

**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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Khan, M.A. and Britz, M.L. Comparison of volatile fatty acids produced by *Megasphaera elsdenii* strains on peptone-yeast medium with glucose, lactate and glucose plus lactate as substrates in batch fermentations with and without pH controlled conditions.

Khan, M.A. and Britz, M.L. Studies on enzymes and sequencing of their respective genes involved in metabolic pathways of *Megasphaera elsdenii* strains following the growth on peptone-yeast-glucose medium.

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	<i>n</i> -butyric acid
$C_{\text{BUTY}}^{\text{b}}$	concentration of <i>n</i> -butyric acid in solution (broth) phase (includes both dissociated and non-dissociated forms), (mM)
$C_{\text{HEX}}^{\text{b}}$	concentration of <i>n</i> -hexanoic acid in solution (broth) phase (includes both dissociated and non-dissociated forms), (mM)
$C_{\text{BUTY}}^{\text{e}}$	effective concentration of <i>n</i> -butyric acid, (mM)
$C_{\text{HEX}}^{\text{e}}$	effective concentration of <i>n</i> -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	<i>n</i> -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	<i>E. coli</i> 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 <i>n</i> -butyric acid, (g/l.h)
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
P_{HEX}	productivity of <i>n</i> -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 <i>n</i> -hexanoic acid from glucose consumed (g <i>n</i> -hexanoic acid/g glucose)

Measurements

bp	base pairs
cm	centimetre
Ci	Curie
$^{\circ}\text{C}$	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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