CAAACAGGTT	CCGGACACTG	ME5
	50	**60**	**70**	**80**	
41	CAGAAGTTAA	GATTGACCCC	GTAAAACATA	CGGTCATCCG	ATCC 25940
41	CAGAAGTTAA	GATTGACCCC	GTAAAACATA	CGGTCATCCG	WT (AF072475)
41	CAGAAGTTAA	GATTGACCCC	GTAAAACATA	CGGTCATCCG	ME5
	90	**100**	**110**	**120**	
81	CGCTGGTGTT	CCTAACATTT	TTAACCCCTT	CGACCAGAAC	ATCC 25940
81	CGCTGGTGTT	CCTAACATTT	TTAACCCCTT	CGACCAGAAC	WT (AF072475)
81	CGCTGGTGTT	CCTAACATTT	TTAACCCCTT	CGACCAGAAC	ME5
	130	**140**	**150**	**160**	
121	GCTTTGGAAG	CAGCTCTCGC	ATTGAAAGAT	GCTGACAAAG	ATCC 25940
121	GCTTTGGAAG	CAGCTCTCGC	ATTGAAAGAT	GCTGACAAAG	WT (AF072475)
121	GCTTTGGAAG	CAGCTCTCGC	ATTGAAAGAT	GCTGACAAAG	ME5
	170	**180**	**190**	**200**	
161	ACGTAAAAAT	CACACTTCTC	TCGATGGGTC	CTGATCAGGC	ATCC 25940
161	ACGTAAAAAT	CACACTTCTC	TCGATGGGTC	CTGATCAGGC	WT (AF072475)
161	ACGTAAAAAT	CACACTTCTC	TCGATGGGTC	CTGATCAGGC	ME5
	210	**220**	**230**	**240**	
201	AAAAGACGTT	CTTCGTGAAG	GCCTCGCAAT	GGGCGCTGAC	ATCC 25940
201	AAAAGACGTT	CTTCGTGAAG	GCCTCGCAAT	GGGCGCTGAC	WT (AF072475)
201	AAAAGACGTT	CTTCGTGAAG	GCCTCGCAAT	GGGCGCTGAC	ME5
	250	**260**	**270**	**280**	
241	GATGCTTATC	TTCTGTCCGA	CCGCAAACTC	GGTGGTTCCG	ATCC 25940
241	GATGCTTATC	TTCTGTCCGA	CCGCAAACTC	GGTGGTTCCG	WT (AF072475)
241	GATGCTTATC	TTCTGTCCGA	CCGCAAACTC	GGTGGTTCCG	ME5
	290	**300**	**310**	**320**	
281	ATACGTTAGC	TACGGGCTAT	GCTTTGGCAC	AGGCTATCAA	ATCC 25940
281	ATACGTTAGC	TACGGGCTAT	GCTTTGGCAC	AGGCTATCAA	WT (AF072475)
281	ATACGTTAGC	TACGGGCTAT	GCTTTGGCAC	AGGCTATCAA	ME5
	330	**340**	**350**	**360**	
321	AAAATTGGCT	GCTGACAAAG	GTATCGAACA	GTTCGATATC	ATCC 25940
321	AAAATTGGCT	GCTGACAAAG	GTATCGAACA	GTTCGATATC	WT (AF072475)
321	AAAATTGGCT	GCTGACAAAG	GTATCGAACA	GTTCGATATC	ME5

	370	380	390	400	
361	ATCCTCTGCG	GCAAACAGGC	TATTGACGGC	GATACCGCAC	ATCC 25940
361	ATCCTCTGCG	GCAAACAGGC	TATTGACGGC	GATACCGCAC	WT (AF072475)
361	ATCCTCTGCG	GCAAACAGGC	TATTGACGGC	GATACCGCAC	ME5
	410	**420**	**430**	**440**	
401	AGGTTGGCCC	GCAGATCGCT	TGCGAACTCG	GTATTCCTCA	ATCC 25940
401	AGGTTGGCCC	GCAGATCGCT	TGCGAACTCG	GTATTCCTCA	WT (AF072475)
401	AGGTTGGCCC	GCAGATCGCT	TGCGAACTCG	GTATTCCTCA	ME5
	450	**460**	**470**	**480**	
441	GATTACGTAT	GCCCGCGACA	TCAAAGTCGA	AGGCGACAAA	ATCC 25940
441	GATTACGTAT	GCCCGCGACA	TCAAAGTCGA	AGGCGACAAA	WT (AF072475)
441	GATTACGTAT	GCCCGCGACA	TCAAAGTCGA	AGGCGACAAA	ME5
	490	**500**	**510**	**520**	
481	GTTACTGTTC	AGCAGGAAAA	CGAAGAAGGC	TACATCGTAA	ATCC 25940
481	GTTACTGTTC	AGCAGGAAAA	CGAAGAAGGC	TACATCGTAA	WT (AF072475)
481	GTTACTGTTC	AGCAGGAAAA	CGAAGAAGGC	TACATCGTAA	ME5
	530	**540**	**550**	**560**	
521	CGGAAGCTCA	GTTCCCTGTT	TTGATCACGG	CTGTTAAAGA	ATCC 25940
521	CGGAAGCTCA	GTTCCCTGTT	TTGATCACGG	CTGTTAAAGA	WT (AF072475)
521	CGGAAGCTCA	GTTCCCTGTT	TTGATCACGG	CTGTTAAAGA	ME5
	570	**580**	**590**	**600**	
561	CTTGAACGAA	CCGCGTTTCC	CGACCATTCG	TGGCACGATG	ATCC 25940
561	CTTGAACGAA	CCGCGTTTCC	CGACCATTCG	TGGCACGATG	WT (AF072475)
561	CTTGAACGAA	CCGCGTTTCC	CGACCATTCG	TGGCACGATG	ME5
	610	**620**	**630**	**640**	
601	AAAGCAAAAC	GCCGCGAAAT	CCCGAACTTG	GACGCTGCTG	ATCC 25940
601	AAAGCAAAAC	GCCGCGAAAT	CCCGAACTTG	GACGCTGCTG	WT (AF072475)
601	AAAGCAAAAC	GCCGCGAAAT	CCCGAACTTG	GACGCTGCTG	ME5
	650	**660**	**670**	**680**	
641	CTGTTGCAGC	TGACGACGCT	CAGATCGGTT	TGTCTGGCTC	ATCC 25940
641	CTGTTGCAGC	TGACGACGCT	CAGATCGGTT	TGTCTGGCTC	WT (AF072475)
641	CTGTTGCAGC	TGACGACGCT	CAGATCGGTT	TGTCTGGCTC	ME5
	690	**700**	**710**	**720**	
681	TCCGACTAAA	GTCCGTAAGA	TTTTCACACC	GCCTCAGAGA	ATCC 25940
681	TCCGACTAAA	GTCCGTAAGA	TTTTCACACC	GCCTCAGAGA	WT (AF072475)
681	TCCGACTAAA	GTCCGTAAGA	TTTTCACACC	GCCTCAGAGA	ME5
	730	**740**	**750**	**760**	
721	TCCGGTGGTC	TCGTTCTCAA	AGTTGAAGAT	GACAACGAAC	ATCC 25940
721	TCCGGTGGTC	TCGTTCTCAA	AGTTGAAGAT	GACAACGAAC	WT (AF072475)
721	TCCGGTGGTC	TCGTTCTCAA	AGTTGAAGAT	GACAACGAAC	ME5
	770	**780**	**790**	**800**	
761	AGGCAATCTT	CGACCAGGTC	ATGGAAAAAC	TGGTTGCCCA	ATCC 25940
761	AGGCAATCTT	CGACCAGGTC	ATGGAAAAAC	TGGTTGCCCA	WT (AF072475)
761	AGGCAATCTT	CGACCAGGTC	ATGGAAAAAC	TGGTTGCCCA	ME5
	810	**820**	**830**	**840**	
		Stop		**Start *etfA***	
801	GAAAATCATT	TAATCTAA GG	AGGAACAGTG	AAA ATGGATT	ATCC 25940
801	GAAAATCATT	TAATCTAA GG	AGGAACAGTG	AAA ATGGATT	WT (AF072475)
801	GAAAATCATT	TAATCTAA GG	AGGAACAGTG	AAA ATGGATT	ME5
	850	**860**	**870**	**880**	
841	TAGCAGAATA	TAAAGGCATT	TATGTAATTG	CTGAACAGTT	ATCC 25940
841	TAGCAGAATA	TAAAGGCATT	TATGTAATTG	CTGAACAGTT	WT (AF072475)
841	TAGCAGAATA	TAAAGGCATT	TATGTAATTG	CTGAACAGTT	ME5

	890	900	910	920	
881	CGAAGGCAAA	TTACGTGATG	TATCTTTCGA	ATTGTTGGGC	ATCC 25940
881	CGAAGGCAAA	TTACGTGATG	TATCTTTCGA	ATTGTTGGGC	WT (AF072475)
881	CGAAGGCAAA	TTACGTGATG	TATCTTTCGA	ATTGTTGGGC	ME5
	930	**940**	**950**	**960**	
921	CAGGCTCGCA	TCTTGGCTGA	CACCATCGGC	GACGAAGTCG	ATCC 25940
921	CAGGCTCGCA	TCTTGGCTGA	CACCATCGGC	GACGAAGTCG	WT (AF072475)
921	CAGGCTCGCA	TCTTGGCTGA	CACCATCGGC	GACGAAGTCG	ME5
	970	**980**	**990**	**1000**	
961	GTGCAATCCT	CATTGGTAAA	GACGTAAAAC	CGTTGGCTCA	ATCC 25940
961	GTGCAATCCT	CATTGGTAAA	GACGTAAAAC	CGTTGGCTCA	WT (AF072475)
961	GTGCAATCCT	CATTGGTAAA	GACGTAAAAC	CGTTGGCTCA	ME5
	1010	**1020**	**1030**	**1040**	
1001	GGAACTTATC	GCTCACGGTG	CTCATAAAGT	ATACGTTTAT	ATCC 25940
1001	GGAACTTATC	GCTCACGGTG	CTCATAAAGT	ATACGTTTAT	WT (AF072475)
1001	GGAACTTATC	GCTCACGGTG	CTCATAAAGT	ATACGTTTAT	ME5
	1050	**1060**	**1070**	**1080**	
1041	GATGATCCTC	AGCTCGAACA	TTACAATACG	ACGGCTTATG	ATCC 25940
1041	GATGATCCTC	AGCTCGAACA	TTACAATACG	ACGGCTTATG	WT (AF072475)
1041	GATGATCCTC	AGCTCGAACA	TTACAATACG	ACGGCTTATG	ME5
	1090	**1100**	**1110**	**1120**	
1081	CAAAAGTTAT	TTGCGATTTC	TTCCATGAAG	AAAAACCGAA	ATCC 25940
1081	CAAAAGTTAT	TTGCGATTTC	TTCCATGAAG	AAAAACCGAA	WT (AF072475)
1081	CAAAAGTTAT	TTGCGATTTC	TTCCATGAAG	AAAAACCGAA	ME5
	1130	**1140**	**1150**	**1160**	
1121	CGTATTCCTC	GTTGGCGCTA	CCAACATCGG	CCGTGACCTC	ATCC 25940
1121	CGTATTCCTC	GTTGGCGCTA	CCAACATCGG	CCGTGACCTC	WT (AF072475)
1121	CGTATTCCTC	GTTGGCGCTA	CCAACATCGG	CCGTGACCTC	ME5
	1170	**1180**	**1190**	**1200**	
1161	GGCCCGCGTG	TCGCTAACTC	CTTGAAGACT	GGCCTCACCG	ATCC 25940
1161	GGCCCGCGTG	TCGCTAACTC	CTTGAAGACT	GGCCTCACCG	WT (AF072475)
1161	GGCCCGCGTG	TCGCTAACTC	CTTGAAGACT	GGCCTCACCG	ME5
	1210	**1220**	**1230**	**1240**	
1201	CTGACTCGAC	GCAGCTCGGC	GTTGACGACG	ACAAAAAGAC	ATCC 25940
1201	CTGACTCGAC	GCAGCTCGGC	GTTGACGACG	ACAAAAAGAC	WT (AF072475)
1201	CTGACTCGAC	GCAGCTCGGC	GTTGACGACG	ACAAAAAGAC	ME5
	1250	**1260**	**1270**	**1280**	
1241	CATCGTATGG	ACCCGTCCGG	CTCTCGGCGG	CAACATCATG	ATCC 25940
1241	CATCGTATGG	ACCCGTCCGG	CTCTCGGCGG	CAACATCATG	WT (AF072475)
1241	CATCGTATGG	ACCCGTCCGG	CTCTCGGCGG	CAACATCATG	ME5
	1290	**1300**	**1310**	**1320**	
1281	GCTGAAATCA	TCTGCCCGGA	CAACCGTCCG	CAGATGGGTA	ATCC 25940
1281	GCTGAAATCA	TCTGCCCGGA	CAACCGTCCG	CAGATGGGTA	WT (AF072475)
1281	GCTGAAATCA	TCTGCCCGGA	CAACCGTCCG	CAGATGGGTA	ME5
	1330	**1340**	**1350**	**1360**	
1321	CTGTCCGTCC	GCATGTCTTC	AAAAAACCGG	AAGCAGATCC	ATCC 25940
1321	CTGTCCGTCC	GCATGTCTTC	AAAAAACCGG	AAGCAGATCC	WT (AF072475)
1321	CTGTCCGTCC	GCATGTCTTC	AAAAAACCGG	AAGCAGATCC	ME5
	1370	**1380**	**1390**	**1400**	
1361	TTCTGCAACT	GGCGAAGTTA	TCGAAAAGAA	AGCTAACCTC	ATCC 25940
1361	TTCTGCAACT	GGCGAAGTTA	TCGAAAAGAA	AGCTAACCTC	WT (AF072475)
1361	TTCTGCAACT	GGCGAAGTTA	TCGAAAAGAA	AGCTAACCTC	ME5

	1410	1420	1430	1440	
1401	TCCGATGCTG	ACTTCATGAC	CAAATTCGTC	GAACTCATCA	ATCC 25940
1401	TCCGATGCTG	ACTTCATGAC	CAAATTCGTC	GAACTCATCA	WT (AF072475)
1401	TCCGATGCTG	ACTTCATGAC	CAAATTCGTC	GAACTCATCA	ME5
	1450	1460	1470	1480	
1441	AATTGGGCGG	CGAAGGCGTT	AAAATCGAAG	ACGCTGACGT	ATCC 25940
1441	AATTGGGCGG	CGAAGGCGTT	AAAATCGAAG	ACGCTGACGT	WT (AF072475)
1441	AATTGGGCGG	CGAAGGCGTT	AAAATCGAAG	ACGCTGACGT	ME5
	1490	1500	1510	1520	
1481	TATCGTTGCT	GGCGGCCGTG	GCATGAACAG	TGAAGAACCG	ATCC 25940
1481	TATCGTTGCT	GGCGGCCGTG	GCATGAACAG	TGAAGAACCG	WT (AF072475)
1481	TATCGTTGCT	GGCGGCCGTG	GCATGAACAG	TGAAGAACCG	ME5
	1530	1540	1550	1560	
1521	TTCAAGACCG	GTATCCTCAA	AGAATGTGCA	GACGTCCTCG	ATCC 25940
1521	TTCAAGACCG	GTATCCTCAA	AGAATGTGCA	GACGTCCTCG	WT (AF072475)
1521	TTCAAGACCG	GTATCCTCAA	AGAATGTGCA	GACGTCCTCG	ME5
	1570	1580	1590	1600	
1561	GCGGCGCTGT	TGGTGCATCC	CGTGCAGCTG	TTGACGCTGG	ATCC 25940
1561	GCGGCGCTGT	TGGTGCATCC	CGTGCAGCTG	TTGACGCTGG	WT (AF072475)
1561	GCGGCGCTGT	TGGTGCATCC	CGTGCAGCTG	TTGACGCTGG	ME5
	1610	1620	1630	1640	
1601	CTGGATCGAT	GCTCTCCATC	AGGTTGGCCA	GACTGGTAAA	ATCC 25940
1601	CTGGATCGAT	GCTCTCCATC	AGGTTGGCCA	GACTGGTAAA	WT (AF072475)
1601	CTGGATCGAT	GCTCTCCATC	AGGTTGGCCA	GACTGGTAAA	ME5
	1650	1660	1670	1680	
1641	ACAGTTGGTC	CGAAGATCTA	CATTGCATGC	GTCATTTCCG	ATCC 25940
1641	ACAGTTGGTC	CGAAGATCTA	CATTGCATGC	GTCATTTCCG	WT (AF072475)
1641	ACAGTTGGTC	CGAAGATCTA	CATTGCATGC	GTCATTTCCG	ME5
	1690	1700	1710	1720	
1681	GTGCTATCCA	GC**AC**TTGGCA	GGCATGACTG	GTTCTGACTG	ATCC 25940
1681	GTGCTATCCA	GCCATTGGCA	GGCATGACTG	GTTCTGACTG	WT (AF072475)
1681	GTGCTATCCA	GC**AC**TTGGCA	GGCATGACTG	GTTCTGACTG	ME5
	1730	1740	1750	1760	
1721	CATCATTGCT	ATCAACAAAG	ACGAAGATGC	TCCGATCTTC	ATCC 25940
1721	CATCATTGCT	ATCAACAAAG	ACGAAGATGC	TCCGATCTTC	WT (AF072475)
1721	CATCATTGCT	ATCAACAAAG	ACGAAGATGC	TCCGATCTTC	ME5
	1770	1780	1790	1800	
1761	AAAGTCTGCG	ACTATGGTAT	CGTAGGCGAT	GTCTTCAAAG	ATCC 25940
1761	AAAGTCTGCG	ACTATGGTAT	CGTAGGCGAT	GTCTTCAAAG	WT (AF072475)
1761	AAAGTCTGCG	ACTATGGTAT	CGTAGGCGAT	GTCTTCAAAG	ME5
	1810	1820	1830	1840	
1801	TTCTCCCGCT	CCTCACGGAA	GCCATCAAGA	AACAGAAAGG	ATCC 25940
1801	TTCTCCCGCT	CCTCACGGAA	GCCATCAAGA	AACAGAAAGG	WT (AF072475)
1801	TTCTCCCGCT	CCTCACGGAA	GCCATCAAGA	AACAGAAAGG	ME5
	1850	1860	1870	1880	
1841	CATTGCATAA	GTTATCTAAC	CGTGCATAAT	AAAAA	ATCC 25940
1841	CATTGCATAA	GTTATCTAAC	CGTGCATAAT	AAAAA	WT (AF072475)
1841	CATTGCATAA	GTTATCTAAC	CGTGCATAAT	AAAAA	ME5
	Stop				

5.4.4 Electron-transferring Flavoprotein gene (*etfA* and *etfB*) amino acid sequence analysis

Compared to the amino acid sequence of *etfA* Accession No: AF072475 with the parental ATCC 25940 and mutant strain ME5, amino acid residue 287 (shown in blue) in both ATCC 25940 and ME5 is Histidine instead of Proline. Except for P287H, all 337 amino acid residues are conserved and show alignment with the database. No change was seen in the amino acid sequence of β-subunit gene (*etfB*) when aligned with the database sequence Accession No: AF072475 indicating that they showed 100% homology as shown in Figure 5.11.

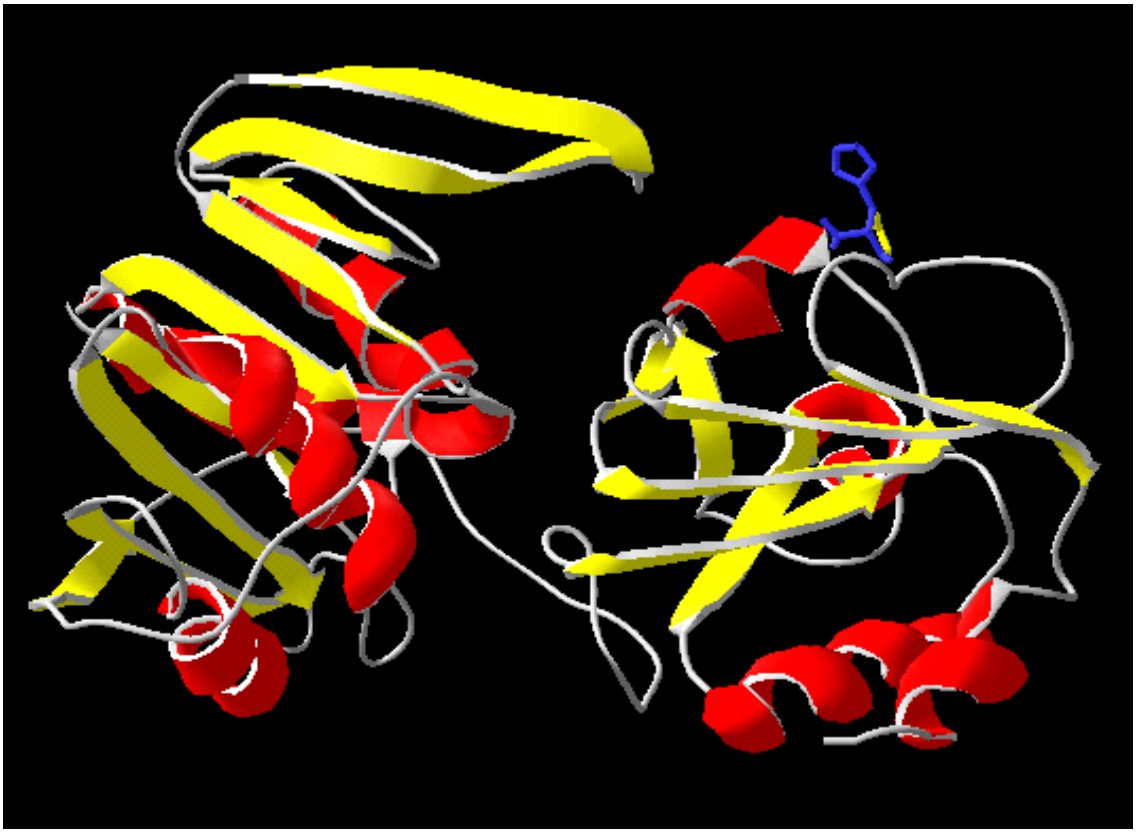
Figure 5.11: Amino acid alignments of Electron-transferring Flavoprotein subunit β (*etfB*) and subunit α (*etfA*) gene sequence of *M. elsdenii* strains ATCC 25940, ME5 and database sequence AF072475 using a multiple comparison program Clustal.

