AN INVESTIGATION OF THE ISOLATION, CHARACTERISATION AND APPLICATION OF HYDANTOINASES FOR THE INDUSTRIAL PRODUCTION OF AMINO ACIDS

THESIS

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Table of Contents

Abstract

This thesis describes a series of investigations into the hydantoin-hydrolysing activity of bacterial strains RU-KM1 and RU-OR, which were previously isolated for their ability to hydrolyse hydantoins to amino acids. The main aim of the study was to develop biotransformations with potential application in the production of enantiomerically pure amino acids using a bioreactor based system utilising the hydantoin hydrolysing enzymes of the two isolated microorganisms. Different substituted hydantoins may be used as substrates by these enzymes for the production of a variety of amino acids. These are not only important for amino acid production, but they may be used for production of other industrially important compounds, such as semisynthetic penicillin/ampicillin, L-aspartame (sweetener), Fluvalinate (insecticide), Enalapril (ACE inhibitor). Thus, the ability of the above-mentioned strains to hydrolyse these substrates was investigated, with the view to utilizing the maximum potential of these biocatalysts.

Hydantoin conversion involves a two-step hydrolysis reaction which yields, initially, an *N*-carbamylamino acid intermediate, and subsequently, an amino acid. The hydantoin-hydrolysing enzymes of a *Pseudomonas* sp. RU-KM1, and an *Agrobacterium* sp. RU-OR were characterised as whole cells and in a crude extract preparation, and reaction conditions for its biocatalytic application were optimised. The optimum conditions for conversion of hydantoin to glycine were found to be 1 hour at 40 °C, with conversion yields greater than 30 % achieved. The enzymes of RU-KM1 demonstrated considerable stability, retaining 80 % of their activity after storage for 2 weeks at 4 °C.

The activities of the enzymes were increased by the addition of a detergent to the extraction medium, suggesting that the enzymes might be membrane-bound. The results of the determination of the metal-dependence of the hydantoinase and *N*-carbamoylase of RU-KM1 suggested that these enzymes required metal ions for activity, with metal ions such as Cu^{2+}, Fe^{2+}, and Co^{2+} resulting in no significant change in enzyme activity, however there was an activation of the enzymes when Mn^{2+} was added to the enzymes. The stereoselectivity of the enzymes was investigated, and the results suggested that the hydantoinase was D-selective, whereas the *N*-carbamoylase was shown to be L-selective by other researchers.

The hydantoin substrate selectivity of RU-KM1 and RU-OR was investigated, and the organisms were shown to be able to hydrolyse all of the seven substrates tested. However, there was a difference in activity levels between crude extract preparations and whole cells, with crude extracts generally showing slightly lower activity than whole cells in RU-KM1, and the whole cells or RU-OR showing the lower activity than its crude extract. Some difference was also observed in the order of preference of substrates between whole cells and crude extracts. The preferred substrate for RU-KM1 whole cells was isopropylhydantoin, whereas the crude extract preparation preferentially hydrolysed *p*-hydroxyphenylhydantoin, achieving 57 % and 52 % conversions respectively. RU-OR whole cells preferred methylhydantoin where as the crude extract preferred isopropylhydantoin, and showed 49 % and 51 % conversions respectively.

The enzymes were characterised in terms of their temperature and pH optima, inducer requirements, and product inhibition studies. The hydantoinase of RU-KM1 was shown to be inducible with low levels of hydantoin, and thermostable upto 75 °C with its optima between 60 and 70 °C. The *N*-carbamoylase was shown to have its optima at 50 °C. The addition of ATP (0.5 mM), DTT (1 mM) and a protease inhibitor (2 $mg.mL^{-1}$) all increased the hydantoinase activity of RU-KM1 crude extract, however they had very little effect on the *N*-carbamoylase activity.

The hydantoinase enzyme from extracts of RU-KM1 was partially purified by development of cell disruption methods using mechanical and lysing enzymes, followed by precipitation and chromatographic resolution. The results obtained showed a hydantoinase enzyme of between 48 and 66 kDa.

RU-KM1 was grown under fermentation conditions using different minimal media. The activity and yields under these conditions were low. Previous attempt to grow the organism in a rich medium had resulted in an increase in biomass but no hydantoinase activity. A rich medium was developed by carbon and nitrogen optimisation and yielded biomass up to 30 $g.L^{-1}$ dry cell weight. The hydantoinase activity was restored by nitrogen starvation in stationary phase. This resulted in high biomass with increased activity. This data is currently in press.

Crude extract and whole cells were immobilised on flat sheet membranes, hollow fibre membranes and in alginate beads. Low hydantoinase activity was measured in bioreactors using membranes in different configurations. A significant increase in hydantoinase activity was measured when the crude extract was immobilised in sodium alginate, as a result of stabilisation of the *N*-carbamoylase. Temperature and pH optima were unaffected by the immobilisation procedure, however the durability of the enzymes increased 2-fold. Different configurations of the bioreactor were investigated, as well as a hydroxyphenylhydantoin as an alternative substrate in this study. The bioreactors showed a near 95 % conversion of the hydantoin to glycine, and a 99 % conversion using HPG.