	10	20	30	40	
1	MDLAEYKGIY	VIAEQFEGKL	RDVSFELLGQ	ARILADTIGD	ATCC 25940
1	MDLAEYKGIY	VIAEQFEGKL	RDVSFELLGQ	ARILADTIGD	WT (AF072475)
1	MDLAEYKGIY	VIAEQFEGKL	RDVSFELLGQ	ARILADTIGD	ME5
	50	**60**	**70**	**80**	
41	EVGAILIGKD	VKPLAQELIA	HGAHKVYVYD	DPQLEHYNTT	ATCC 25940
41	EVGAILIGKD	VKPLAQELIA	HGAHKVYVYD	DPQLEHYNTT	WT (AF072475)
41	EVGAILIGKD	VKPLAQELIA	HGAHKVYVYD	DPQLEHYNTT	ME5
	90	**100**	**110**	**120**	
81	AYAKVICDFF	HEEKPNVFLV	GATNIGRDLG	PRVANSLKTG	ATCC 25940
81	AYAKVICDFF	HEEKPNVFLV	GATNIGRDLG	PRVANSLKTG	WT (AF072475)
81	AYAKVICDFF	HEEKPNVFLV	GATNIGRDLG	PRVANSLKTG	ME5
	130	**140**	**150**	**160**	
121	LTADCTQLGV	DDDKKTIVWT	RPALGGNIMA	EIICPDNRPQ	ATCC 25940
121	LTADCTQLGV	DDDKKTIVWT	RPALGGNIMA	EIICPDNRPQ	WT (AF072475)
121	LTADCTQLGV	DDDKKTIVWT	RPALGGNIMA	EIICPDNRPQ	ME5
	170	**180**	**190**	**200**	
161	MGTVRPHVFK	KPEADPSATG	EVIEKKANLS	DADFMTKFVE	ATCC 25940
161	MGTVRPHVFK	KPEADPSATG	EVIEKKANLS	DADFMTKFVE	WT (AF072475)
161	MGTVRPHVFK	KPEADPSATG	EVIEKKANLS	DADFMTKFVE	ME5
	210	**220**	**230**	**240**	
201	LIKLGGEGVK	IEDADVIVAG	GRGMNSEEPF	KTGILKECAD	ATCC 25940
201	LIKLGGEGVK	IEDADVIVAG	GRGMNSEEPF	KTGILKECAD	WT (AF072475)
201	LIKLGGEGVK	IEDADVIVAG	GRGMNSEEPF	KTGILKECAD	ME5
	250	**260**	**270**	**280**	
241	VLGGAVGASR	AAVDAGWIDA	LHQVGQTGKT	VGPKIYIACA	ATCC 25940
241	VLGGAVGASR	AAVDAGWIDA	LHQVGQTGKT	VGPKIYIACA	WT (AF072475)
241	VLGGAVGASR	AAVDAGWIDA	LHQVGQTGKT	VGPKIYIACA	ME5
	290	**300**	**310**	**320**	
281	ISGAIQ**H**LAG	MTGSDCIIAI	NKDEDAPIFK	VCDYGIVGDV	ATCC 25940
281	ISGAIQPLAG	MTGSDCIIAI	NKDEDAPIFK	VCDYGIVGDV	WT (AF072475)
281	ISGAIQ**H**LAG	MTGSDCIIAI	NKDEDAPIFK	VCDYGIVGDV	ME5
	330	**340**			
321	FKVLPLLTEA	IKKQKGIA			ATCC 25940
321	FKVLPLLTEA	IKKQKGIA			WT (AF072475)
321	FKVLPLLTEA	IKKQKGIA			ME5

5.4.5 Comparison of protein structures of ATCC 25940 and the *M. elsdenii* strain in database Accession no. AF072475 for subunit α (*etfA*)

The tertiary structure of electron-transferring flavoprotein subunit α (*etfA*) for wild-type strain was overlayed with the *etfA* structure of the amino acid sequence of the *M. elsdenii* strains ATCC 25940 and ME5 deduced from this study. The amino acid histidine at the position 287 (shown in blue) rather than proline did not cause any change in the backbone of the structure, and no significant differences were observed between the strains studied. No change was seen in the amino acid sequence of β-subunit as shown in Figure 5.12.

Figure 5.12: Tertiary structure of electron-transferring flavoprotein subunit α (*etfA*) from *M. elsdenii*. Overlay of the wild-type strain with mutant P287H. Histidine residue 287 is shown in blue.



5.5 Attempted PCR cloning and sequencing of the gene coding for Lactate Dehydrogenase (LDH) from *M. elsdenii* parental ATCC 25940 and mutant ME5 strains

5.5.1 Construction of primers

Primers for the amplification of Lactate Dehydrogenase (LDH) genes from total genomic DNA isolated from *M. elsdenii* parental ATCC 25940 and mutant ME5 strains were designed as listed in Table 5.4.

Table 5.4: Synthetic oligonucleotide primers developed from other species employed in the PCR amplification of genomic DNA of *M. elsdenii* strain for Lactate Dehydrogenase (LDH).

Primer	Sequence 5' to 3'	Position (nucleotide number)	Length (nucleotide)	Hybridize to
MEF–8	GGTGACGGTGCCGTAGGTTCT	371–391	21	LDH
MER–9	ACCGTAGAATGTAGCACC	1019–1036	18	LDH

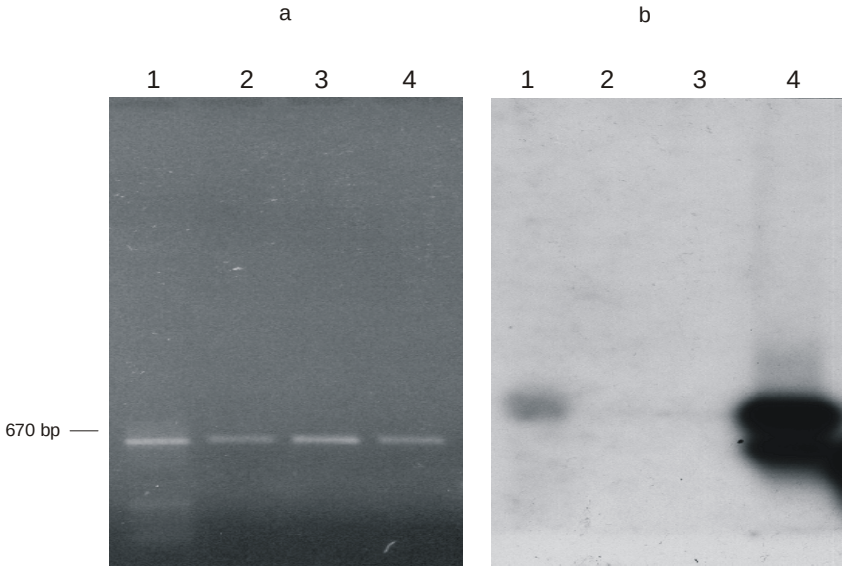
F and R indicating forward and reverse primers respectively and were designed in this study from the coding region of published LDH gene sequences of different genera that was homologus to LDH gene sequence of *Pediococcus acidilactici* Accession no: X70927 (Garmyn *et al*., 1995).

The primers were designed in this study from the published LDH gene sequence from different genera: *P. acidilactici* Accession no: X70927 (Garmyn *et al*., 1995) because LDH gene in *M. elsdenii* has not been sequenced yet. The reason to select *P. acidilactici* L-LDH was the sequence similarity to other bacterial L-LDHs using gene "pileup" program through NCBI database *via* Australian National Genomic Information Services (ANGIS). Primers were designed within the coding region of LDH gene and the positions of the primer MEF–8 starts from (371–391) and primer MER–9 starts from (1019–1036) were used as amplification primers for full-length (665 bp) PCR product.

5.5.2 PCR amplification products

These oligonucleotides then served as primers (MEF–8 and MER–9) in PCR using genomic DNA from *M. elsdenii* strains ATCC 25940, and ME5. PCR amplification products (665 bp) of the Lactate Dehydrogenase (LDH) gene were obtained by using AmpliTaq DNA polymerase. The conditions for the PCR were followed as described by Garmyn *et al*., 1995. Initial denaturation for 10 min at 95^{o}C; annealing, 60 s at 50^{o}C; elongation, 1 min at 72^{o}C for one cycle, followed by denaturation for 60 s 92^{o}C; annealing, 60 s at 50^{o}C; elongation, 1 min at 72^{o}C for 24 cycles followed by the final elongation cycle for 10 min at 72^{o}C. The PCR mixture was analysed by 1% agarose gel electrophoresis as shown in Figure 5.13a.

Figure 5.13: PCR amplification products of 665 bp LDH gene from *M. elsdenii* strains using genomic DNA a) agarose gel electrophoresis and b) analysis of the LDH gene by Southern hybridisation using 665 bp LDH gene probe from *P. acidilactici*.



Panel a and b:

Lane 1: ME5 (665 bp PCR product using primers MEF–8 and MER–9)

Lane 2: ATCC 25940 (665 bp gene cleaned PCR product using primers MEF–8 and MER–9)

Lane 3: ME5 (665 bp PCR product using primers MEF–8 and MER–9)

Lane 4: Positive control (665 bp PCR product of *P. acidilactici* using primers MEF–8 and MER–9)

5.5.3 Southern hybridisation analysis of LDH gene

After analysing the PCR products on an agarose gel, the gel was transferred on Hybond^{TM}-N^{+} membrane overnight by the method described in the section 2.6.11.1. The 665 bp PCR fragment from *P. acidilactici* was labelled with ^{32}P as described in the section 2.6.11.2 and used as a probe for hybridising the membrane. The membrane was autoradiographed as shown in Figure 5.13b. PCR product of the strain ME5 (Lane 1) showed a 665 bp band corresponds with the PCR fragment from *P. acidilactici* (Lane 4) as a positive control. Strain ATCC 25940 (Lane 2) and strain ME5 (Lane 3) gene-clean PCR products did show very feint hybridising signals. However, an intense signal with ME5 PCR product prompted further experimentation to clone the 665 bp fragment in to the vector for screening out the clones and for sequencing the gene product.

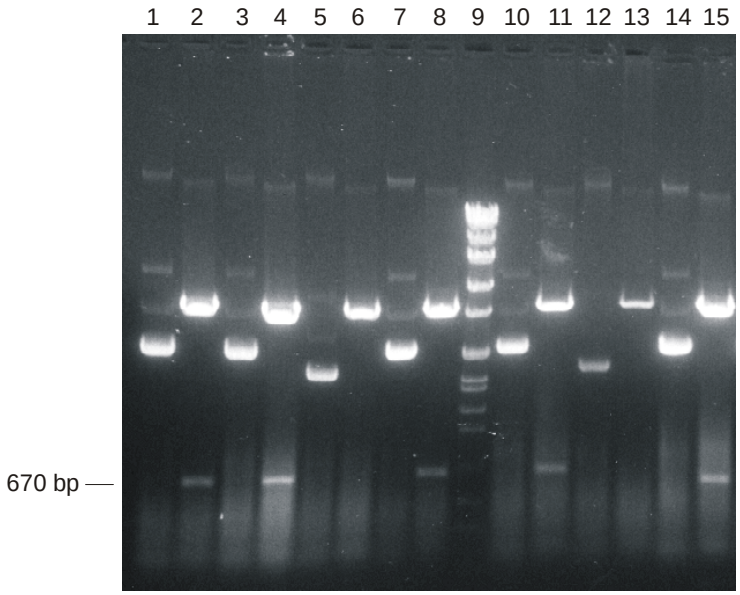
5.5.4 Cloning of lactate dehydrogenase (LDH) gene

PCR amplification products (665 bp) for the lactate dehydrogenase (LDH) gene from the genomic DNA of strain ATCC 25940 and ME5 of *M. elsdenii* are shown in Figure 5.13a. The 665 bp LDH gene products from the strains of *M. elsdenii* ATCC 25940

and ME5 were cloned into pGEM-T Easy Vector system (Figure 2.3). This vector system allowed blue/white selection of clones with and without insert, as described in the section 2.6.10. Plasmid DNA was isolated from four selected clones named as clone 2 and 7 from ME5 strain and clone 4 and 16 from ATCC 25940 appeared as white colonies on X-GAL/IPTG selection.

Plasmid DNA with cloned fragment of 665 bp from ATCC 25940 and ME5 strains was screened by digesting with restriction enzyme *Eco*RI and samples were run on 1% agarose TAE gel as shown in the Figure 5.14. The gel suggested the 665 bp *Eco*RI fragment was cloned in to pGEMT-easy vector and DNA from selected clones were subjected to Wizard Minipreps DNA Purification System (section 2.6.1.4) to purify the DNA for sequencing.

Figure 5.14: Agarose gel electrophoresis of plasmid pGEM-T Easy with 665 bp fragment of putative LDH gene digested with the restriction endonuclease *Eco*RI to screen the clones with LDH gene inserts from *M. elsdenii*.



Lane 1: ATCC 25940 (clone 4) undigested
Lane 2: ATCC 25940 (clone 4) digested
Lane 3: ATCC 25940 (clone 16) undigested
Lane 4: ATCC 25940 (clone 16) digested
Lane 5: pGEMT-Easy vector (no insert) undigested
Lane 6: pGEMT-Easy vector (no insert) digested
Lane 7: ME5 (clone 2) undigested
Lane 8: ME5 (clone 2) digested
Lane 9: SPP molecular weight markers
Lane 10: ME5 (clone 7) undigested
Lane 11: ME5 (clone 7) digested
Lane 12: pGEMT-Easy vector (no insert) undigested
Lane 13: pGEMT-Easy vector (no insert) digested
Lane 14: *P. acidilactici* (clone P1) undigested
Lane 15: *P. acidilactici* (clone P1) digested

5.5.5 Lactate dehydrogenase (LDH) gene sequence analysis

After screening the clones with an insert, the DNA was prepared and purified to sequence the gene by using T7 (forward) and SP6 (reverse) primers (Promega Manual, TM042). The nucleotide sequence of these purified PCR fragments of both strains was determined using the ABI-PRISM Dye Terminator Cycle Sequencing Kit in an automated DNA sequencer as described in section 2.6.12. The specificity of the primers designed was validated by sequencing the product from *P. acidilactici.* Sequences for LDH gene of *M. elsdenii* strains ATCC 25940 and ME5 were obtained and comparison of sequences from mutant strain ME5 and *P. acidilactici* using gene "pileup" program as shown in appendix-4. Forward and reverse sequences with their respective primers had no start codon for the translation product; means there was no protein synthesis for LDH gene and the clones (2, 4, 7 and 16) selected had ambiguity and were artefacts. A sequence similarity search based on the NCBI database was performed using ANGIS and BLASTN analysis, which showed the unsuccessful attempt to sequence the LDH gene and there is no homology with the database sequences of LDH, which was performed by using a multiple comparison program (Clustal).

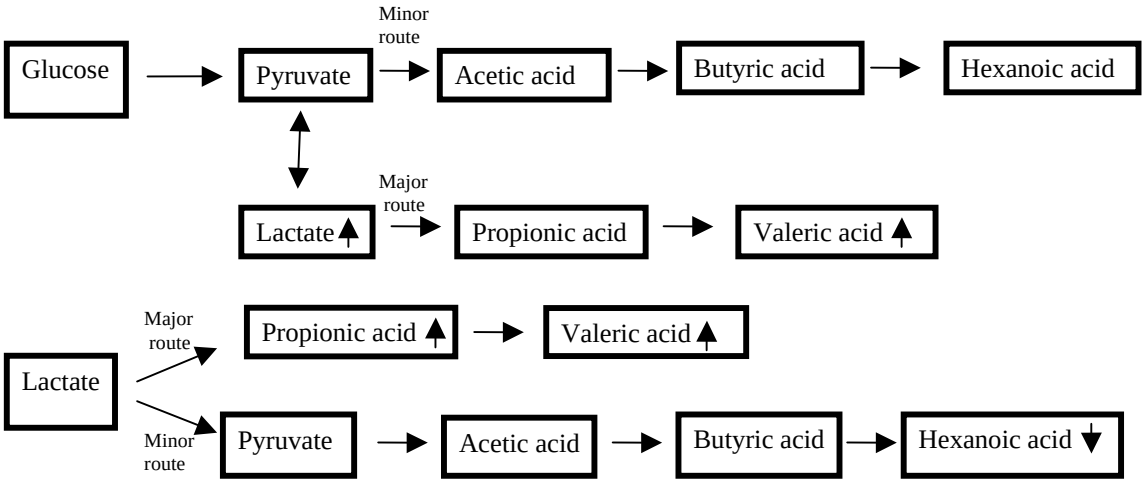
5.6 Discussion

Pyruvate dehydrogenase (PDH) activity was measured in cell free extracts of parental strain ATCC 25940 and mutants ME5 and ME7 grown in peptone-yeast-glucose as shown in Table 5.1. The highest PDH activity was observed in strain ATCC 25940, which was 100% more than its mutant ME5 and 4% more than mutant ME7 strain. However, no PDH activity was seen in control experiments (without cell extracts) implies that the activity detected was only due to the enzyme-substrate reaction. The ATCC 25940 showed relative resistance to pyruvic acid compared to its mutant strains ME5 and ME7 on THBAG plates (Table 4.1). However, both mutant strains ME5 and ME7 showed nine times more resistance to pyruvic acid compared to ATCC 25940 on THBAL plates (Table 4.2). The parental strain ATCC 25940 and mutant ME7 showed two to three times more pyruvic acid production compared to strain ME5 when grown in fermenters with pH control at 48 h sampling period with a glucose substrate. Despite an active PDH specific activity in ATCC 25940 and ME7, pyruvate was still accumulated in culture fluids but not as much as lactate, in both small-volume culture (uncontrolled and non-stirred) and in one-litre volume fermenters (pH controlled and stirred). This correlated with the higher production of long chain fatty acids such as hexanoic and butyric acids during fermentation in the presence of glucose media for ATCC 25940 and ME7.

Contrary to this, parental strain ATCC 25940 and mutant ME7 showed relatively low lactate dehydrogenase (LDH) specific activity (Table 5.1) compared to strain ME5. LDH activity in strain ME5 was 400 times more than parental strain ATCC 25940 and

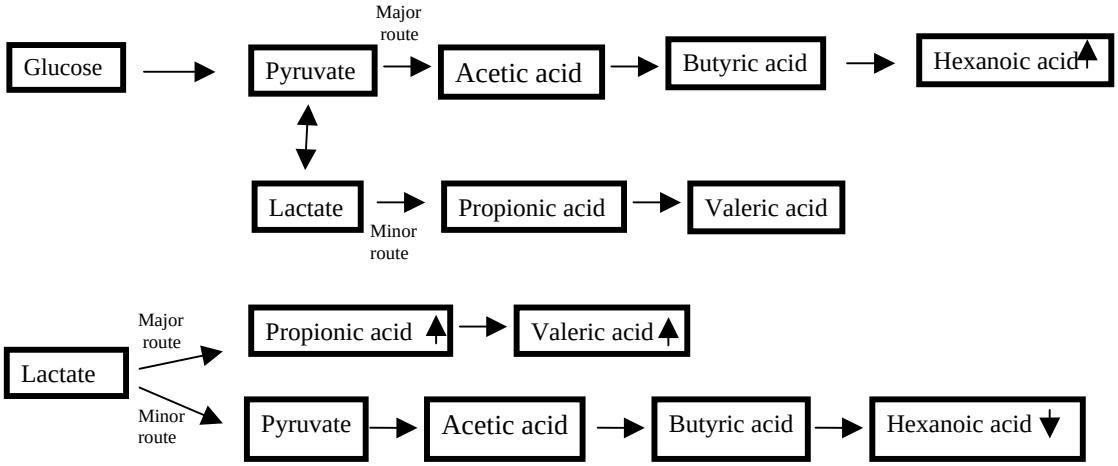
215 times more than mutant ME7. Mutant strain ME5 produced more valeric acid compared to its parental strain ATCC 25940. Strain ME5 was seven times and two times more resistant to 3-FP on THBAG and THBAL plates respectively compared to ATCC 25940. Hino and Kuroda (1993) reported the activity of D-lactate dehydrogenase (D-LDH) was shown not only in cell extracts from *M. elsdenii* grown on DL-lactate, but also in cell extracts from glucose-grown cells, although glucose-grown cells contained approximately half as much D-LDH as DL-lactate-grown cells. The activity of both enzymes was higher during the logarithmic phase of the growth, and then this gradually decreased and was low during stationary phase. Enzyme activity of D-iLDH (NAD independent DLDH), L-nLDH (NAD dependent L-LDH) and D-nLDH (NAD dependent D-LDH) has been reported from the cell extracts of *Selenomonas ruminantium* obtained from different stage of growth on glucose. Both nLDH activities peaked during exponential growth, when glucose was being converted to lactate, although activity could be detected throughout the stationary phase of growth (Gilmour *et al*., 1994). Strain ME5 showed resistance to 3-FP and MIC to 3-FP was 3mg/ml in the presence of glucose compared to its parental strain ATCC 25940 (Table 4.1). The toxicity of fluoropyruvate perhaps depends on its ability to influence pyruvate dehydrogenase complex and pyruvate carboxylase; therefore it is responsible for varied product formation profile. Kinetic studies were undertaken by measuring K_m and V_{max} values for pyruvate and NADH by using the cell-free extract of mutant ME5 (Figure 5.1a and 5.1b). The K_m and V_{max} studies were not done for ATCC 25940 and ME7 because of the low activity of lactate dehydrogenase seen and measurement of the K_m and V_{max} using cell-free extracts of either ATCC 25940 or mutant ME7 was difficult. The production of pyruvic and lactic acids was measured from the glucose grown cells of parent strain ATCC 25940 and mutant strain ME5 and ME7 (Figure 5.2a and 5.2b) obtained from the Applicon fermenters. The equilibrium position of the iD-LDH reaction in *M. elsdenii* strongly favours pyruvate formation; the reverse reaction should occur if the ratio of pyruvate to D-lactate becomes high enough under certain conditions. Such a situation may be brought about if D-lactate is readily metabolised to propionate (Hino and Kuroda, 1993). The lactic acid produced by mutant ME5 was very high compared to the traces found in the parental strain ATCC 25940 and mutant strain ME7 (Figure 5.2a) and that is partially due to higher activity of lactate dehydrogenase as shown in Table 5.1. LDH involved in the conversion of pyruvate to lactate in the presence of a cofactor NADH suggested a difference in major and minor metabolic route for ME5 grown on peptone-yeast-glucose medium (Figure 5.15) compared to parent strain ATCC 25940. This mutant also showed higher production of valeric acid through pyruvate to lactate thence propionate, compared to ATCC 25940 as shown in Figure 5.16.