In conclusion, the hydantoin-hydrolysing enzymes of RU-KM1 have been shown to be possibly membrane associated, which is a novel finding. This study has shown that the hydantoinase of RU-KM1 is D-stereoselective, with high temperature stability. A growth medium was developed for the rapid production of active biomass. A bioreactor was developed using a single and a dual biocatalyst configuration, which was capable of hydrolysing hydantoin and monosubstituted hydantoins to produce amino acids. To our knowledge this system is the first such dual biocatalyst system reported for the production of amino acids.

List of Figures

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

Chapter 7:

List of Tables

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

Chapter 7:

List of Schemes

Chapter 1:

Chapter 4:

List of Plates

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Abbreviations

ACE	acetylcholine esterase
ATP	adenosine triphosphate
CE	capillary electrophoresis
CER	carbon evolution rate
CMP	chiral mobile phase
CSP	chiral stationary phase
CTAB	cetyltrimethyl-ammonium bromide
CHAPS	3-chloroaminopropyldimethyl-amino-1-propane sulfonate
DCW	dry cell weight
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DSP	downstream processing
EB	elution buffer
EDTA	ethylenediaminetetraacetic acid
EPS	extracellular polysaccharide
ELISA	enzyme linked immunosorbant assay
GC	gas chromatography
HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
p-HPG	*p*-hydroxyphenyl hydantoin
MM (H)	minimal media (hydantoin)
NCG	*N*-carbamyl glycine
OUR	oxygen utilisation rate
^{1H}NMR	proton nuclear magnetic resonance
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
POE-W1	polyoxyethylene ether-W1
RNA	ribonucleic acid

rRNA	ribosomal RNA
TLC	thin layer chromatography
TFHFMB	Transverse Flow Hollow Fibre Membrane Bioreactor
UFBB	up-flow fluidised-bed bead bioreactor
vvm	volume per volume per minute (aeration)

Chapter 1

1. Introduction

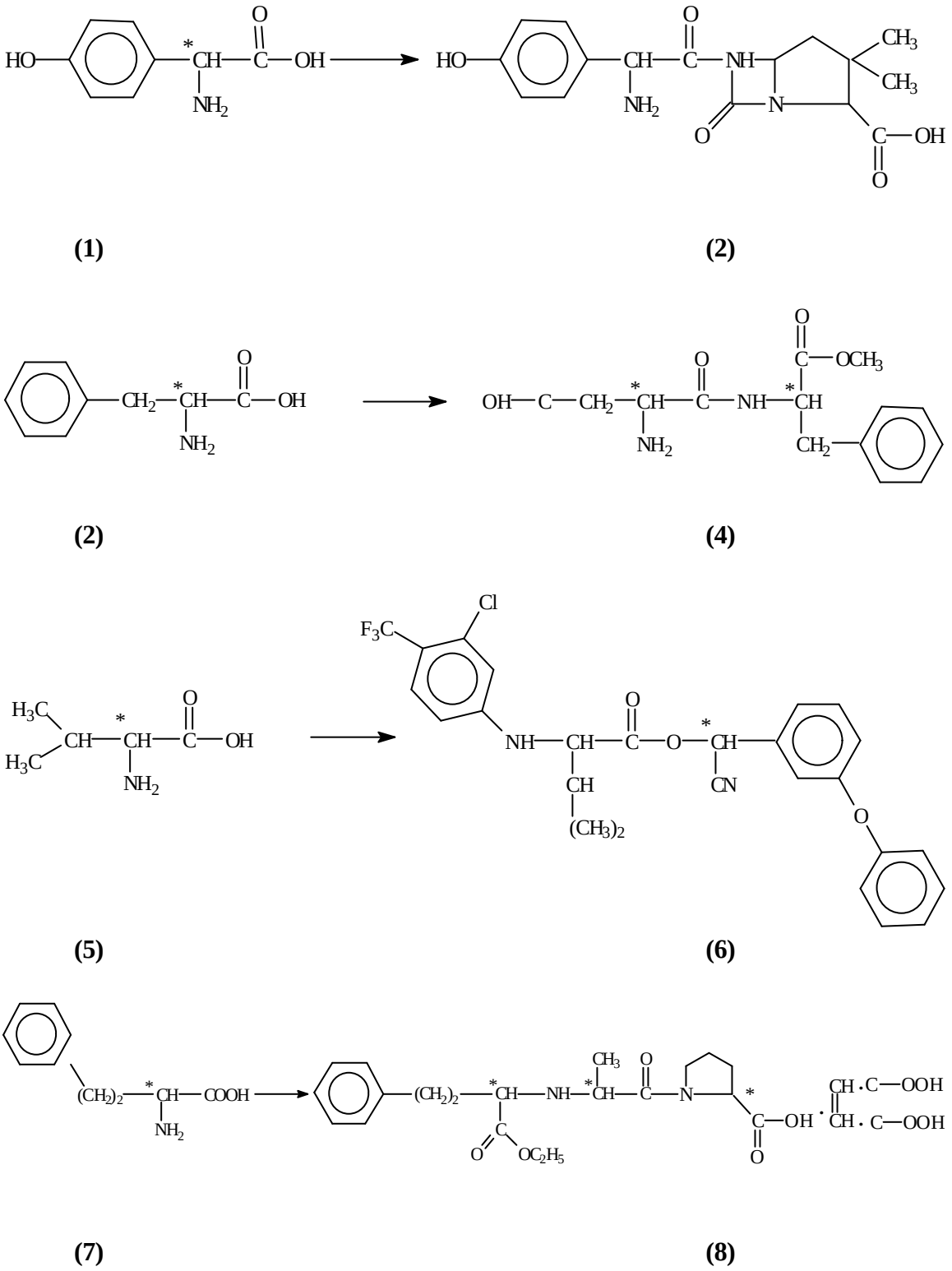
1.1 General background

This thesis describes an investigation into the hydantoin-hydrolysing activity of a locally isolated bacterial strain, RU-KM1. The focus of this research was to isolate, characterise and purify an hydantoinase enzyme from this organism for use in the development of a biocatalytic process to produce enantiomerically pure amino acids.

Over the last 10 to 15 years, amino acids have attracted wide interest in the pharmaceutical industry, largely towards the large-scale synthesis of optically pure D- or L-α-amino acids (Boesten *et al.*, 1986; Syldatk *et al.*, 1990a; Hermes *et al.*, 1993). The reason for this interest arises from the numerous applications of amino acids, including:

- additives in food and feedstocks; (Syldatk *et al.*, 1990a; Ryu *et al.*, 1997; Wagner *et al.*, 1996),
- intermediates in semi-synthetic antibiotic synthesis (Syldatk *et al.*, 1990a, 1998; Kim and Kim, 1993; Lee *et al.*, 1994; Burton *et al.*, 1998; Hartley *et al.*, 1998; Ikenaka *et al.*, 1998b; Ogawa and Shimizu, 1999),
- starting materials for peptide and peptide hormones synthesis (Kim and Kim, 1993; Gokhale *et al.*, 1996; Grifantini *et al.*, 1996; Drauz, 1997; Lee and Kim, 1998),
- building blocks in the synthesis of pesticides (Kim *et al.*, 1994; Grifantini *et al.*, 1996; Ikenaka *et al.*, 1998b; Sudge *et al.*, 1998; May *et al.*, 1998d),
- starting materials in the synthesis of acetylcholine esterase (ACE) inhibitors, such as enalapril (Keil *et al.*, 1995; Chien and Hsu, 1996; Tsuji *et al.*, 1997).

Examples are D-phenylglycine and D-*p*-hydroxyphenylglycine, which are precursors for semisynthetic antibiotics (Hermes *et al.*, 1993 and 1994; van den Tweel, 1993); L-valine, for the production of Cyclosporin A (Hermes *et al.*, 1993); isovaline, a natural component of several antibiotics (Hermes *et al.*, 1994); and D-valine, used as an intermediate in the production of Fluvalinate, an insecticide (Hermes *et al.*, 1993; van den Tweel, 1993). α-Alkyl-α-amino acids have also become valuable pharmaceutical compounds (van den Tweel, 1993). For example, L-α-methyl-3,4-dihydroxyphenylalanine is an important drug for the treatment of hypertension. Scheme 1.1 shows some examples of optically pure amino acids and the economically important products derived from them (Kamphius *et al.*, 1990).



Scheme 1.1: Structural relationships between some optically pure amino acids and products derived from them (adapted from Kamphuis *et al*., 1990)

(1) D-(*p*-hydroxy)phenylglycine, (2) semisynthetic penicillin/ampicillin, (3) L-phenylalanine, (4) L-aspartame (sweetener), (5) D-valine, (6) Fluvalinate (insecticide), (7) L-homophenylalanine, (8) Enalapril (ACE inhibitor)

1.2 Methods available for amino acid synthesis

1.2.1 Biocatalytic vs chemical synthesis

There is a growing demand for biologically active chemicals such as amino acids and their derivatives, which are specific in their mode of action, whose products have no toxic side effects, and whose production is environmentally benign (Kamphuis *et al*., 1990). Many of these products can be produced with biocatalysts, and use of microorganisms as a source of the enzyme biocatalysts holds definite advantages. They are readily produced in large fermentations, and in high yields. Furthermore, from a single fermentation, a variety of enzymes may be produced, that are capable of catalysing many reactions.