Figure 5.15: Metabolic pathways and end-products of strain ME5.



ATCC 25940 and mutant ME7 produced 2 to 3 times more pyruvic acid when compared to the strain ME5. The parental strain ATCC 25940 and mutant ME7 showed 58 mM and 53 mM of hexanoic acid respectively compared to mutant ME5 (49 mM).

Figure 5.16: Metabolic pathways and end-products of strain ATCC 25940.



The change in lactate and pyruvate dehydrogenase activity in cell free extract of parental ATCC 25940 and its mutant ME5 seen are responsible for the change in their metabolic end-products and the phenotypes seen. Among rumen bacteria, *M. elsdenii* possesses a constitutive D-iLDH involved in lactate oxidation (Brockman and Wood, 1975; Hino and Kuroda, 1993). However, the data in this thesis shows that LDH activity can be greatly increased in 3-FP^r mutants and this can significant influence the carbon flow to acidic end-products.

Lactic and pyruvic acids were also detected in *M. elsdenii* strains (ATCC 25940, ME5, ME7, T-81, B159, ME9, ME10, ME12, ME14 and 28.7B) grown on 50 ml peptone-yeast-glucose medium (Figure 5.3a and 5.3b). To relate the sensitivity towards the anti-metabolites for these strains with the volatile fatty acids and the enzyme activities observed in small-volume cultures, the detection of lactic and pyruvic acids was conducted as shown in Figure 5.3a and 5.3b. Strain T-81 was three times more sensitive to pyruvic acid in terms of minimal inhibitory concentration compared to other parental strains ATCC 25940 and B-159 in the presence of glucose media. Mutant strain ME5 produced the highest concentration of lactic acid in uncontrolled pH small-volume culture and showed high resistance to 3-fluoropyruvic acids compared to other strains studied in the presence of glucose (Table 4.1). However, no significant difference was observed in terms of pyruvic acid production amongst the strains studied (Figure 5.3b). These results indicate that the changes in end-products seen for ME5 when compared to ATCC 25940 were due to actual relative production of PDH and LDH, resulting in ME5 channelling pyruvate to lactate, propionate and valerate in favour of butyrate and hexanoate as seen in parent strains.

The first step in the fatty-acid oxidation (β-oxidation) cycle is catalyzed by a family of enzymes named fatty acyl-CoA dehydrogenases (Crane *et al*., 1956). To understand the differences in the ratio of metabolic products profile during fermentation, it was important to study the organism at a molecular genetic level by sequencing the genes involved in the metabolic routes. Short-chain acyl-CoA dehydrogenase (SCAD) also known as butyryl-CoA dehydrogenase (BDH) is the only fatty acyl-CoA dehydrogenase present in *M. elsdenii* (Engel and Massey, 1971; Engel, 1981). It functions primarily as an enoyl-CoA reductase by reducing unsaturated short-chain (C_3-C_6) acyl-CoA thioesters as a means of disposing of excess reducing equivalents (Elsden and Lewis, 1953; Brockman and Wood, 1975). In *M. elsdenii*, electron transfer flavoprotein (ETF) transfers two electrons simultaneously from NADH to SCAD after which SCAD reduces enoyl-CoA (Becker, 1994; Stankovich and Soltysik, 1987). Short-chain acyl-CoA dehydrogenase is an important enzyme in the metabolic pathway and is involved in the production of butyric acid that leads to the formation of hexanoic acid. It was important to sequence the SCAD gene of parental strain ATCC 25940 and its mutants ME5 and ME7 to compare any change in their sequences they may account for the altered end-products observed. The amplified PCR products of 1.22 Kb SCAD gene of strains ATCC 25940, ME5 and ME7 was sequenced and multiple sequence alignment of the SCAD gene sequence showed 100% similarity compared to database sequence of *M. elsdenii* Accession no. L04528 as shown in Figure 5.7. Due to the changes observed in the activity of the enzymes (particularly in LDH activity) in parental and mutant strains of *M. elsdenii*

and as in the literature spectral evidence showed that D-lactate dehydrogenase, when reduced by D-lactate, was able to reduce butyryl-CoA dehydrogenase but only in the presence of the electron-transferring flavoprotein. Whitfield and Mayhew (1974) sequenced the flavin-containing enzymes *etfA* (subunit α) and *etfB* (subunit β) involved in catalysis of a variety of reactions, ranging from the dehydrogenation of amino acids to 'DNA damage repair'. The sequencing of amplified PCR products of the strain ATCC 25940 and ME5 showed the 100% similarity in the sequence (Figure 5.10) of subunit β (*etfB*). However, the sequence of subunit α (*etfA*) showed a difference in one codon in both ATCC 25940 and ME5 strains compared to the sequence of *M. elsdenii etfA* in the database. In both the strains, the amino acid residue 287 is histidine instead of proline (Figure 5.11). By comparing the tertiary structure (Figure 5.12) of electron-transferring flavoprotein subunit α (*etfA*) for wild-type strain with the *etfA* structure of the amino acid sequence of the *M. elsdenii* strains ATCC 25940 and ME5, there were no changes in the backbone of the structure with this change from proline to histidine so it unlikely that this was involved in the changed metabolism seen. This enzyme serves as an electron donor to butyryl-CoA dehydrogenase, and it also has NADH dehydrogenase activity (O'Neill *et al*., 1998). NADH-dependent lactate dehydrogenase (LDH) is a key enzyme in the fermentative metabolism of lactic acid bacteria. LDH catalyses reoxidation of NADH into NAD^+, which is required for glycolysis, through accompanying reduction of pyruvate to lactate. Attempts to sequence LDH gene in ATCC 25940 and ME5 strains of *M. elsdenii* by designing a PCR primer set based on the most conservative regions of the LDH gene from *P. acidilactici* (Accession no: X70927) and *Streptococcus bovis* (Accession no: U60997) were unsuccessful as there was no homology compared to the sequences of LDH in databases, indicating that the *M. elsdenii* is not similar to other currently sequenced LDH genes.

CHAPTER 6

6 CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

This thesis described the studies on the processes that may lead to adipic acid production through hexanoic acid synthesis by the anaerobic bacterium *M. elsdenii* and its subsequent utilization by *Pseudomonas* strains. The most apparent conclusion to be drawn from the research described in chapter three provides the first report of significant formation of *n*-octanoic acid (up to 38 mM) production in *Pseudomonas* strains with and without OCT plasmid grown on *n*-octanol vapours in the presence and absence of neutralized *n*-hexanoic acid targeted long-term incubated small-volume culture (up to 52 weeks). At no time was adipic acid detected as an intermediate, in contrast to previous reports by du Pont (Kunz and Weimer, 1983) and by Thijsse (1964). Because these studies failed to detect adipic acid at any stage of growth, including very old cultures, studies on adipic acid production by *Pseudomonas* strains was not pursued further.

The research work involved in second part of the thesis is described in chapters four and five and this involved the intermediary metabolism of the anaerobic bacterium *M. elsdenii*. Ten different parent and mutant strains of *M. elsdenii* that showed altered sensitivity to various antimetabolites and their end-products were examined in context of their altered biochemical properties as described in chapter four. Minimal inhibitory concentrations (MICs) were determined for growth on glucose and lactate to determine whether the major carbon source influenced their growth. The antimetabolites were all more growth inhibitory than their volatile fatty acid counterparts. The phenotype of mutant strain ME5 was verified in that it was consistent with previous unpublished data, which implied that this strain might be a metabolic variant: the most important trait was resistance to 3-fluoropyruvate.

All the parental and mutant strains were studied further for the end-product formation in terms of volatile fatty acids in small-volume cultures (50 ml) containing glucose or lactate and with the combination of glucose plus lactate using uncontrolled pH conditions without stirring for 144 h growth periods. In the presence of glucose, hexanoic acid was the major end-product produced by all the strains studied, however, mutant strain ME5 produced 10 times more valeric acid compared to its parental strain ATCC 25940. The major end-products on lactate medium were valeric, butyric and propionic acids for all strains studied, however, propionic acid almost eight times more than produced in the presence of mixed substrates. Strain ME5 produced relatively more butyric and valeric acids than its parental strain ATCC 25940. In the presence of glucose combined with lactate, valeric and butyric acids were the major end-products while acetate and propionate was the minor end-products produced by all the strains studied, however, strain ME5 produced relatively

more valeric acid than its parental strain ATCC 25940. These results showed that same mutants resistant to 3-FP showed different proportion of end-products compared to their parent strains.

This chapter also described the experiments to confirm the metabolic capacity of the mutants in fermenters. The first step was to optimize the pH for growth, the most important parameter affecting growth and end-product formation during fermentation for the parental strain ATCC 25940 and mutants ME5 and ME7 when grown in the presence of peptone-yeast extract-glucose medium. The optimal pH in terms of growth rate and hexanoic and butyric acid production was at pH 6.5.

Parent strain ATCC 25940 and its mutants ME5 and ME7 were further tested for end-product formation under controlled pH fermenters with continuous stirring in the presence of glucose, lactate, and glucose plus lactate medium at pH 6.5. Uncontrolled pH cultures of *M. elsdenii* strains with or without stirring produced lower amount of acids than the comparable controlled pH and stirred cultures. Total acids produced were greater in the presence of mixed substrates compared to single substrate used. The composition of its fermentation end-products was not the same in media with different energy sources, evidently due to different metabolic pathways involved in glucose and lactate metabolism. Changes in metabolism seen for these mutants may have been attributed to changes in levels of enzymes involved in pyruvate or lactate metabolism, an aspect studied later in this work.

The work described in chapter four was also concerned with the efficient recovery of fermentation products leads to higher productivity by adsorption to anion exchange resins. Initially, Amberlite IRA-93 (weak base anion-exchange resin) and Amberlite IRA-400 (strong base anion-exchange resin) resins were tested for their ability to adsorb and desorb butyric and hexanoic acids from water and peptone-yeast-glucose media. Results showed that both resins adsorbed more *n*-hexanoic acid than *n*-butyric acid, which suggested that the VFA chain length may affect the degree of adsorption. However, both acids were desorbed more effectively by esterification from Amberlite IRA-93 resin, which indicated that Amberlite IRA-93 might have some attractive features above Amberlite IRA-400 for product recovery in the form of esters. Studies were also carried out on the adsorption and desorption of both acids on fermentation level in peptone-yeast extract-glucose media under pH controlled and uncontrolled pH conditions for the parent strain ATCC 25940. Results indicated that using the Amberlite IRA-93 had an advantage in that it exerted a buffering effect on the fermentation broth and reducing the amount of alkali used to maintain the optimum pH. The use of resins was not carried with the mutants because the results for ATCC 25940 indicated that the ratio of products did not change greatly from those seen in pH-controlled, stirred fermenters.

Studies on the analysis of selected enzymes and genes involved in intermediary metabolism were carried out in parental strain ATCC 25940 and its mutants as described in chapter five of this thesis. Different proportions of the end-products such as valeric acid seen for some $3FP^r$ mutants may have contributed to changes in levels of enzymes such as pyruvate dehydrogenase (PDH), lactate dehydrogenase (LDH) and butyryl CoA dehydrogenase (BDH) involved in pyruvate or lactate

metabolism. Pyruvate dehydrogenase activity was measured for the first time in cell-free extracts of parental strain ATCC 25940 and its mutants ME5 and ME7 grown in peptone-yeast extract-glucose media. Strain ME5 showed 100 times less pyruvate dehydrogenase activity compared to its parental strain ATCC 25940, however, lactate dehydrogenase activity was 400 times more than its parental strain ATCC 25940 and mutant ME5 was, indeed, very different in these activities relative to the parent strain ATCC 25940 and mutant ME7. Contrary to this, high PDH and low LDH specific activities were observed in strains ATCC 25940 and ME7 and despite an active PDH specific activity in ATCC 25940 and ME7, pyruvate was still accumulated in culture fluids but not as much as lactate, in both small-volume cultures (uncontrolled and not stirred) and in one-litre fermenters (pH controlled and stirred). The activity of both enzymes was higher during the logarithmic phase of the growth, and then this gradually decreased and was low during stationary phase. The data in this thesis showed that LDH activity was greatly increased at least in one of the 3-FP^r mutants and that this change significantly influenced the carbon flow to acidic end-products.

The detection of lactic and pyruvic acids was conducted for both small- and large-volume cultures grown in peptone-yeast extract-glucose medium to relate the sensitivity towards the anti-metabolites for *M. elsdenii* strains with the volatile fatty acids and the enzyme activities observed. The results described in chapter five indicated that the changes in end-products seen for ME5 when compared to ATCC 25940 were due to actual relative production of PDH and LDH, resulting in ME5 channelling pyruvate to lactate, propionate and valerate in favour of butyrate and hexanoate as seen in parent strains.

It was important to study the organism at a molecular genetic level by sequencing the genes to understand the differences in the ratio of metabolic products during fermentation as explained in chapter five. Short-chain acyl-CoA dehydrogenase (SCAD) is an important enzyme in the metabolic pathway and involved in the production of butyric acid that leads to the formation of hexanoic acid. SCAD gene was sequenced for parental strain ATCC 25940 and its mutants ME5 and ME7 that showed 100% similarity when compared with database sequence of *M. elsdenii* Accession no. L04528 using multiple sequence alignment. Due to the changes observed in the activity of the enzymes (particularly in LDH activity) in parental and mutant strains of *M. elsdenii*, it was necessary to sequence LDH gene. As in the literature spectral evidence showed that D-lactate dehydrogenase, when reduced by D-lactate, was able to reduce butyryl-CoA dehydrogenase but only in the presence of the electron-transferring flavoprotein. The sequencing of two subunits of electron-transferring flavoprotein from the parental strain ATCC 25940 and its mutant ME5 was carried out and sequencing data showed 100% similarity in the sequence of subunit β (*etfB*). However, the sequence of subunit α (*etfA*) showed a difference in one amino acid (histidine instead of proline) in both ATCC 25940 and ME5 strains compared to the sequence of *M. elsdenii etfA* database Accession no. AF072475. By comparing the tertiary structure of electron-transferring flavoprotein subunit α (*etfA*) for wild-type strain with the *etfA* structure of the amino acid sequence of the *M. elsdenii* strains ATCC 25940 and ME5, there were no changes in the backbone of

the structure with this change from proline to histidine so it is unlikely that this was involved in the changed metabolism seen. After sequencing electron-transferring flavoprotein, attempts were made to sequence LDH gene in parental strain ATCC 25940 and its mutant ME5 from PCR products obtained on the basis of primers generated after comparing the coding regions of LDH genes from different organisms as described in the section 5.5.1. To sequence LDH gene was unsuccessful, as the sequence comparison showed no homology to the sequences of LDH in databases, indicating that the *M. elsdenii* is not similar to other currently sequenced LDH genes.

6.2 Recommendations for future work

1. The observation that the *Pseudomonas* stains studied continued to accumulated *n*-octanoic acid over extended periods of incubation was an interesting observation that may have broader implications. Presumably, *n*-octanoic did not accumulate in normal, short-term cultures as it was degraded by normal metabolic processes including β–oxidation. In extended incubations, cells presumably remained viable or at least metabolically active, accumulating *n*-octanoic when normal physiological processes (including β–oxidation) ceased to operate as cells aged and died. This hypothesis could be investigated by monitoring the viability and metabolic activity of cells, including expression of stationary-phase proteins, changes in cellular ultrastructure, during extended periods of culture, preferably under more controlled environments in fermenters. Outcomes would include validation of the observations made in shake-flask; small-scale cultures used in the present study and provide insights into the underlying mechanisms that gave rise to accumulation of a metabolic intermediate.

2. The use of antimetabolites to select mutants with altered intermediary metabolism has been rarely investigated in anaerobic bacteria. The chance isolation of 3FP resistant mutants, and the subsequent characterization of one of the mutants in terms of altered ratios of enzymes involved in utilizing pyruvate either towards hexanoic acid or propionate/valeric acids, indicates that is may be possible to isolate other mutants with altered carbon flow patterns. It may be possible, for example, to isolate strains that accumulate acrylic acid or other products of potential commercial value. However, the strategy used may require a greater understanding of which analogues would be of value and how they could be used. For example, it may be useful to explore other analogues, including ones for lactate, in terms of their inhibition and use these to select mutants with resistance to these. This would involve developing appropriate mutagenic procedures, using classical random mutagenesis or applying insertional/transposon mutagenesis approaches, given that there is increasing interest in the molecular genetics of this species and the development of appropriate genetic tools is evolving. As only one mutant was evaluated in detail in the present study, it may be useful to isolate and evaluate more 3FP resistant mutants to determine the general utility of using antimetabolites in this manner. More quantitative approaches such as mass balances in conjunction with theoretical yields, stoichiometry and

metabolic flux analysis should be used to understand the metabolite transformation and an assessment of the true potential for (over) production of a given product or intermediates.

3. This study failed to detect LDH gene using PCR approaches based on known genes from different organisms. Given that this gene was key for channelling carbon from pyruvate to propionate and valerate, it would be useful to characterize this gene further. However, it is not possible to investigate the transcriptional regulation of the LDH gene and its function in metabolism in *M. elsdenii*. *M. elsdenii* is not a viable host for altered gene regulation because current technologies available for gene expression and gene transformation studies are inadequate. One possible approach would be to isolate the enzyme and obtain some amino acid sequence information using classical protein purification approaches (given the ease of assaying this activity) and use this information to determine a strategy for characterizing the gene in *M. elsdenii*. Another possible approach would be to investigate the altered sensitivity to inhibition by end-products; *M. elsdenii* would be grown in the presence and absence of known inhibitors or anti-metabolites. Cell extracts from *M. elsdenii* will then be harvested and prepared for proteomics analysis to identify the proteins that affect the regulation of LDH activity.

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APPENDIX–1: Sources of chemicals and reagents

Chemical/Reagent	Source
Acetic acid galacial	BDH
Acrylamide	Biorad
Acrylic acid	Ajax
Adipic acid	Ajax
Agarose DNA grade	Progen
Ammonium persulphate	Biorad
Bis, N",N Methylene bis acrylamide	Biorad
Bovine serum albumin	Sigma
Bromoacetic acid	Aldrich
2-Bromobutyric acid	Aldrich
4-Bromobutyric acid	Aldrich
6-Bromohexanoyl chloride	Aldrich
2-bromomethyl acrylic acid	Aldrich
Bromophenol blue	BDH
2-Bromopropionyl bromide	Aldrich
Butyric acid	Aldrich
4-Chlorobutyryl chloride	Aldrich
Chloroform	BDH
3-Chloropropionic acid	Sigma
Coenzyme A	ICN
Cysteine-HCl	Aldrich
Defibrinated horse blood	CSL
Diethyl ether	BDH
Dithiothreitol	Sigma
DNA grade Agarose	Progen
EDTA (di-sodium salt)	Ajax
Ethidium bromide	Sigma
Ethyl butyrate	Aldrich
Ethyl caproate	Aldrich
3-Flouropyruvate	Aldrich
D-Glucose anhydrous	BDH
Heptanoic acid	Sigma
Hexanoic acid	Sigma
High Prime	Boehringer
IPTG	Promega
Isoamyl alcohol	BDH
Iso butyric acid	Sigma
Isopropanol	Sigma
Isovaleric acid	Aldrich
Lactate dehydrogenase	Boehringer
Lactic acid	BDH
2-Mercaptoethanol	BDH

Chemical/Reagent	**Source**
2-Methyl butyric acid	Sigma
Methyl *n*-butyrate	Alltech
Methyl *n*-caproate	Alltech
Methyl *n*-heptanoate	Alltech
2-methyl pentanoic acid	Sigma
Methyl viologen	Sigma
NAD^+	ICN
NADH	ICN
n-Octane	BDH
n-Octanol	BDH
n-Octanoic acid	Sigma
Phenol	ICN
Propionic acid	Sigma
Pyrogallol	Sigma
Pyruvic acid	ICN
Resazurin	Sigma
Sodium dodacyl sulphate	Sigma
Sodium pyruvate	BDH
TEMED (N,N,N',N'-tetramethylenediamine)	Biorad
Tris base	Sigma
Valeric acid	Sigma
X-gal	Progen

APPENDIX-2:

Figure A-1: Schematic diagram of replica plates shows the positions of the *M. elsdenii* strains applied during MICs on THBAG and THBAL medium plates.