Several methods have been reported in the literature for the production of optically active amino acids, including chemical or enzymatic processes, or a combination of the two (Volkel and Wagner, 1995; Louwrier and Knowles, 1996). Four processes are commonly used:

- chemical extraction of the amino acids from plant or animal hydrolysates
- chemical conversion of cheap substrates such as sugars and/or molasses
- chemical synthesis using aminonitriles as intermediates
- enzymatic catalysis using stereoselective biocatalysts to convert amino acid amides (Morin *et al.*, 1990).

Biocatalysts are able to perform most known chemical reactions, under mild conditions of temperature, pH and pressure. In addition, regio-, enantioselectivity and substrate specificity of biocatalysts are high, and these characteristics can be exploited in developing efficient biotransformations (Polastro, 1989). Enzymes are considered to be superior to chemical catalysts, as they possess high specificity allowing the production of

a pure product, if the reactants are contaminant free (Tramper, 1985). This useful characteristic removes the necessity for a chemical resolution step after synthesis. Thus, biocatalytic processes utilising hydrolysing enzymes for the production of enantiomerically pure amino acids are often preferred to chemical and/or chemoenzymatic methods.

The two enantiomers of an amino acid are identical in chemical and most physical properties. However, in a chiral environment such as the human body, the enantiomers may act with different biological activities (Martens and Bhushan, 1989). For example, the treatment of Parkinson's disease requires L-DOPA (3,4-dihydroxyphenylalanine) whereas the D- form has no effect. Similarly the antibiotic D-penicillamine exhibits antimicrobial activity *in vivo*, whereas the L-isomer is toxic (Martens and Bhushan, 1989).

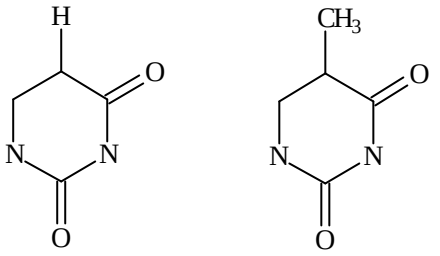
One route for the sythesis of amino acids for pharmaceutical applications is the hydrolysis of hydantoin. Numerous disadvantages are associated with the chemical hydrolysis of hydantoins, including the toxicity of the starting reactants, high temperatures associated with the reaction, poor mass yields of the products, and the large amount of energy required, which prohibits the development of large-scale production of amino acids using this method (Yamashiro *et al.*, 1989). Enzymatic hydrolysis of the hydantoin is regarded as a feasible option, offering the advantage that the reaction occurs under mild reaction conditions, which can result in a cheaper industrial process (Polastro, 1989; Hartley *et al.*, 1998).

1.2.2 Enzymatic production of amino acids from hydantoins

An enzymatic system for the production of amino acids from 5-substituted hydantoins that is both enzymatic and enantioselective has been reported (Olivieri *et al.*, 1979; Kim and Kim, 1993 and Ogawa *et al.*, 1994b). This two-step enzymatic process involves the hydrolysis of the hydantoin derivative by an hydantoinase enzyme, and the further hydrolysis of the intermediate *N*-carbamyl amino acid, by an *N*-carbamylamino acid amidohydrolase (*N*-carbamoylase) (Olivieri *et al.*, 1979; Möller *et al.*, 1988; Kim and Kim, 1993; Ogawa *et al.*, 1994b; Ikenaka *et al.*, 1998a). A number of microorganisms from various genera have been reported to produce both an hydantoinase and an *N*-carbamoylase. Examples include *Flavobacterium* (Nishida *et al.*, 1987), *Pseudomonas* (Chevalier *et al.*, 1989; Ishikawa *et al.*, 1993), *Arthrobacter* (Syldatk *et al.*, 1987), *Bacillus* (Yamashiro *et al.*, 1989), and *Agrobacterium* species (Hartley *et al.*, 1998).

2. Distribution and ecology of hydantoin-hydrolysing enzymes

Hydantoin hydrolysing activity is widely distributed through microbial, plant, and animal kingdoms (Bernheim and Bernheim, 1946; Yamada *et al.*, 1978; Syldatk *et al.*, 1990b; Chien *et al.*, 1998). Hydantoin is an inducible catabolic enzyme thought to be involved in the degradation of pyrimidine nucleotides (Syldatk *et al.*, 1990a; Siemann *et al.*, 1999), and it is suggested that the natural substrates for hydantoinase are 5,6-dihydrouracil and 5,6-dihydrothymine (Siemann *et al.*, 1999) (Scheme 1.2).



(i) (ii)

Scheme 1.2: Natural substrates of hydantoinase (i) 5,6-dihydrouracil and (ii) 5,6-dihydrothymine

Hydantoinase stereospecificity may be D-, L-, or non-selective (Siemann *et al.*, 1999), and thus *N*-carbamyl-D- and/or L-amino acids (NCAA) could be produced. The D-specific enzymes are the most widely distributed, having been isolated from several microbial, plant and animal sources (Syldatk *et al.*, 1990a).

In most microorganisms, hydantoinase is regarded as identical to dihydropyrimidinase. However, it has been shown that in some microorganisms the hydantoin-hydrolysing activity is distinct from pyrimidine hydrolysing activity. Runser and Ohleyer (1990) reported that the substrate dihydrouracil was poorly hydrolysed by the D-hydantoinase of *Agrobacterium* spp. Runser and Meyer (1993) also reported that when dihydrouracil was used as a substrate for resting cells of *Agrobacterium* IP I-671, the dihydropyrimidinase activity disappeared on heating, and the hydantoinase activity remained, suggesting that the hydantoinase and dihydropyrimidinase of this bacterium were different enzymes.

An *N*-carbamoylase is involved in the second step for the production of amino acids from hydantoins. The hydrolysis of the NCAA results in the corresponding amino acid (Sano *et al.*, 1977; Oliveri *et al.*, 1981; Chien *et al.*, 1996; Ikenaka *et al.*, 1998b). This enzyme is

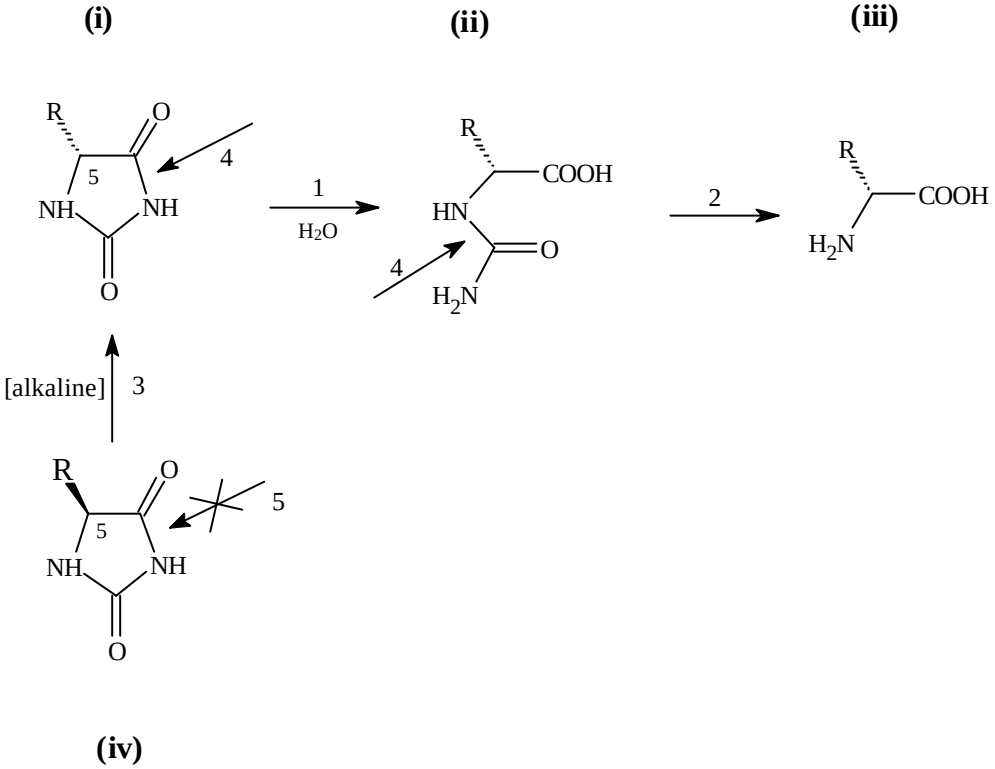
found to co-exist with an hydantoinase in some microorganisms, but it is also found in microorganisms that do not produce hydantoinase, such as *Comamonas* sp. E222c (Ogawa *et al.*, 1993). The *N*-carbamoylase is thought to be identical to β-ureidopropionase (EC 3.5.1.6) (Möller *et al.*, 1988; Ogawa *et al.*, 1994b; May *et al.*, 1998c).