PYG (peptone-yeast-glucose), PYL (peptone-yeast-lactate), strains ATCC 25940, ME5, ME7, B159, ME8, ME9, T-81, ME12, ME14, and 28.7B are applied on these positions for MICs experiments.

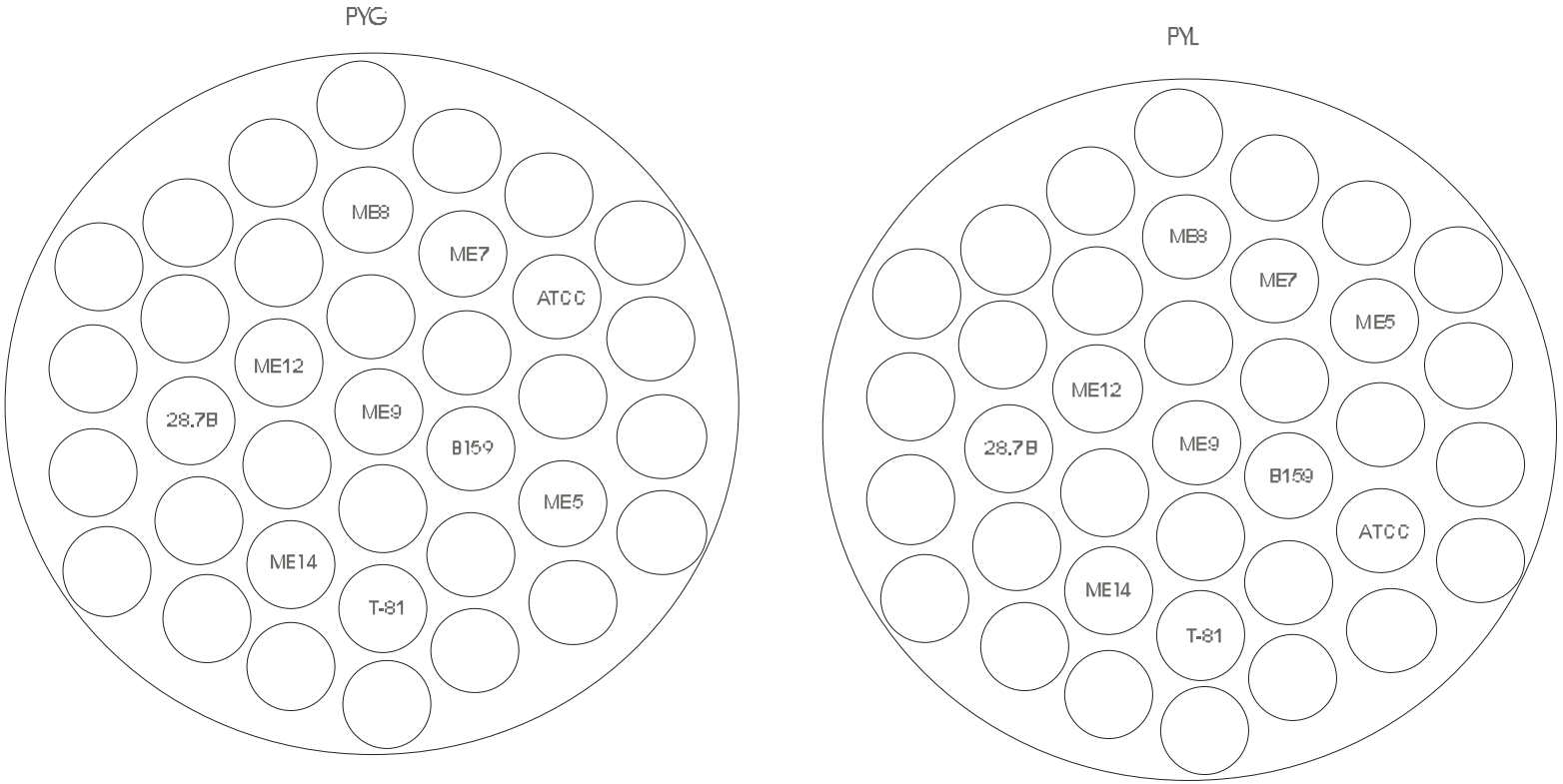


Figure A-2: Control growth of *M. elsdenii* strains on THBAL (top) and THBAG (bottom) plates using the replica plate technique. The details of each strain are described in the key shown in appendix-2 Figure A-1.

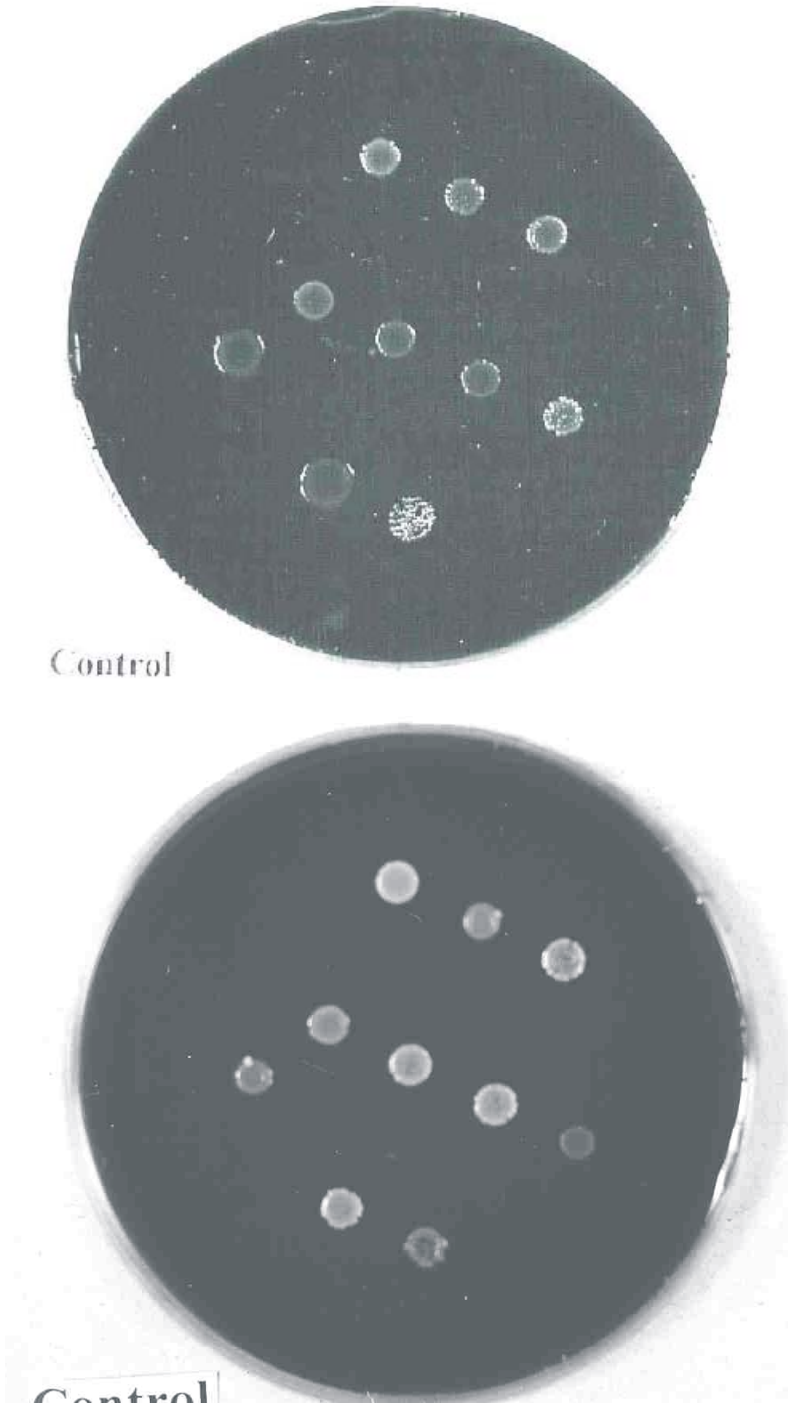
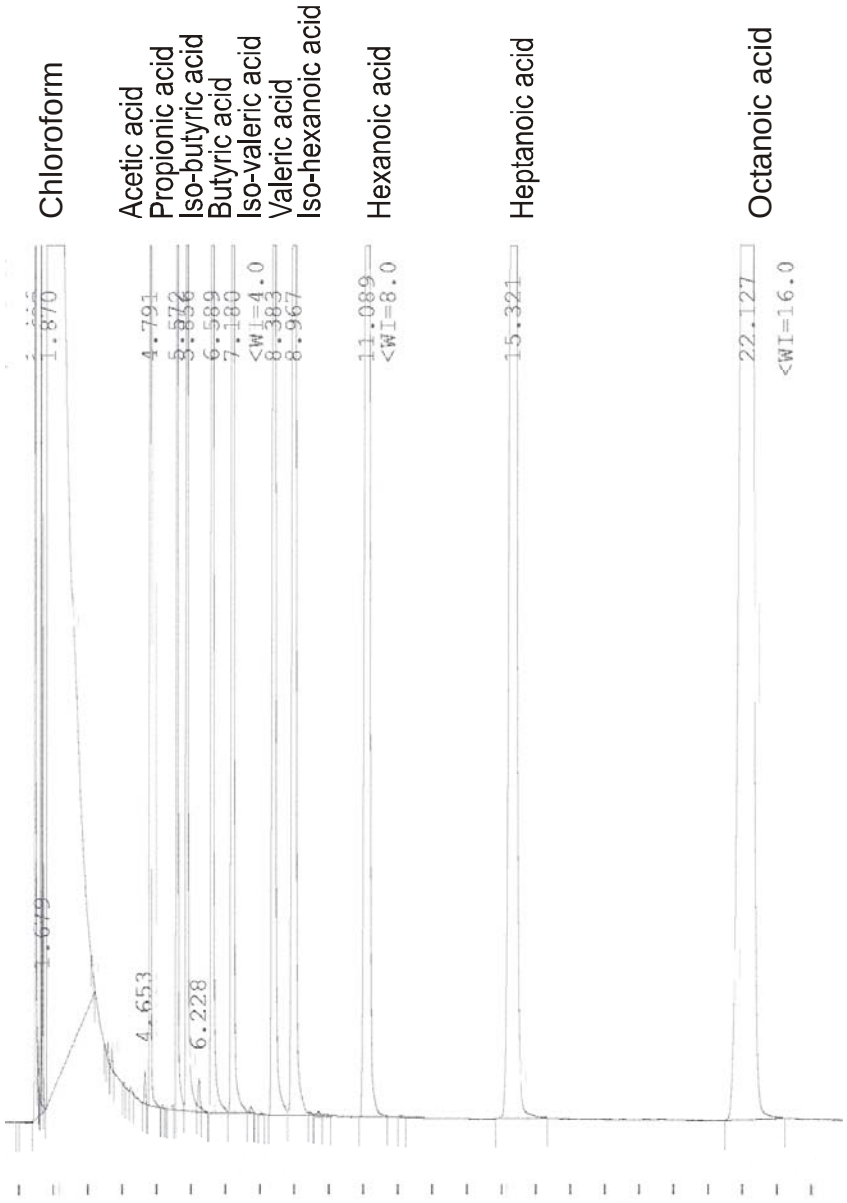


Figure A-3: Example of gas chromatogram from the standards methyl esters of VFAs including organic acid extracted in chloroform solution as described in section 2.3.2. This chromatogram shows 11 peaks starting at 1.87 min as solvent (chloroform) peak.



Peak-1:	Chloroform	Peak-2:	Acetic acid
Peak-3:	Propionic acid	Peak-4:	Iso-butyric acid
Peak-5:	Butyric acid	Peak-6:	Iso-valeric acid
Peak-7:	Valeric acid	Peak-8:	Iso-hexanoic acid
Peak-9:	Hexanoic acid	Peak-10:	Heptanoic acid
Peak-11:	Octanoic acid		

Figure A-4: Example of gas chromatogram from the standards Volatile Fatty Acids extracted in Diethyl ether solution as described in section 2.4.2.

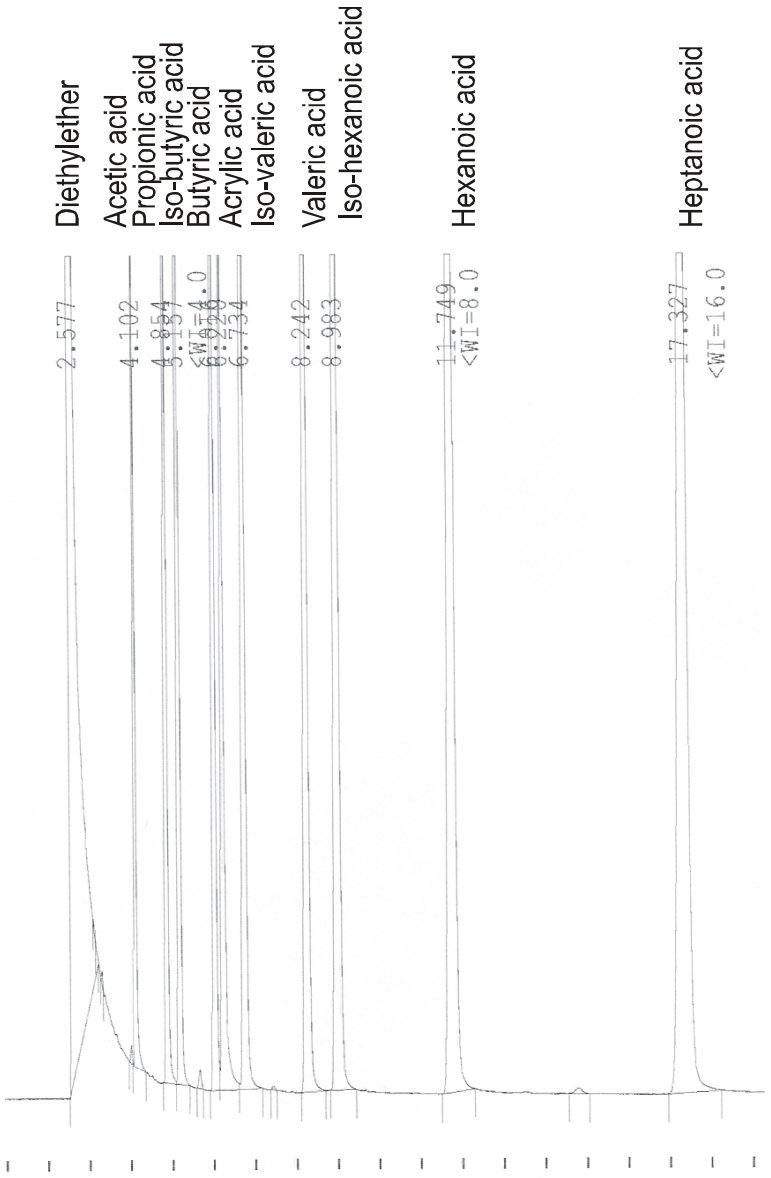


Figure A-5: Standard curve for the determination of the concentration of esterified heptanoic acid by GC extracted in chloroform as described in Materials and Methods section 2.3.2.

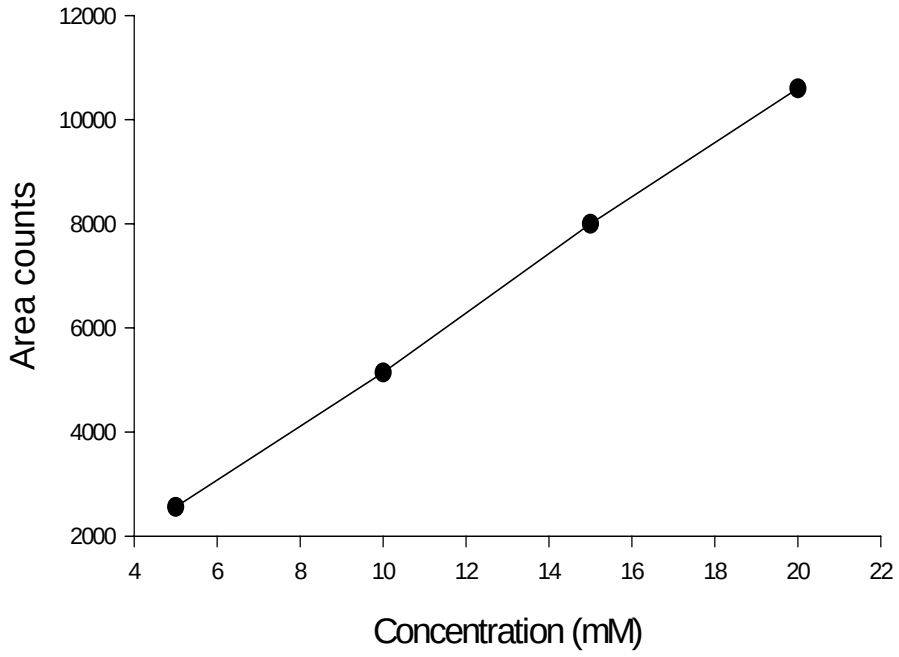


Figure A-6: Standard curve for the determination of the concentration of esterified hexanoic acid by GC extracted in chloroform as described in Materials and Methods section 2.3.2.

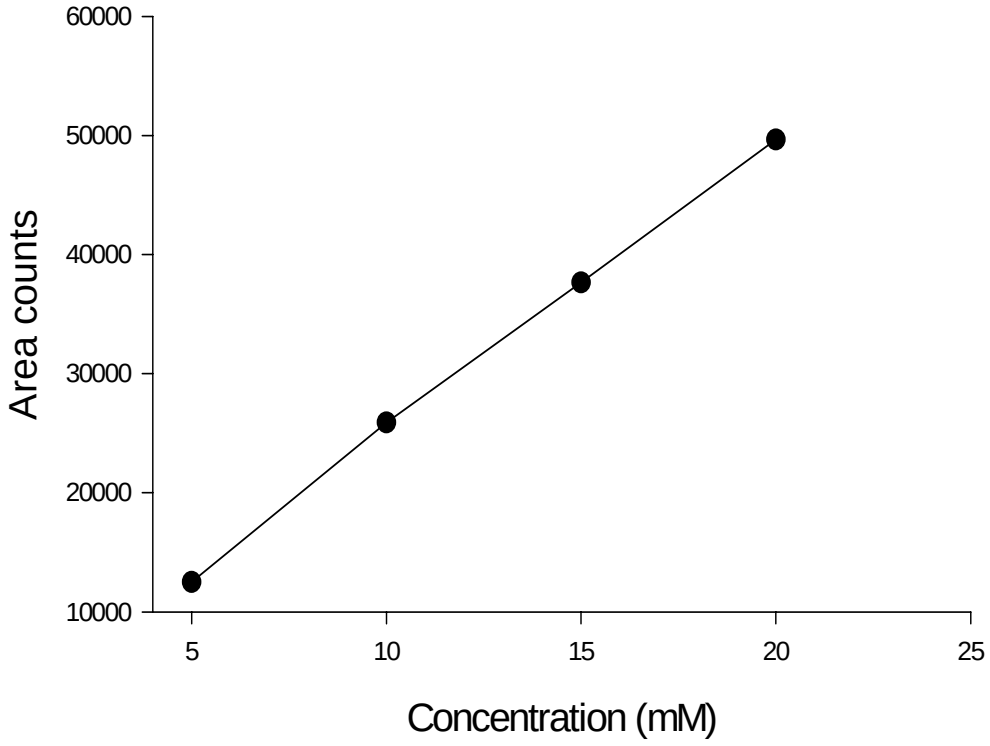


Figure A-7: Standard curve for the determination of concentration of lactic acid dissolved in 0.0075 N H_2SO_4 by using HPLC as described in Materials and Methods section 2.4.4.4.

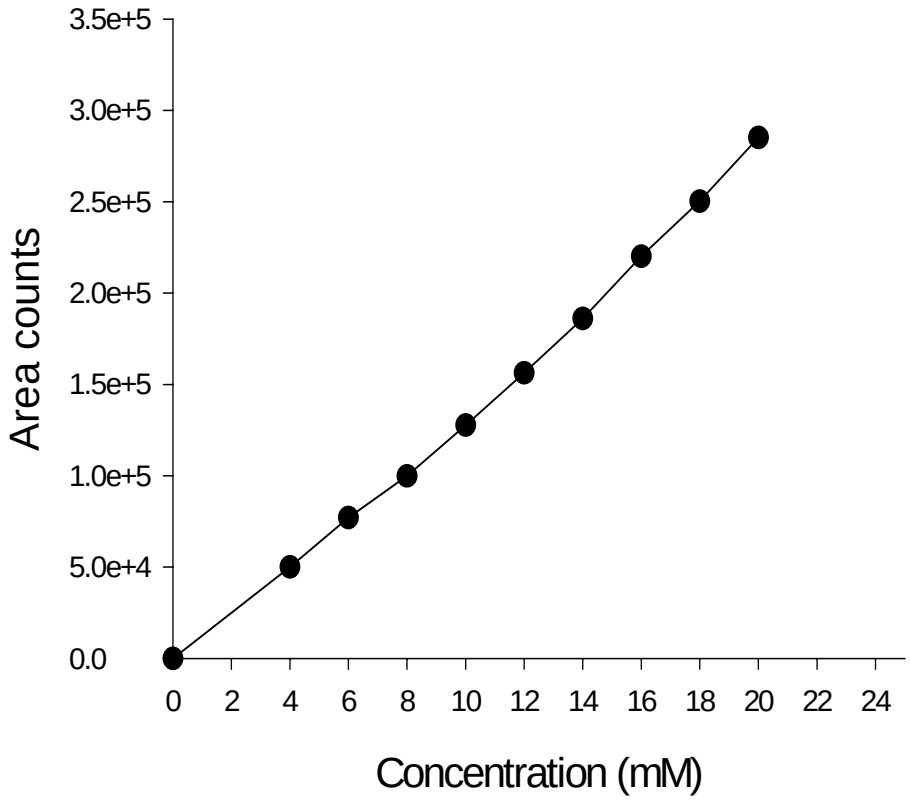


Figure A-8: Standard curve for the determination of concentration of pyruvic acid dissolved in 0.0075 N H_2SO_4 by using HPLC as described in Materials and Methods section 2.4.4.4.