3. The hydantoin-hydrolysing activities

3.1 The hydantoin-hydrolysing reactions

The substrates for the hydantoinase reaction are 5-substituted hydantoins or their derivatives (Syldatk *et al.*, 1990c), the systematic names of these compounds being imidazolidine-2,4-diones or 2,4-diketotetrahydroimidazoles (Drauz and Waldman, 1995). The stereoisomerism arises from the orientation of the side chain at the chiral carbon at position 5 in the ring (Scheme 1.3). The hydantoinase enzyme catalyses the initial ring-opening step in the biological hydrolysis of the hydantoin (Scheme 1.3). The reaction may be D-selective (LaPointe *et al.*, 1994), or L-selective (Sano *et al.*, 1977; Nishida *et al.*, 1987; Yamashiro *et al.*, 1989; Syldatk *et al.*, 1986; Syldatk *et al.*, 1990a), or non-selective (Louwrier and Knowles, 1997; Möller *et al.*, 1988; Watabe *et al.*, 1992b). The ring-opening cleavage yields the intermediate *N*-carbamyl amino acid as the D- or L- form. The second cleavage step is catalysed by an L-specific (Yokozeki *et al.*, 1987c) or D-specific (Olivieri *et al.*, 1979; Yokozeki *et al.*, 1987a,b) *N*-carbamyl amino acid amidohydrolase (*N*-carbamoylase), which hydrolyses the intermediate to the optically pure amino acid with ammonia and carbon dioxide as by-products. D-hydantoinase and *N*-D-carbamoylase have been reported to be similar to the dihydropyrimidinase and the β-ureidopropionase enzymes responsible for the degradation of cytosine or uracil, and pyrimidine, respectively (Yamada *et al.*, 1978; Yokozeki and Kubota, 1987).

A racemase enzyme, which converts an hydantoin of the "incorrect" chirality to the "correct" isomer, may be present in some organisms. Spontaneous racemisation of L-hydantoins, at temperatures in excess of 60 °C and under alkaline conditions, has also been shown to occur by keto-enol tautomerism (Bovarnick and Clark, 1938 cited in Tsuji *et al.*, 1987; Takahashi *et al.*, 1978). It is thus theoretically possible to obtain 100 % conversion of the racemic substrate to the amino acid by a stereoselective enzyme system.



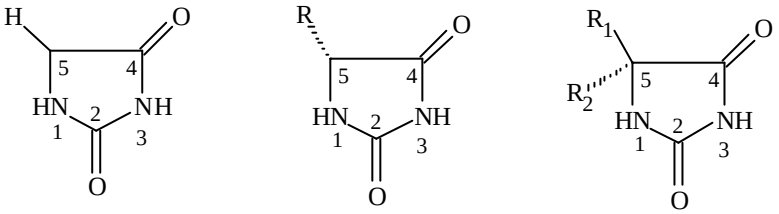
Scheme 1.3: Enzymatic hydrolysis of 5-monosubtituted hydantoins

Key: (1) = Hydantoinase; (2) = N-Carbamoylase); (3) = Racemase; (4) = Site of enzyme cleavage; (5) = No cleavage incorrect isomer; (i) = 5-monosubstituted hydantoin; (ii) = intermediate N-carbamyl D- or L- amino acid; (iii) D- or L- amino acid and (iv) = 5-monosustituted hydantoin of incorrect stereospecificity

3.2 Substrates for hydantoin-hydrolysing enzymes

3.2.1 Synthesis of 5-monosubstituted hydantoins

Since its discovery by Baeyer in 1861, the hydantoin ring system has been intensively studied (Finkbeiner, 1965; Drauz *et al.*, 1995), with the main focus in two areas: the natural occurrence of hydantoins, and synthesis of compounds from them for use as pharmaceuticals (Finkbeiner, 1965). A wide spectrum of different hydantoin, 5-mono- and 5,5-disubstituted hydantoin derivatives of industrial and pharmacological interest have been described in the literature (Scheme.1.4. (i), (ii) and (iii)). 5-Monosubstituted hydantoins, the cyclic ureides of α-amino acids, are important precursors for the synthesis of α-amino acids (Yokozeki *et al.*, 1987c; Syldatk *et al.*, 1990b; Drauz *et al.*, 1995). 5,5-Disubstituted hydantoin derivatives have been used for production of drugs important in the treatment of Parkinson's disease (Drauz *et al.*, 1995).



(i) **(ii)** **(iii)**

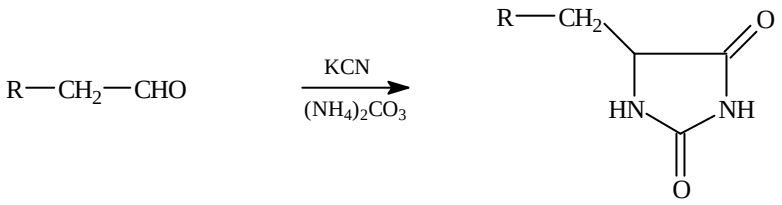
Scheme 1.4: Structures of hydantoin (i), 5-monosubstituted hydantoin (iii), and 5,5-disubstituted hydantoin

Various methods are currently employed for the chemical synthesis of 5-monosubstituted hydantoins (Drauz *et al.*, 1995; Syldatk *et al.*, 1990b). They include the Bucherer-Bergs synthesis from carbonyl compounds (Bucherer and Steiner, 1934), and the condensation of aldehydes (Syldatk *et al.*, 1990a) to form 5-arkylidine hydantoins. The subsequent reduction was reported by Wheeler and Hoffman in 1911 (Finkbeiner, 1965), and this

condensation / reduction reaction has since been utilised in the synthesis of different amino acids. The applicability of the method depends on the nature of the C-5 residue required and on the availability of precursors to allow the appropriate cost-effective introduction of the 5-substituent (Syldatk *et al.*, 1990c).

3.2.1.1 Bucherer-Bergs synthesis

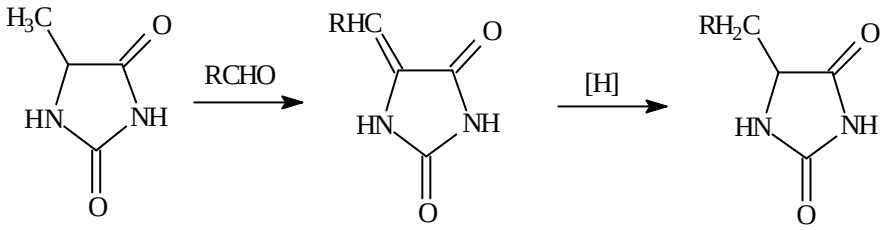
The Bucherer-Bergs synthesis is the most commonly used method for the synthesis of different hydantoins due to its relative ease and scalability of the process to produce large quantities of the desired hydantoin. Carbonyl compounds are the precursors for this synthesis (Scheme 1.5). During the Bucherer-Bergs synthesis (Scheme 1.5), where aliphatic or aromatic aldehydes are treated with potassium cyanide (KCN) and ammonium carbonate ($(NH_4)_2CO_3$) under mild conditions.



Scheme 1.5: Bucherer-Bergs synthesis of C-5 substituted hydantoins (Adapted from Syldatk *et al*., 1990c)

3.2.1.2 Condensation of aldehydes with hydantoin

The 5-methylene group at position C-5 on the ring is active and allows for a substitution to occur. In the example below, the methyl group is substituted and then condensation occurs between the aldehyde and the ring structure (Scheme 1.6)



Scheme 1.6: Condensation of hydantoin and an aldehyde to produce a 5-monosubstituted hydantoin (adapted from Syldak *et al*., 1990a)

These methods have also been modified for the synthesis of other hydantoin substrates. For example, 5-(4-hydroxyphenyl)-hydantoin (*p*-HPH) is synthesised *via* the amidoalkylation of phenol with glyoxylic acid and urea under acid conditions (Ohashi *et al.*, 1981). *p*-HPH is an important precursor in the synthesis of D-2-(4-hydroxyphenyl)glycine, which is in turn an important precursor for semi-synthetic penicillins and cephalosporins. The advantage of this method is the exclusion of sodium cyanide and 4-hydroxybenzaldehyde, which are toxic reactants, and which can undergo product-contaminating side reactions. This method has been optimised for the exclusion of *o*-HPH, which is co-produced with the *p*-HPH (Ohashi *et al.*, 1981).

3.2.1.3 Chemical synthesis of amino acids

Alkaline chemical hydrolysis has been used to convert substituted hydantoins to α-amino acids (Syldatk *et al.*, 1990a,b; Watabe *et al.*, 1992a,b; Sudge *et al.*, 1998), but the process yields a racemic mixture of the amino acids, requiring further resolution to produce the optically active amino acid (Syldatk *et al.*, 1990b; Sudge *et al.*, 1998). Since the synthesis of biologically active compounds requires enantiomerically pure amino acids as building blocks, this further resolution is important (Runser *et al.*, 1990).

3.2.2 Substrate selectivity and specificity of hydantoinases

It has been reported that hydantoinases from different sources have relatively wide substrate selectivities, and varied stereoselectivity characteristics. Olivieri and co-workers (Oliveri *et al*., 1981) showed the hydantoinase and *N*-carbamoylase from intact cells of *Agrobacterium radiobacter* hydrolysed a wide range of 5-substituted hydantoins, including aliphatic and aromatic substrates. Syldatk and co-workers (Syldatk *et al*., 1987) reported a similar substrate selectivity for the hydantoinase from *Arthrobacter* sp. BH20, which showed no stereoselectivity between D- and L- hydantoins. An hydantoinase from *Arthrobacter crystallopoietes* AM2, showed hydrolysis of a wide range of hydantoins, but was strictly D-specific (Möller *et al.*, 1988). Ishikawa *et al*. (1994) partially purified a D-hydantoinase from *Bacillus stearothermophilus* NS1122A, and showed that the enzyme hydrolysed D-hydantoins several-fold more efficiently than L-hydantoins. It was later reported that the D-specific hydantoinase showed no activity toward 2-thiouracil and 2,4-thiouracil and preferentially hydrolysed hydantoins (Lee *et al.*, 1995; Akamatsu, 1960).