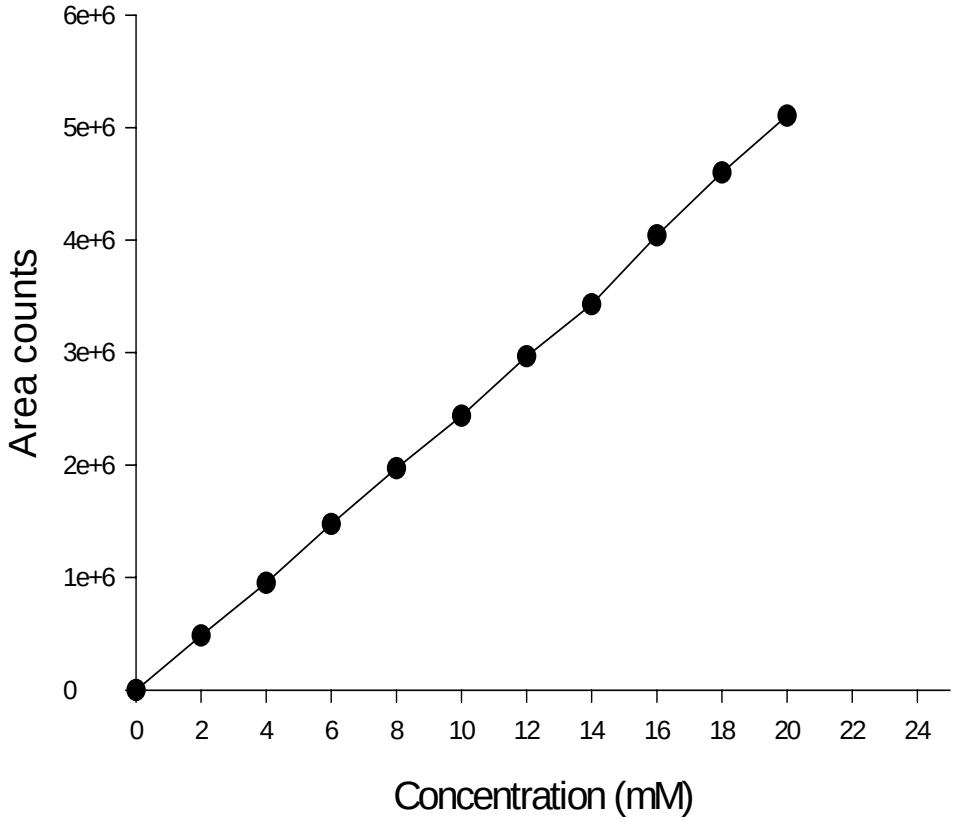


Figure A-9: Standard curve for the determination of protein concentration by Lowry method as described in Materials and Methods section 2.5.1.

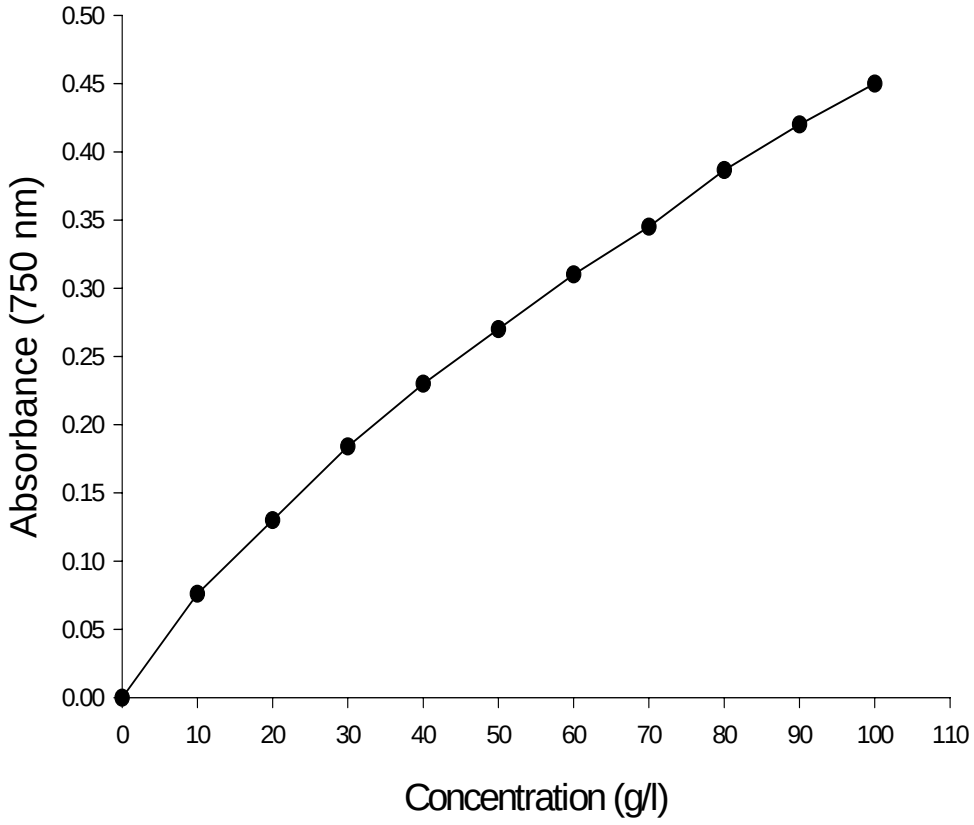


Figure A-10: Standard curve for the determination of glucose concentration by the DNS method as described in Materials and Methods section 2.5.2.

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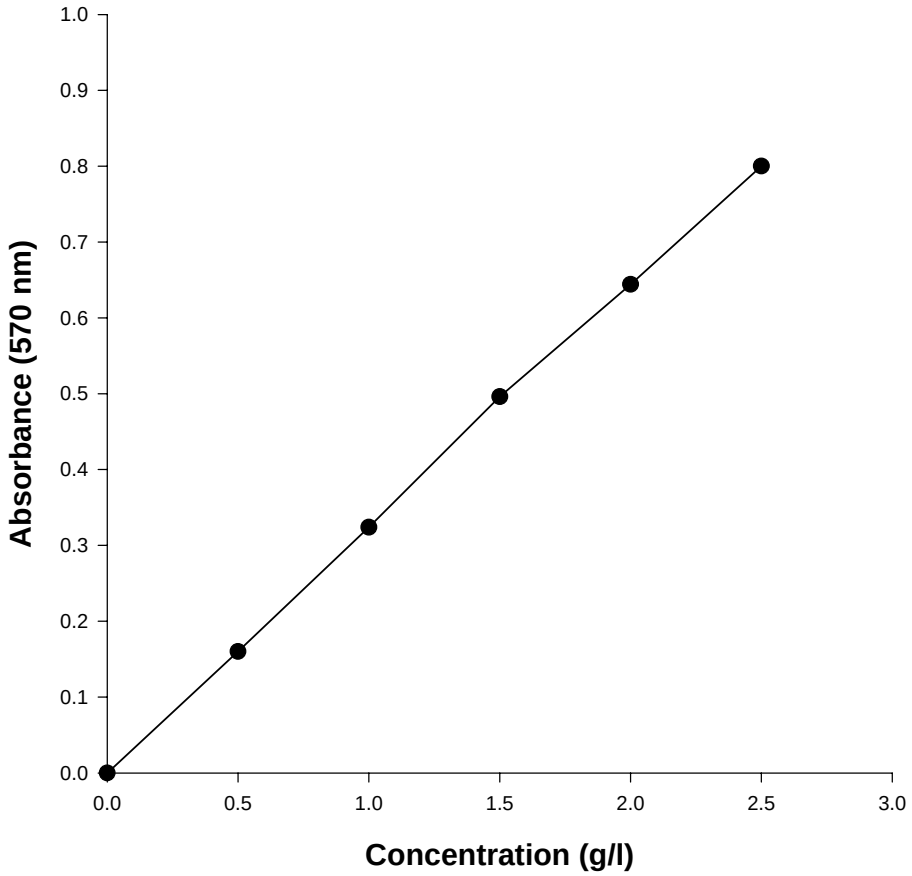


Figure A-11: Rate of NADH oxidation (absorbance 340 nm) in 10 µl cell-free extract of *M. elsdenii* strains ATCC 25940, ME5 and ME7 at 24 h incubation. Control 1 represents the absorbance taken in the presence of buffer (as described in Materials and Methods section 2.5.3.2) with the addition of NADH (reduced form of nicotinamide adenine dinucleotide). Control 2 represents the same as control 1 with the addition of cell-free extracts of strain ME5.

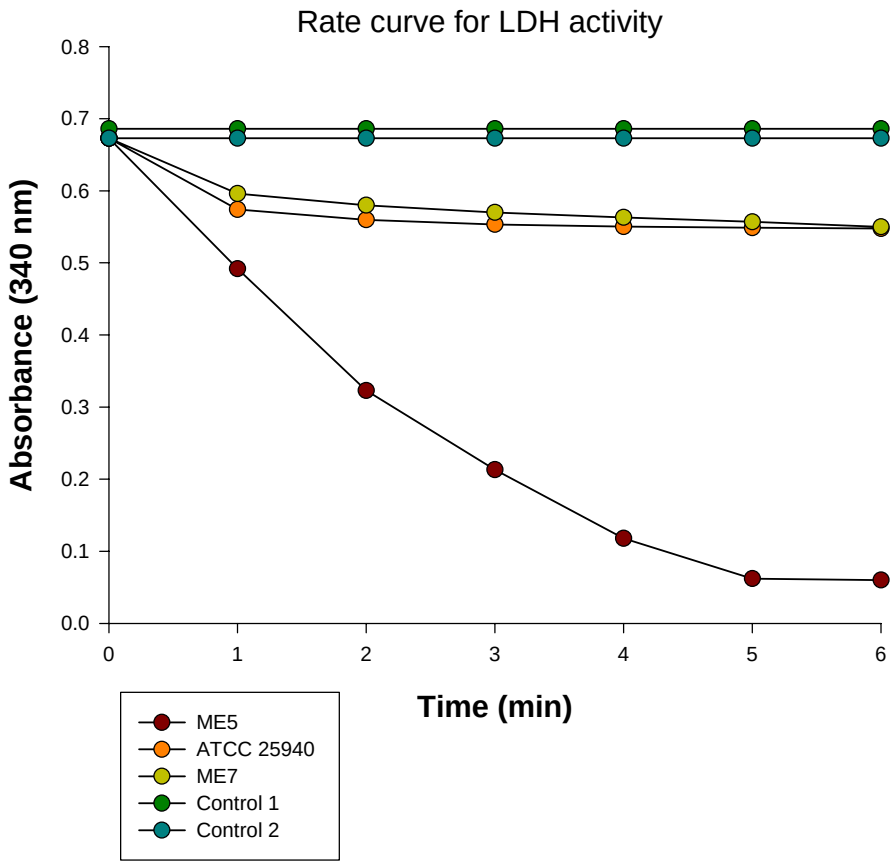


Figure A-12: Rate of methyl viologen reduction indicated by increases in absorbance at 600 nm in 10 µl cell-free extract of *M. elsdenii* strains ATCC 25940, ME5 and ME7 at 24 h incubation. Control 1 represents the absorbance taken in the presence of buffer (as described in Materials and Methods section 2.5.3.3) with the addition of NAD (nicotinamide adenine dinucleotide). Control 2 represents the same as control 1 with the addition of cell-free extracts of strain ATCC 25940. Control 3 was same as control 2 with the addition of pyruvate.

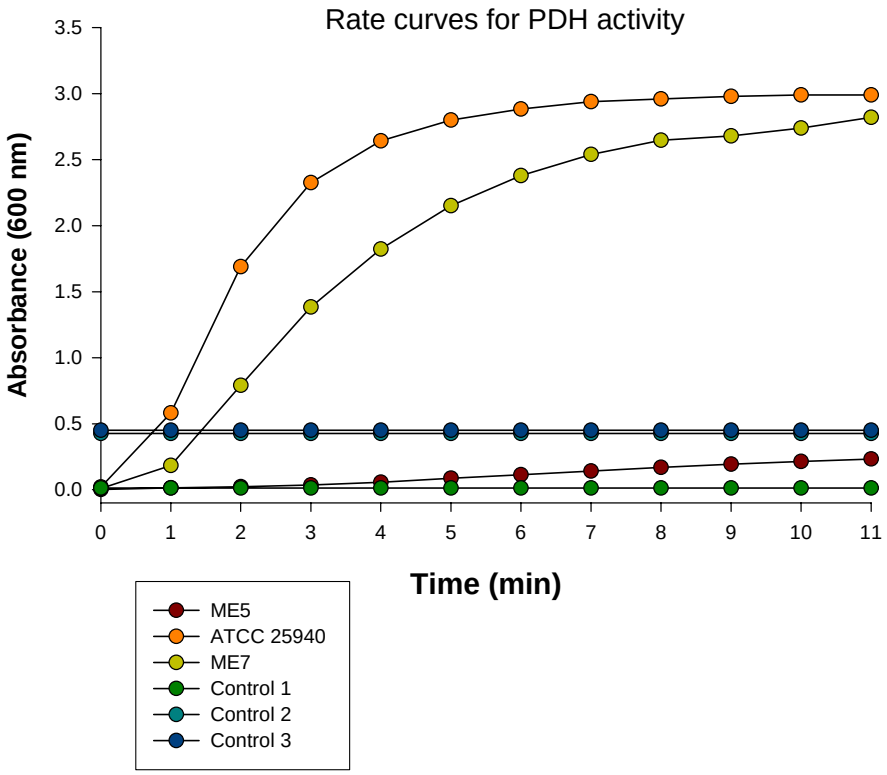


Figure A-13: Example of gas chromatogram from the standard 10 mM concentration of adipic acid extracted in chloroform solution as described in Materials and Methods section 2.3.2.

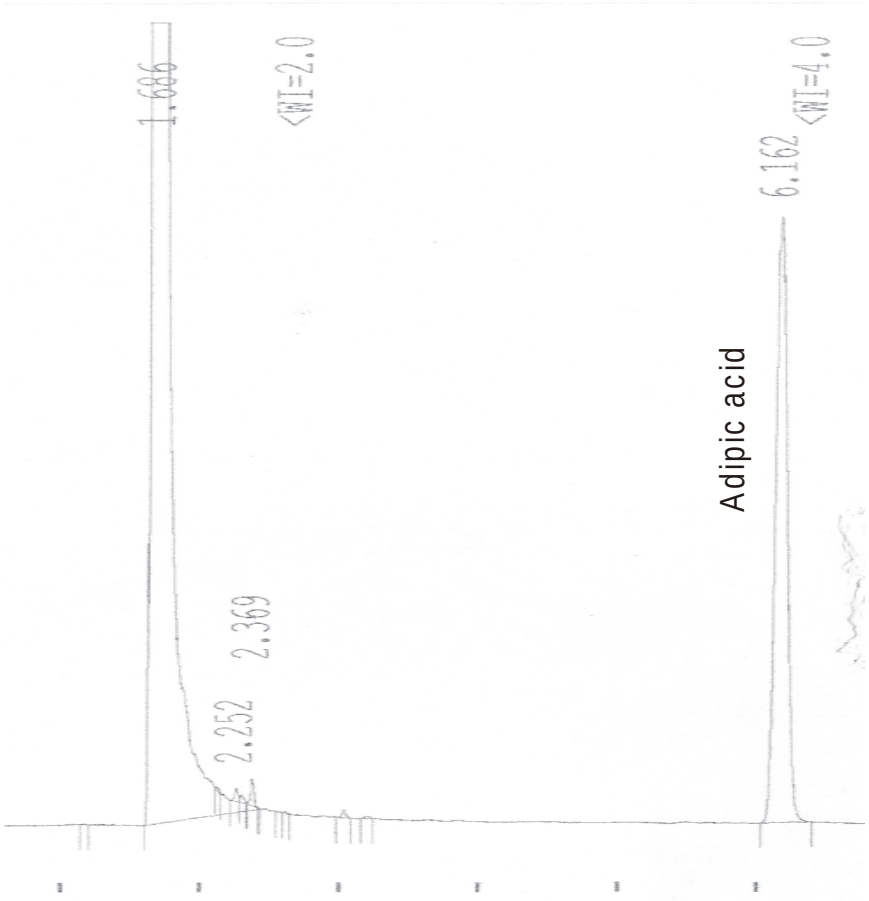


Figure A-14: Various *M. elsdenii* strains were grown on THBAG plates containing 0.6 – 3.0 mg/ml pyruvic acid (P.A.) to determine the minimum inhibitory concentration (MIC). MIC was determined using the replica plate technique. The details of each strain are described in the key shown in appendix-2 Figure A-1.

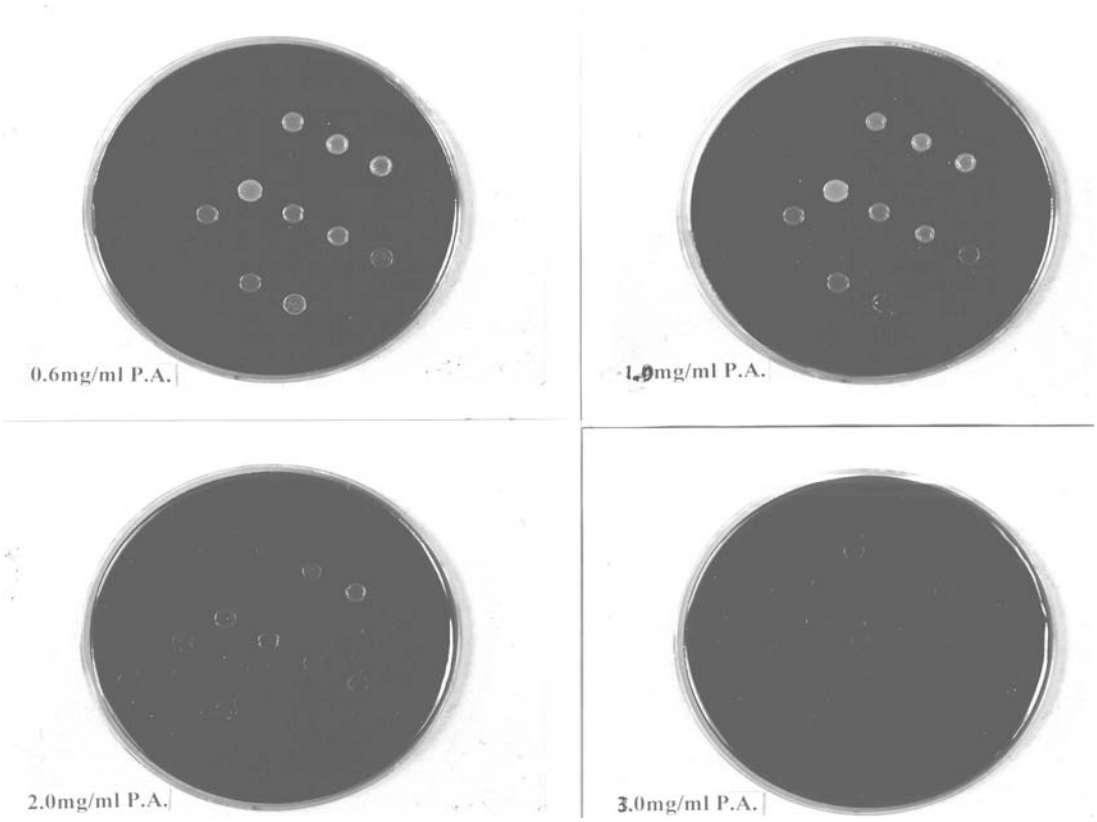


Figure A-15: Various *M. elsdenii* strains were grown on THBAL plates containing 0.5 – 5.0 mg/ml pyruvic acid (P.A) to determine the minimum inhibitory concentration (MIC). MIC was determined using the replica plate technique. The details of each strain are described in the key shown in appendix-2 Figure A-1.

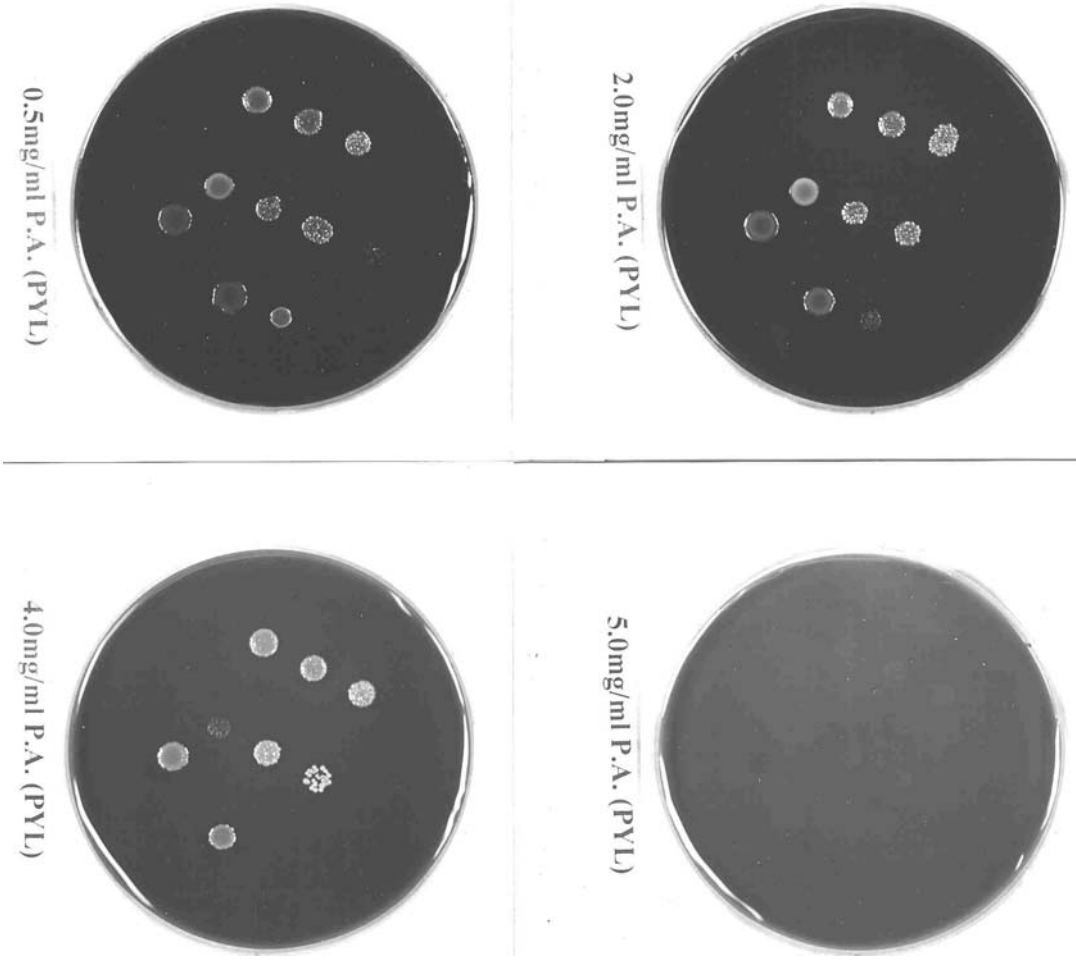


Figure A-16: Various *M. elsdenii* strains were grown on THBAG plates containing 0.5 – 3.0 mg/ml 3-fluoropyruvate (3-FP) to determine the minimum inhibitory concentration (MIC). MIC was determined using the replica plate technique. The details of each strain are described in the key shown in appendix-2 Figure A-1.

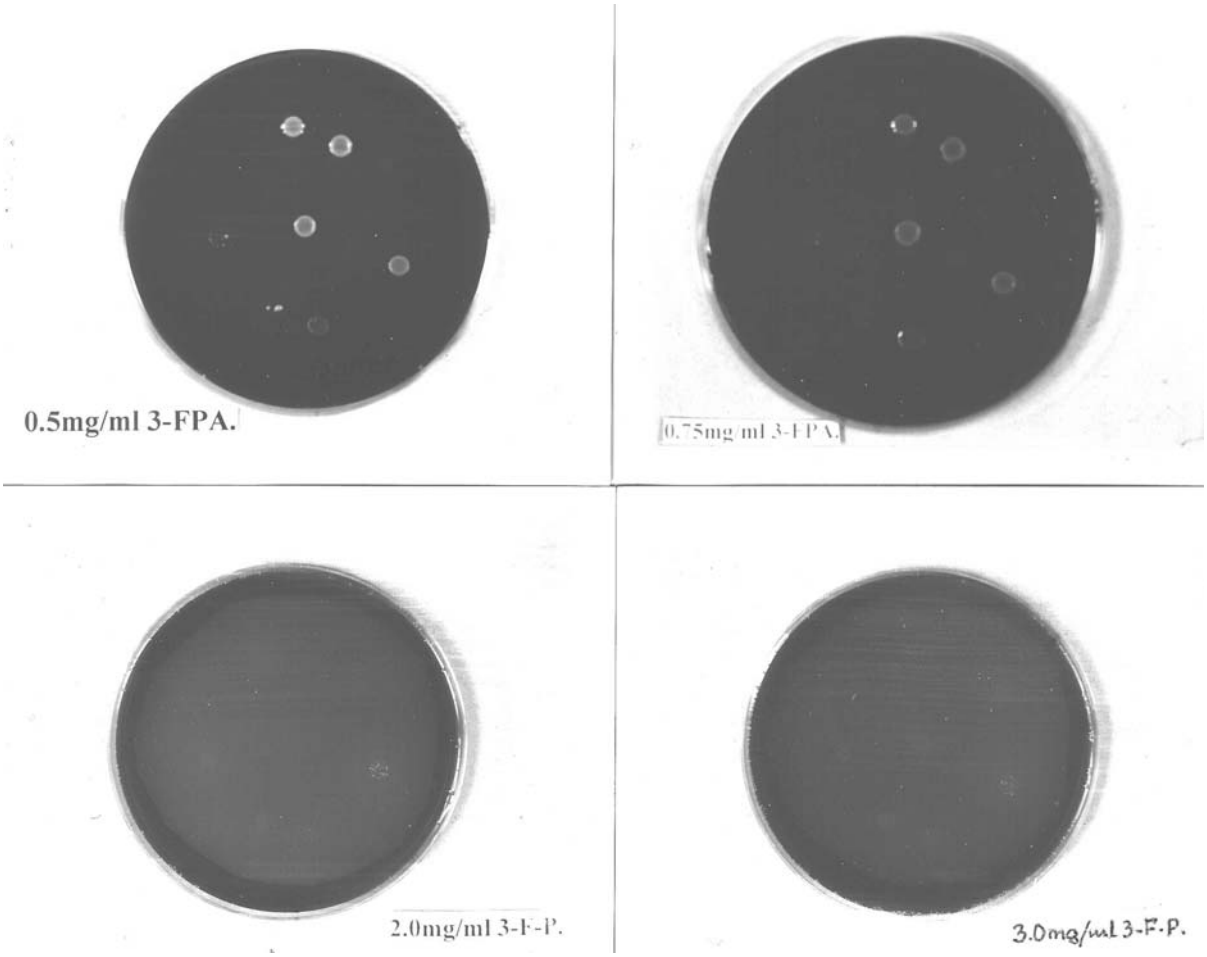


Figure A-17: Various *M. elsdenii* strains were grown on THBAL plates containing 0.5 – 2.0 mg/ml 3-fluoropyruvate (3-FP) to determine the minimum inhibitory concentration (MIC). MIC was determined using the replica plate technique. The details of each strain are described in the key shown in appendix-2 Figure A-1.

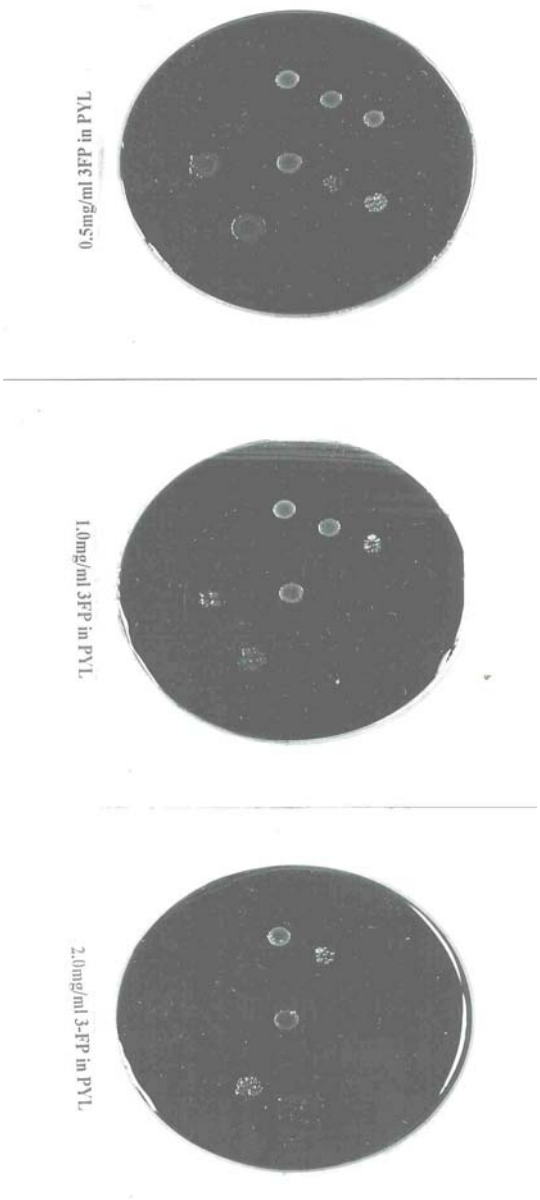


Figure A-18: Various *M. elsdenii* strains were grown on THBAG plates containing 0.2 – 3.0 mg/ml hexanoic acid (H.A) to determine the minimum inhibitory concentration (MIC). MIC was determined using the replica plate technique. The details of each strain are described in the key shown in appendix-2 Figure A-1.

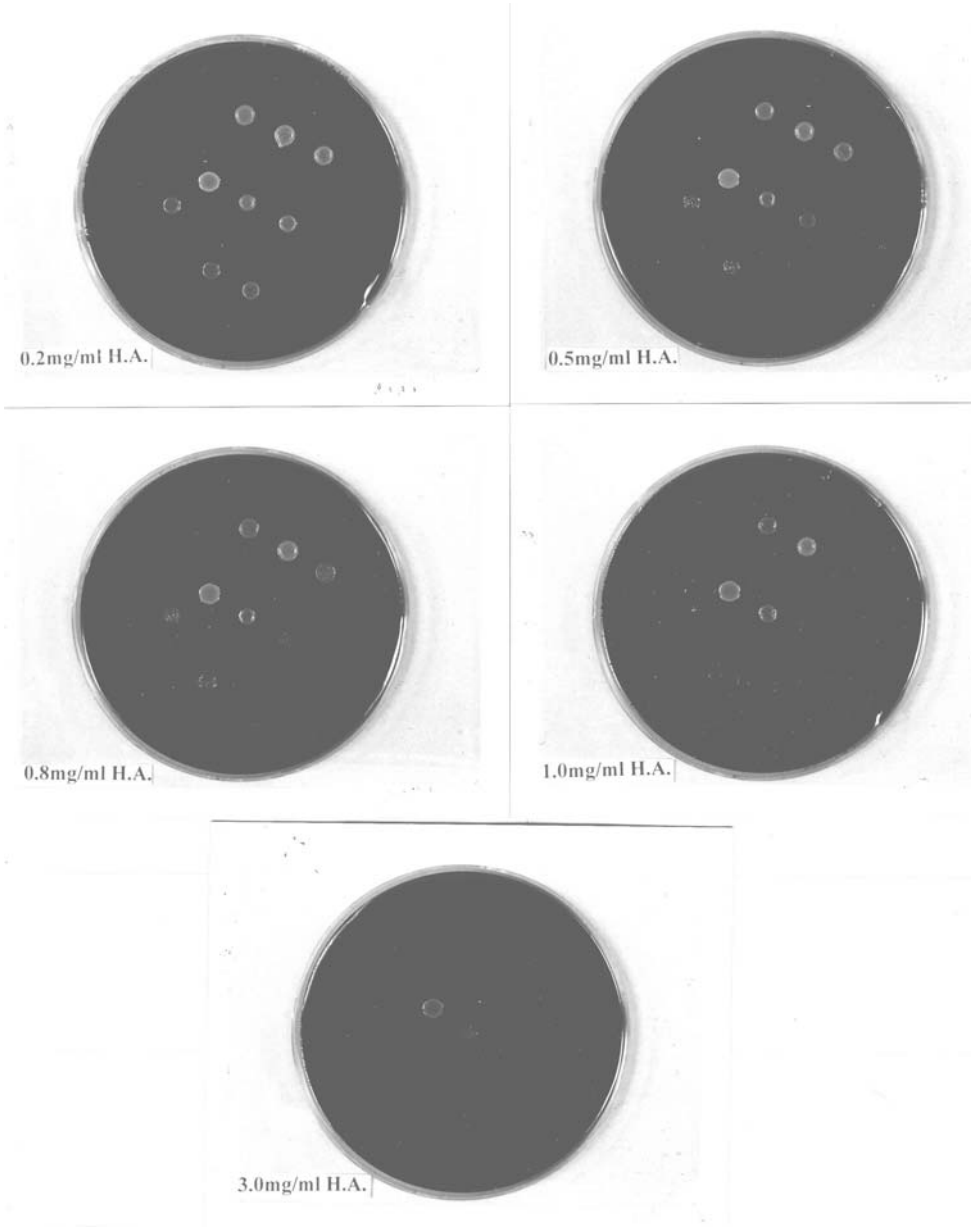


Figure A-19: Various *M. elsdenii* strains were grown on THBAL plates containing 0.1 – 3.0 mg/ml hexanoic acid (H.A.) to determine the minimum inhibitory concentration (MIC). MIC was determined using the replica plate technique. The details of each strain are described in the key shown in appendix-2 Figure A-1.

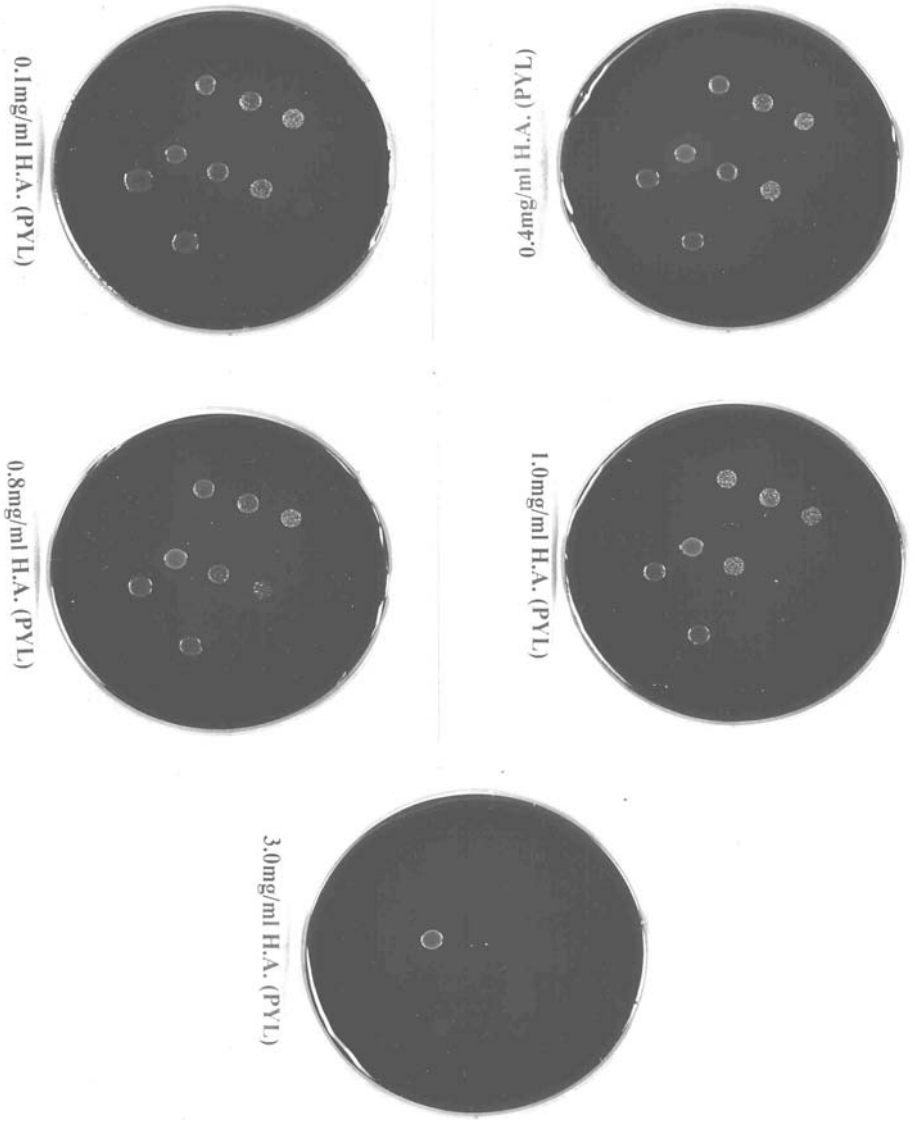


Figure A-20: Various *M. elsdenii* strains were grown on THBAG plates containing 0.5 - 5.0 mg/ml acetic acid (A. Acid) to determine the minimum inhibitory concentration (MIC). MIC was determined using the replica plate technique. The details of each strain are described in the key shown in appendix-2 Figure A-1.

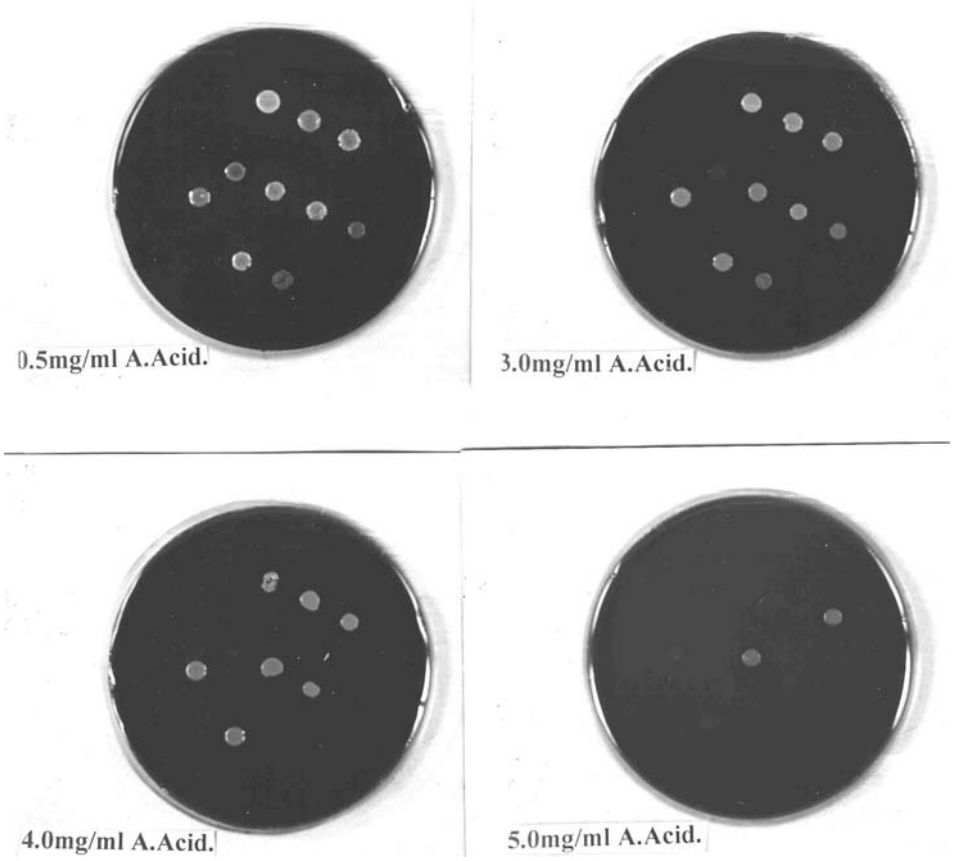


Figure A-21: Various *M. elsdenii* strains were grown on THBAL plates containing 0.5 – 5.0 mg/ml acetic acid (A.A.) to determine the minimum inhibitory concentration (MIC). MIC was determined using the replica plate technique. The details of each strain are described in the key shown in appendix-2 Figure A-1.

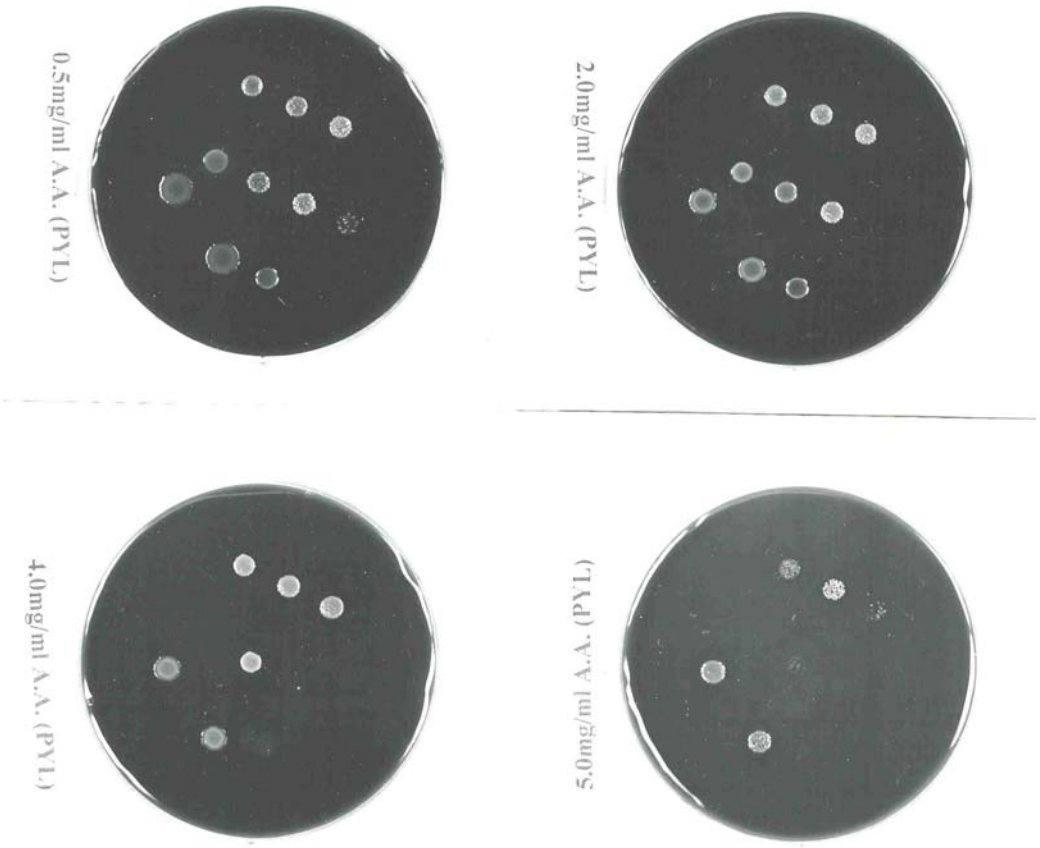


Figure A-22: Various *M. elsdenii* strains were grown on THBAG plates containing 0.1 – 3.0 mg/ml butyric acid (B.A.) to determine the minimum inhibitory concentration (MIC). MIC was determined using the replica plate technique. The details of each strain are described in the key shown in appendix-2 Figure A-1.

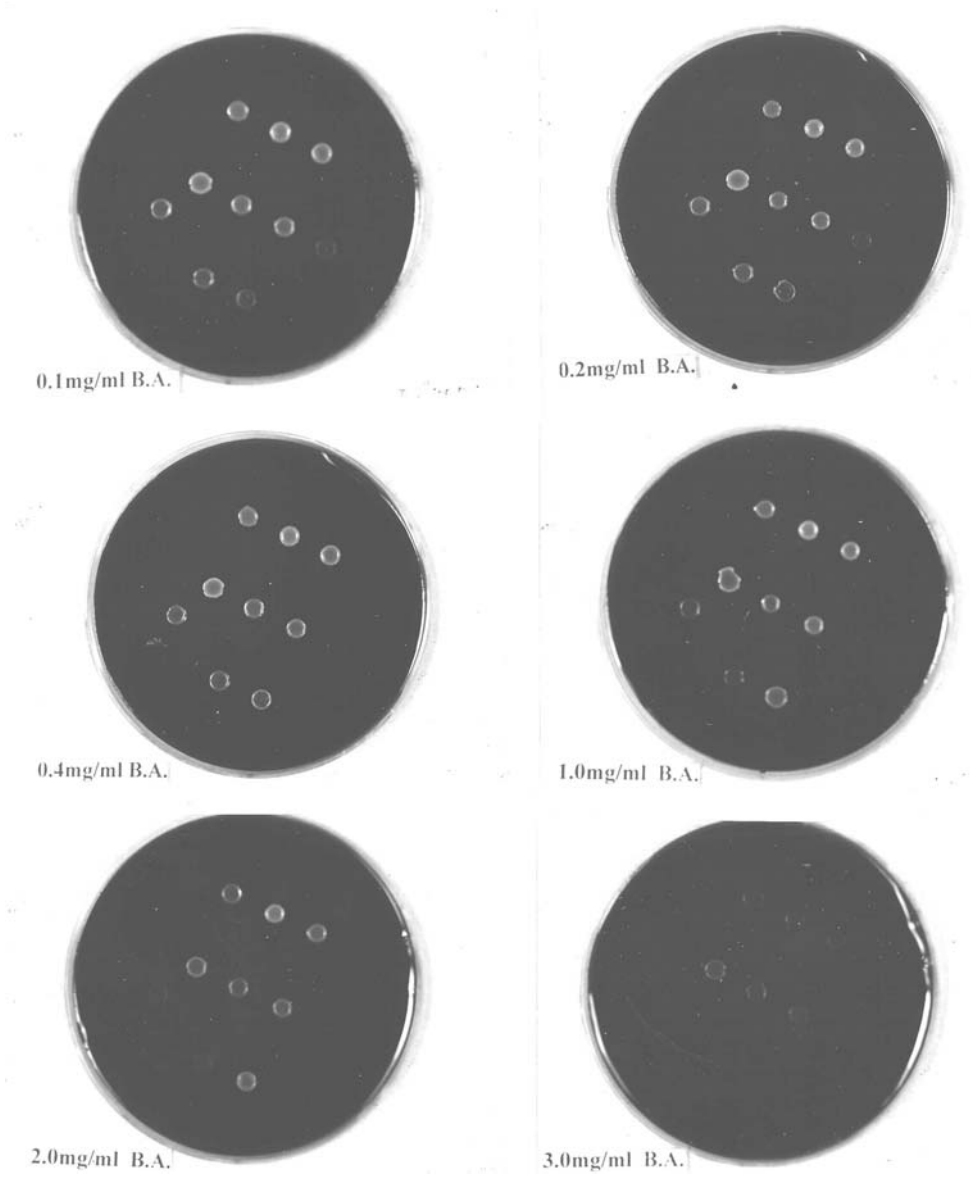


Figure A-23: Various *M. elsdenii* strains were grown on THBAL plates containing 0.1 – 5.0 mg/ml butyric acid (B.A.) to determine the minimum inhibitory concentration (MIC). MIC was determined using the replica plate technique. The details of each strain are described in the key shown in appendix-2 Figure A-1.

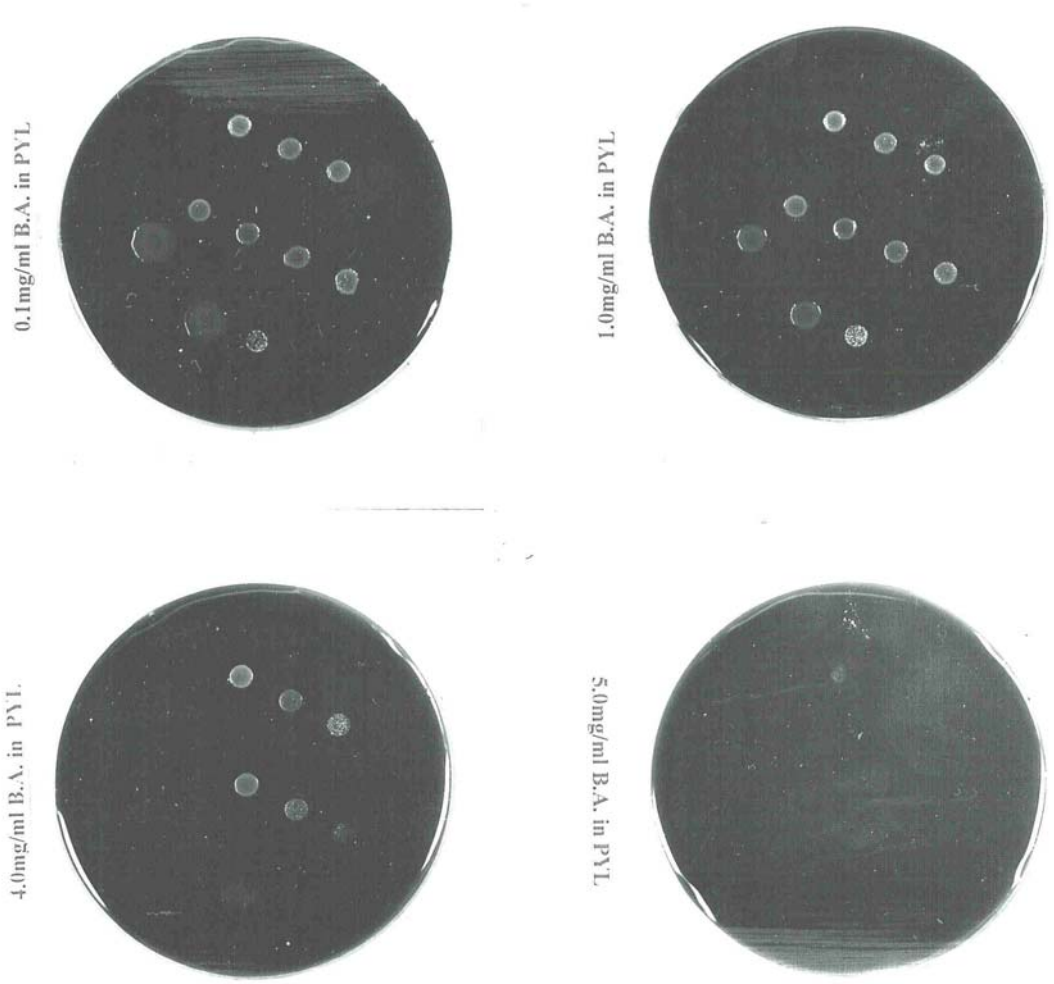
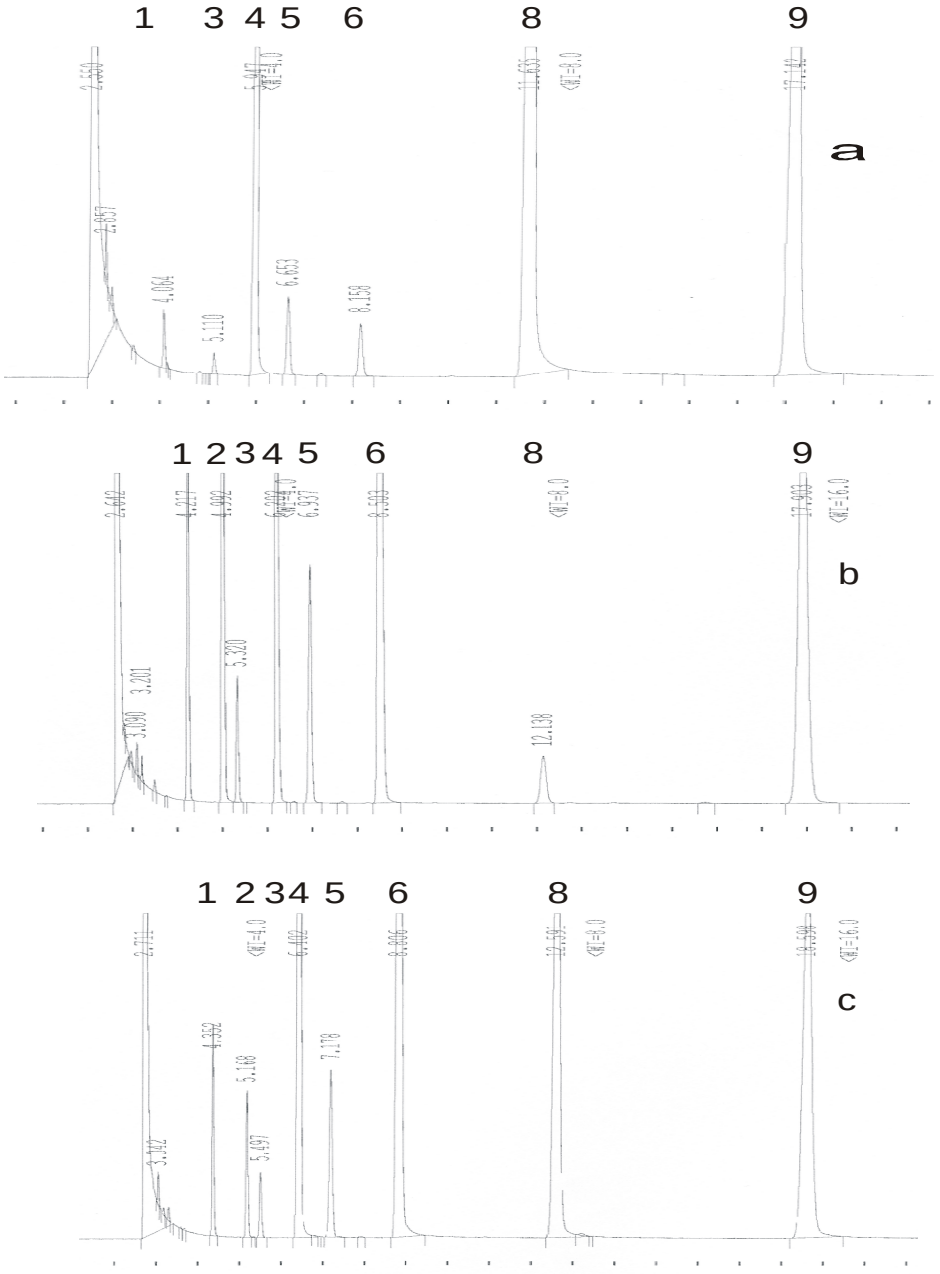
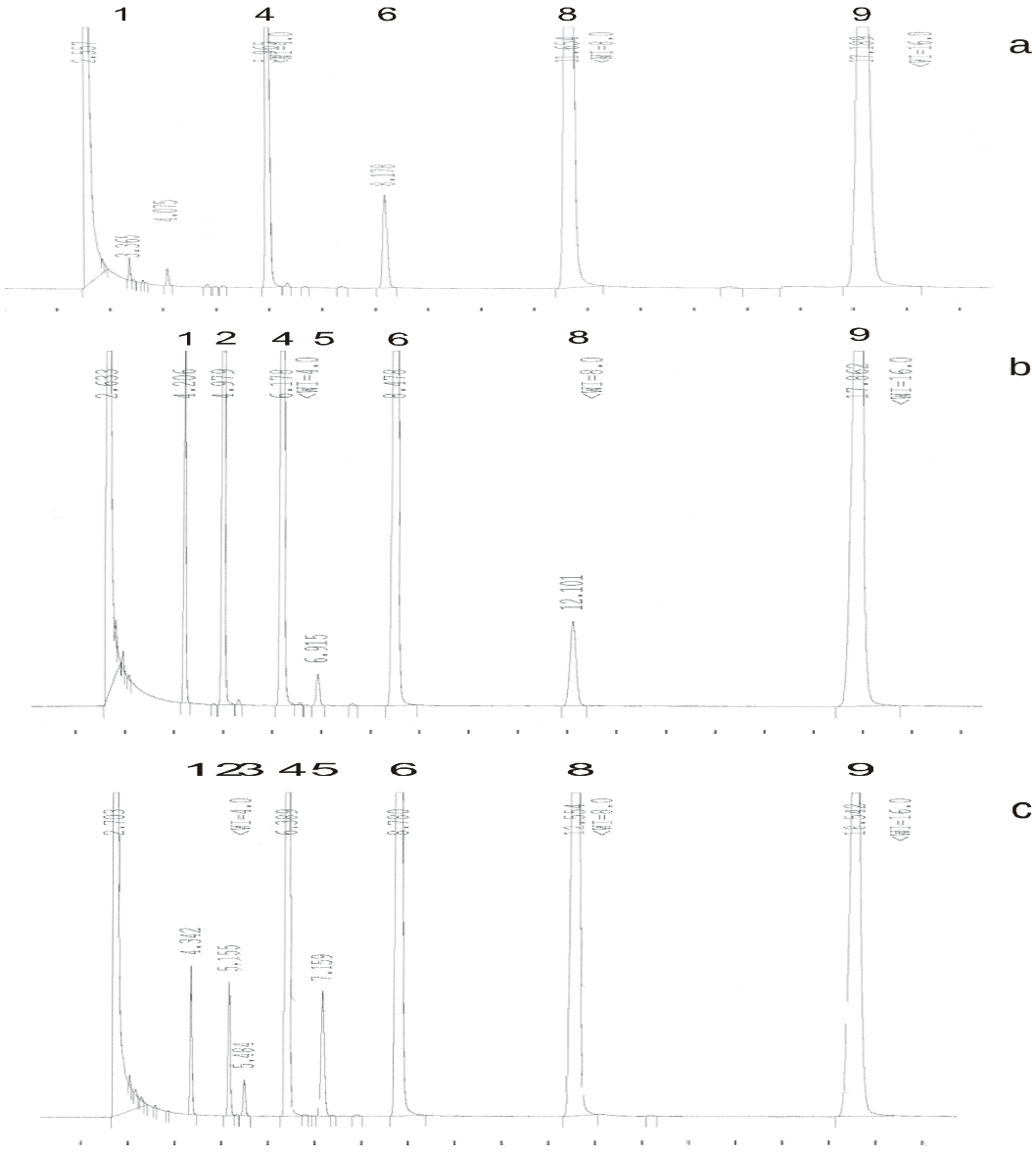


Figure A-24: Example of gas chromatogram of volatile fatty acids following growth of *M. elsdenii* parental strain ATCC 25940 in peptone-yeast medium plus a) glucose b) lactate c) glucose plus lactate after extraction in diethyl ether solution as described in section 2.4.2. Peaks scheme for following chromatograms are:



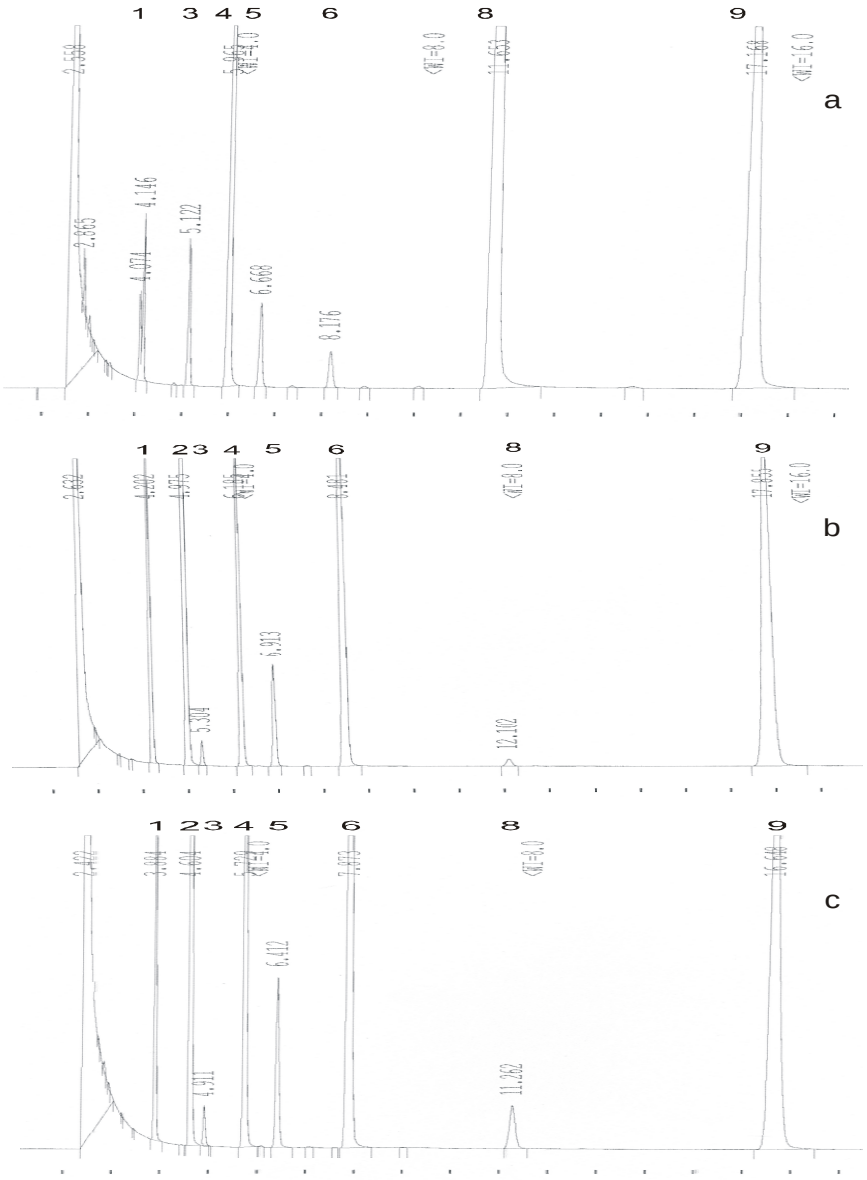
Peak-1:	Acetic acid	Peak-2:	Propionic acid
Peak-3:	Iso-butyric acid	Peak-4:	Butyric acid
Peak-5:	Iso-valeric acid	Peak-6:	Valeric acid
Peak-8:	Hexanoic acid	Peak-9:	Heptanoic acid

Figure A-25: Example of gas chromatogram of volatile fatty acids following growth of *M. elsdenii* strain ME5 in peptone-yeast medium plus a) glucose b) lactate c) glucose plus lactate after extraction in diethyl ether solution as described in section 2.4.2. Peaks scheme for following chromatograms are:



Peak-1:	Acetic acid	Peak-2:	Propionic acid
Peak-3:	Iso-butyric acid	Peak-4:	Butyric acid
Peak-5:	Iso-valeric acid	Peak-6:	Valeric acid
Peak-8:	Hexanoic acid	Peak-9:	Heptanoic acid

Figure A-26: Example of gas chromatogram of volatile fatty acids following growth of *M. elsdenii* strain ME7 in peptone-yeast medium plus a) glucose b) lactate c) glucose plus lactate after extraction in diethyl ether solution as described in section 2.4.2. Peaks scheme for following chromatograms are:



Peak-1:	Acetic acid	Peak-2:	Propionic acid
Peak-3:	Iso-butyric acid	Peak-4:	Butyric acid
Peak-5:	Iso-valeric acid	Peak-6:	Valeric acid
Peak-8:	Hexanoic acid	Peak-9:	Heptanoic acid

Figure A-27: Time course for the end-products detected in peptone-yeast plus a) glucose b) lactate c) glucose plus lactate medium following growth of *M. elsdenii* strain ATCC 25940 during 144 h in one litre volume Applicon fermenters with stirring at automated controlled pH 6.5. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.

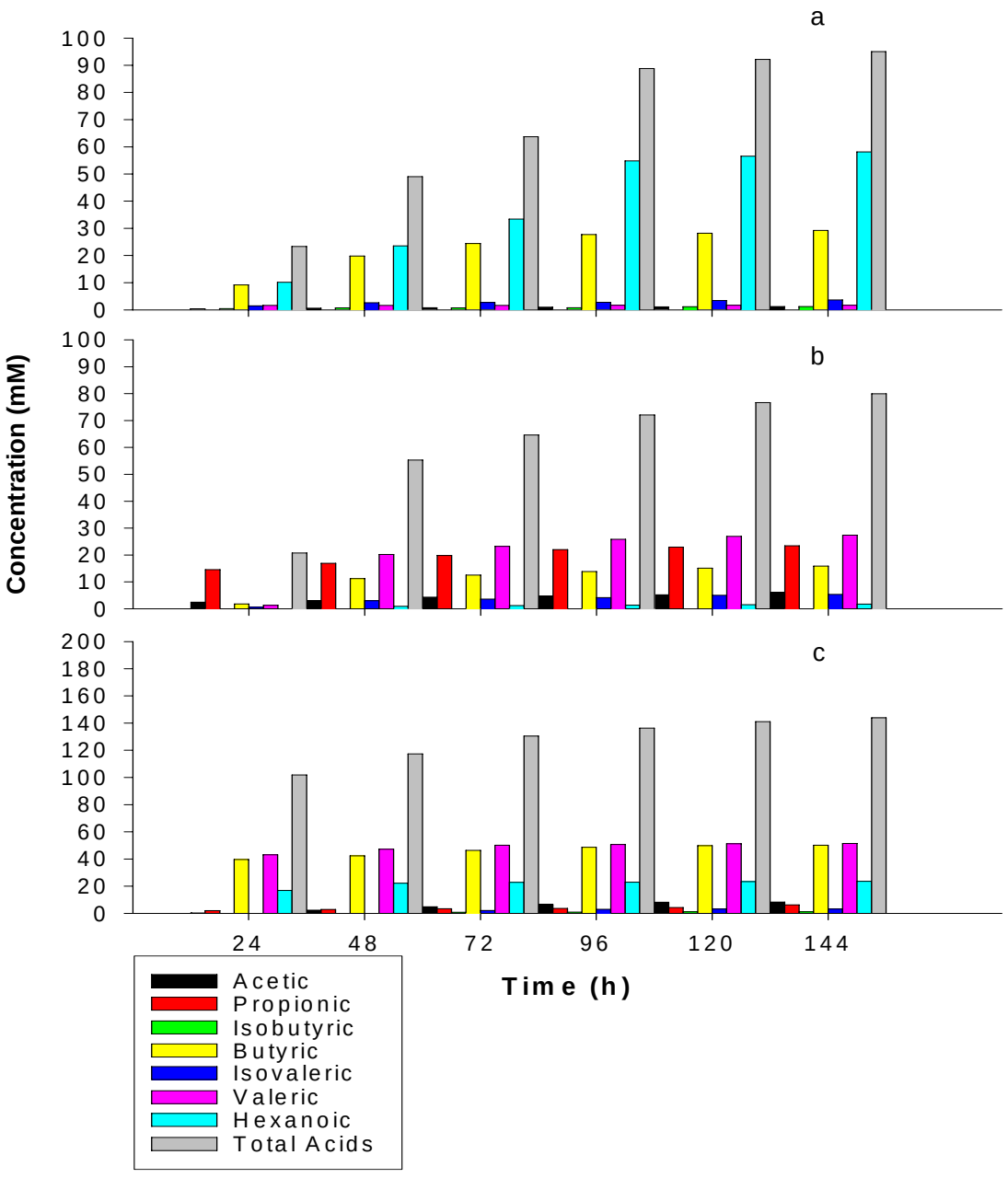


Figure A-28: Time course for the end-products detected in peptone-yeast plus a) glucose b) lactate c) glucose plus lactate medium following growth of *M. elsdenii* strain ME5 during 144 h in one litre volume Applicon fermenters with stirring at automated controlled pH 6.5. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.

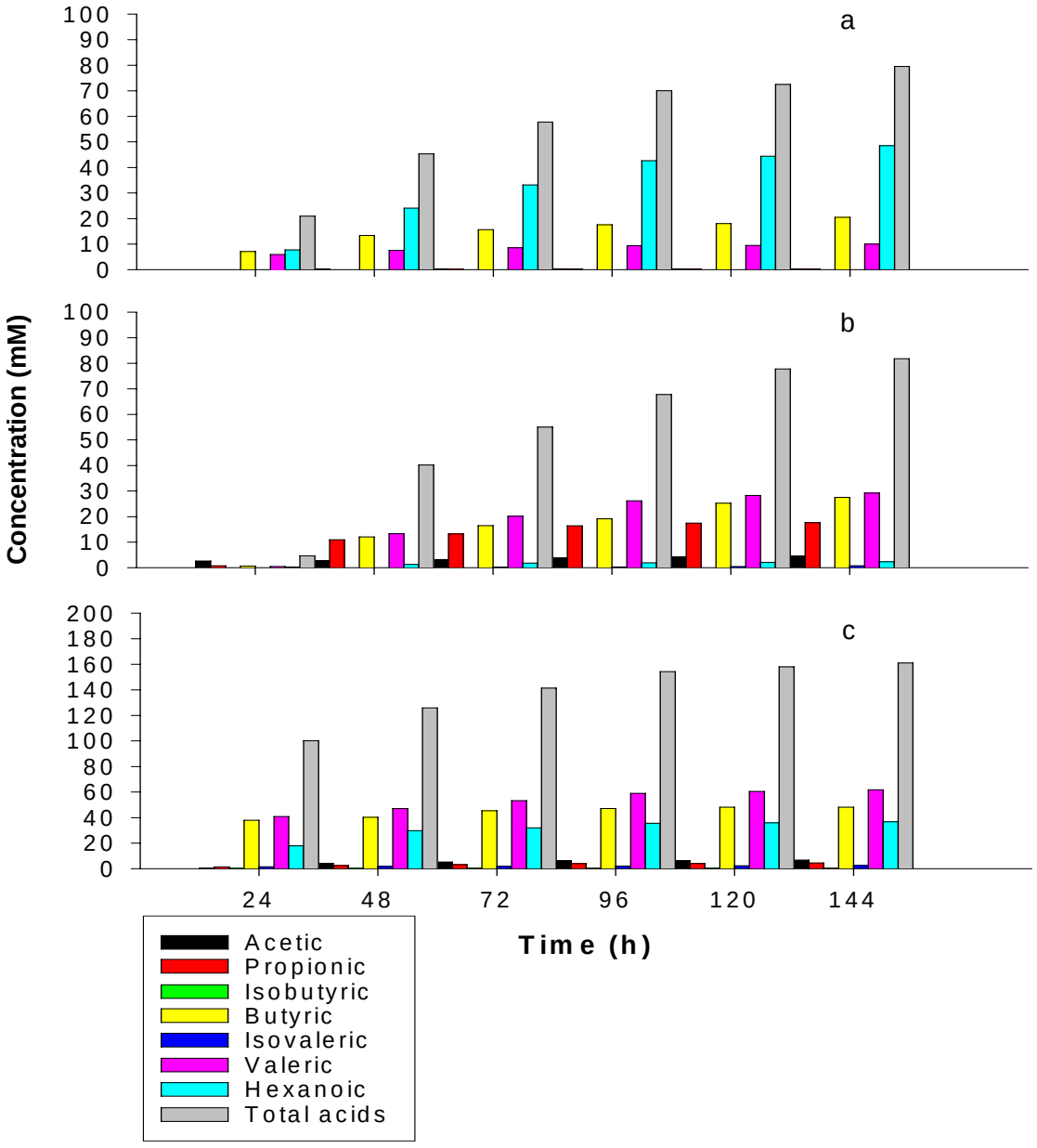


Figure A-29: Time course for the end-products detected in peptone-yeast plus a) glucose b) lactate c) glucose plus lactate medium following growth of *M. elsdenii* strain ME7 during 144 h in one litre volume Applicon fermenters with stirring at automated controlled pH 6.5. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.

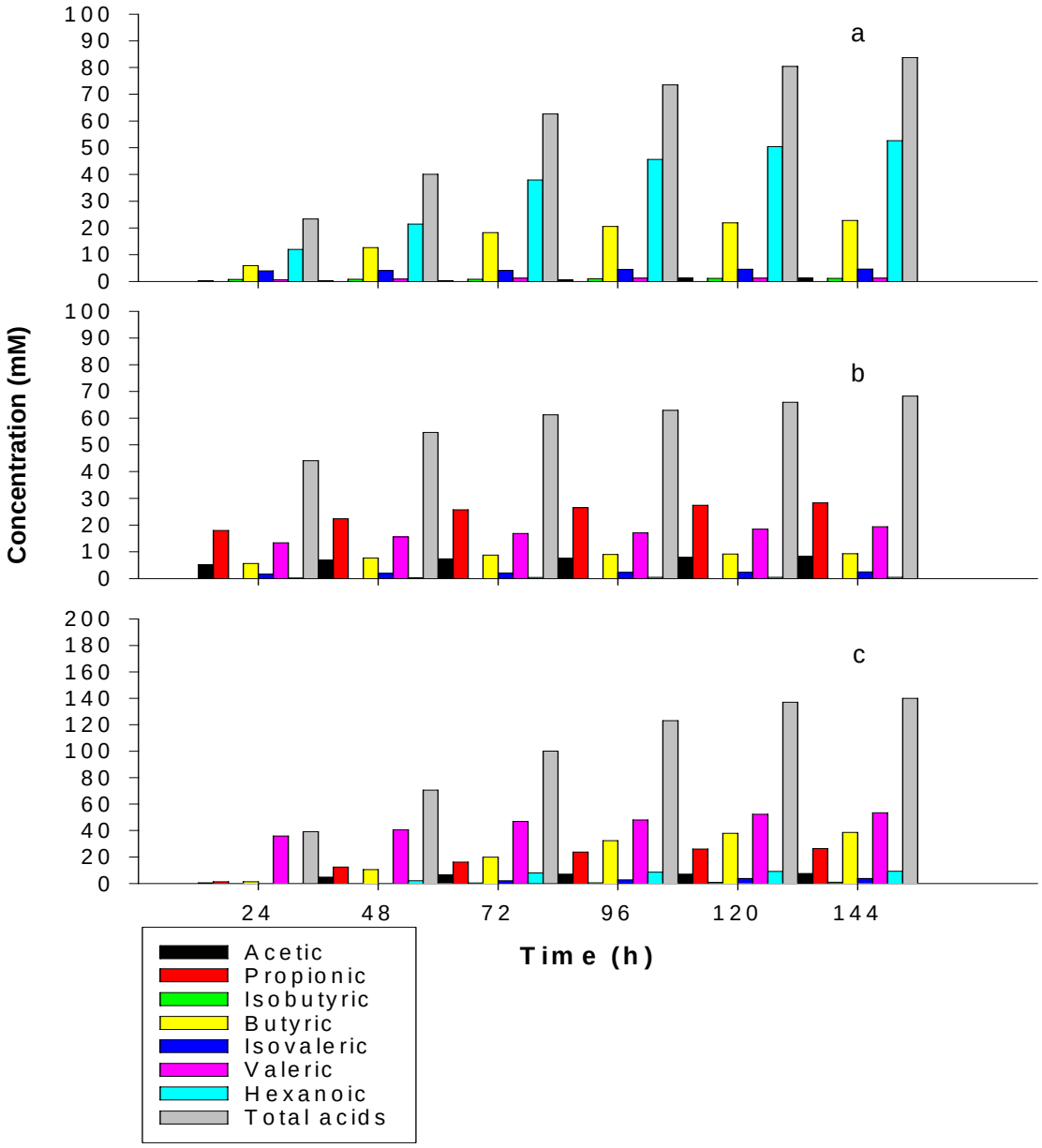
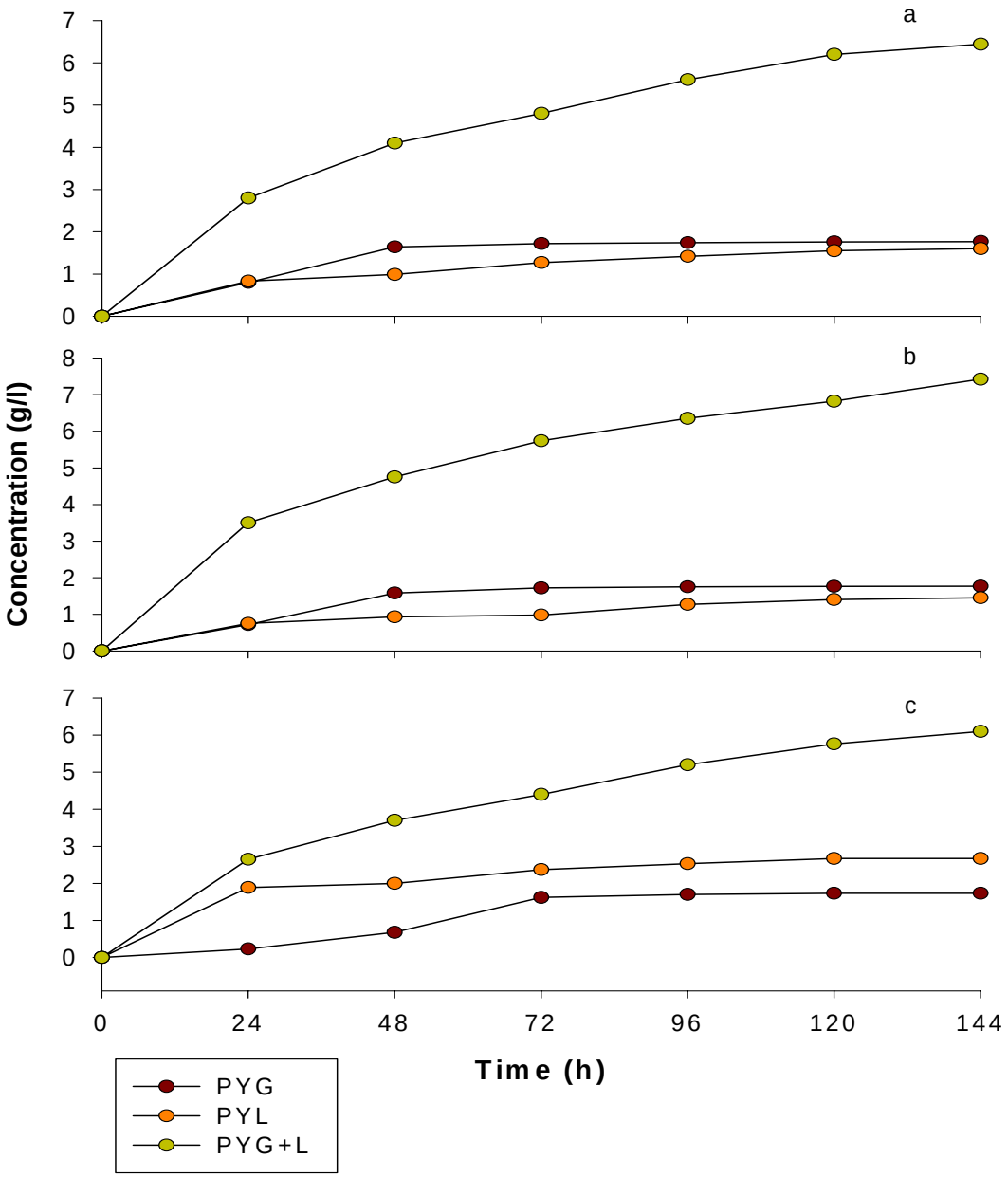


Figure A-30: Time course for protein concentration estimated in peptone-yeast-glucose medium following growth of *M. elsdenii* strains a) ATCC 25940 b) ME5 and c) ME7 during 144 h in one litre volume Applicon fermenters with stirring at automated controlled pH 6.5. Figures represent the average and standard deviation of triplicate experiments, where the standard deviation was less than 5%.



APPENDIX–3: Formulas for the fermentation experiments

From the results of analysis of VFAs and desorption of VFAs by esterification, the effective concentrations of *n*-hexanoic acid (C^e_{HEX}) and of *n*-butyric acid (C^e_{BUTY}) were determined using the following equations:

$C^e_{HEX} = C^b_{HEX} + A^r_{HEX}/V_b$, (mM)

$C^e_{BUTY} = C^b_{BUTY} + A^r_{BUTY}/V_b$, (mM)

Where A^r_{HEX} and A^r_{BUTY} represent the amount of *n*-hexanoic acid and *n*-butyric acid adsorbed to the resin (mmole) respectively; they were obtained from the esterification results assuming that all the VFAs adsorbed to the resin were desorbed as either their esters or acids. C^b_{HEX} and C^b_{BUTY} represent the concentration of *n*-hexanoic and *n*-butyric acid in the broth, V_b represents the volume of the broth.

The yield of *n*-hexanoic acid ($Y_{p/s}$) and productivity of *n*-hexanoic acid (P_{HEX}), were determined from the effective concentration of *n*-hexanoic acid, glucose consumption, and biomass concentration as follows.

$Y_{p/s} = C^e_{HEX} . M^r_{HEX} / (1000. S)$, (g/g)

$P_{HEX} = C^e_{HEX} . M^r_{HEX} / (1000. T_h)$, (g/l.h)

Where

V_b = broth volume (l)

M^r_{HEX} = molecular weight of *n*-hexanoic acid (116)

M^r_{BUTY} = molecular weight of *n*-butyric acid (88)

S= glucose consumption (g/l)

T_h = harvest time (h)

APPENDIX–4: Comparison of attempted LDH gene sequence from *M. elsdenii* strain ME5 with *P. acidilactici* sequence using gene pileup program

LDH pileup.msf MSF: 799 Type: N May 25, 19104 01:40 Check: 6816 ..

Name: *ME5* Len: 816 Check: 1028 Weight: 1.00
Name: *P. acidilactici* Len: 816 Check: 5788 Weight: 1.00

//

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                1                                                  50
ME5             TGCTCCCGCC GCCATGGCGG CCGCGGGAAT TCGATTGGTG ACGGTGCCGT
P.acidilactici  . . .. ... . .. . . ... . . . . . .. . . . . .. . . . . GGTG ACGGTGCCGT

               51                                                 100
ME5             AGGTTCTTCT GAC . CATTAT  GTCTGGGATG GATCGAAAAA TGTCCAC TAT
P.acidilactici  AGGTTC TAGT TACGCATTCG CGATGGCACA ACAAGGAATC GCTGAAGAAT

              101                                                 150
ME5             GCTCAGCAGA CCCGCCAGAT TACCAATGTA GCCGCCGGGA AAGAAGATAC
P.acidilactici  TCGTCAT TGT CGACGT TGTT AAGGATCGTA CAGTTGGGGA CGCATTGGAC

              151                                                 200
ME5             . . . GGATGCC GTCAACGTGG CCCAGCTGAA ACAGGTGGTT AATCTGGTCA
P.acidilactici  CTTGAAGATG CTACTCCA T T CACAGCTCCA AAGAACAT CT ACTCTGGTGA

              201                                                 250
ME5             ACAATGGCGG GGGAAG . . .  . . . . CGGAAC CGGCGGCAGC GGTGTCCACG
P.acidilactici  AT ACTCAGAC  TGCAAG GATG CTGACTTA GT TGT TAT CACA GCTGGCGCAC

              251                                                 300
ME5             ATTACAGCGT AAACTCTGTA GATTCGACCA CCGATAGCAA CTACAACAAT
P.acidilactici  CACAAAAGCC AGGTGAAACA CGTCTTGACC TTGTTAACAA GAACTTAAAC

              301                                                 350
ME5             GCCGGTGCCA CCGGGAGTAA CGCCCTGGCT GCGGGGGTCA GTGCCTCGGC
P.acidilactici  ATCC TTT CAA CAA TTG TTAA ACCAG TTGTT GA TTCTGG TT  TTG ATGGTAT

              351                                                 400
ME5             AACGGGCGAA AATGCCGTAG CTATCGGTAC CGGAGCCAAA GCGGATGGTG
P.acidilactici  CTTCC TTG TT GCTGCTAACC CAGTTGATAT CC TTAC T TAC GCAACATGGA
```

401 450

ME5 TAGGCGCTAC GGTCATCGGC CAGTATGGCA CAGCTTCCGG . . . .TCGCTA

P.acidilactici AATTC TCTGG CT TCCCTAAG GAAAAAGT TA TCGGTTCAGG TATCTCACTT

451 500

ME5 TGCGTTGGCC TTTGGAGGCC AACAGACGGA CAATACGGCT CCAGCAGCCA

P.acidilactici GACACAGCTC GTTTGCGCGT AGCTC TTGGT AAGAAATTCA ACG TTAGCCC

501 550

ME5 ACAATCAAGC CATCGGAGAT AATTCAGTGG CCTTCGGGGA ACGGAGTAAG

P.acidilactici AGAATC TGTA GATGCTTACA TCTTAGGTGA ACATGGTGAC AGTGAAT TTG

551 600

ME5 GCCAGCGGCA GCAATTCCAC GGCCTTCGGG CAGGGAACCC AGGCCACGGA

P.acidilactici . . . . CTG C TT TCTCATCAGC TACAATC GG . . TAC AAAGCC AT TGC TTGAA

601 650

ME5 AAAGCGGGCC ACGGCTTTTG GCCAGCGGAC CCAGGCGACC CAATCCAA TG

P.acidilactici A TCGCTAAAG AAGAANGCGT TTCAACT GAC GAAT TGG CTG AAATCGAA . .

651 700

ME5 CCACGGCTTT TGGTATCGAT ACTNTNGGCT TTCGGGCAAG AAACC TTACN

P.acidilactici . GACAGCGTA CGTAACAAAG CTTATGAAAT CATC AACAAG AAAGGGTGC .

701 750

ME5 GGCACCGTTC ACCAATCAAC TNAGGTGGAA AATTCGCGGG GCCCGCCTTG

P.acidilactici . . . . . TAC ATCTACGGTA ATCACT AGTG AATTCGCGG . . . . . .

751 799

ME5 CAANGNTCGA ACCCATTTTT GGGGAAAAAG CTTCCCAAAA CGCCGTTTT

P.acidilactici . . . . . .