In contrast to the results of Lee e*t al*. (1995), Durham and Weber (1995) showed that dihydrouracil was the preferred substrate for a D-hydantoinase of an *Agrobacterium tumefaciens* strain. An hydantoinase of *Bacillus brevis* No. 102 was reported by Yamashiro *et al*. (1989) to possess relaxed substrate selectivity, which resulted in the production of a variety of L-amino acids. The D-hydantoinase of *Bacillus circulans* also exhibited wide substrate selectivity, particularly for D,L-5-substituted hydantoins with longer aliphatic side chains (Luška *et al.*, 1997). However, the most readily hydrolysed substrates were hydantoin and 5-phenylhydantoin.

The hydantoinase extracted from the green hulls of the pea plant, *Pisum sativum*, displayed varying activities towards different hydantoin derivatives. The greater the steric demand of the substitutent at the C-5 position the lower the hydrolysis yield (Table 1.1) (Morin, 1993; Morin *et al.*, 1995a).

Table 1.1: The effect of substitution on the activity of the hydantoinase from the pea plant, *Pisum sativum* (Morin, 1993; Morin *et al.*, 1995a)

5-sustituted hydantoin	Relative conversion (%)
Hydantoin	95
5-methylhydantoin	93
5-methylmercaptoethylhydantoin	74
5-isopropylhydantoin	53

3.2.3 Substrate specificity of *N*-carbamoylase

The *N*-carbamoylase from an *Agrobacterium* species was reported to have wide substrate selectivity, and exhibited a strict D-stereospecificity as it hydrolysed a number of D-*N*-carbamylamino acids, both aliphatic and aromatic, but no L-*N*-carbamylamino acids (Louwrier and Knowles, 1996). A further D-specific *N*-carbamoylase was reported for *Agrobacterium* sp. KNK712, which hydrolysed a variety of substrates (Ikenaka *et al.*, 1999). In contrast, an L-specific *N*-carbamoylase was purified from *Alcaligens xylosoxidans* which was capable of hydrolysing a number of substrates, but the relative reaction rates were low, with *N*-carbamylalanine hydrolysis giving the highest yield, but the V_{max}/ K_M values indicating that *N*-carbamyl-L-valine was the best substrate for this enzyme (Ogawa *et al.*, 1995c). An *N*-carbamoylase from *Pseudomonas* sp. strain NS671

showed strict L-stereospecificity, and hydrolysed a wide range of aliphatic substrates, as well as the aromatic *N*-carbamylphenylalanine (Ishikawa *et al.*, 1996).

3.3 Induction of hydantoin-hydrolysing enzymes

Durham and Weber (1995) used an *Agrobacterium tumefaciens* strain 47C to investigate the production of hydantoinase, and found that the enzyme was produced in low quantities unless an inducer was incorporated into the growth medium. The highest titers of enzyme were obtained when either D,L-hydantoin or D,L-5-methylhydantoin were included in the growth medium as inducer, but when 2,4-dithiouracil was used as an inducer, no effect on hydantoinase production was observed. It has also been reported that the substitution at the chiral centre of an hydantoinase inducer may be of importance for the efficiency of the inducer. Runser and Meyer (1993) showed that the hydantoinase of this bacterium was induced to a greater extent by *p*-hydroxyphenylhydantoin than by hydantoin. However, Möller *et al*. (1998) investigated the effect of the addition of different hydantoin derivatives as inducers of hydantoinase by *Arthrobacter crystallopoietes*, and found that maximum D,L-5-methylhydantoin transformation was achieved when hydantoin was used as an inducer.

3.4 Quantification of reaction products

Any investigation of hydantoin-hydrolysing activity requires both the detection and quantification of reaction products. In this study, the products are *N*-carbamyl amino acids from the hydantoinase reaction and amino acids from the *N*-carbamyl amino acid amidohydrolase (*N*-carbamoylase) reaction. Detection and quantification of the hydantoinase activity is most commonly achieved using Ehrlich's reagent. This reagent,

acidified *p*-dimethylaminobenzaldehyde, reacts with the *N*-carbamyl amino acids to form yellow compounds that are visible and can be quantified colorimetrically at OD_{420}, by comparison to known concentrations of specific *N*-carbamyl amino acids (Cecere *et al.*, 1975). A similar colorimetric reaction using ninhydrin, (triketohydrindene hydrate), is used to visualise and quantify amino acids at $OD_{595.}$ In this reaction the activity of the *N*-carbamoylase enzyme is determined by the reaction of the ninhydrin and α-amino acids to afford a purple-coloured complex from the decarboxylated amino acid, CO_2 and aldehydes, over pH range 4-8 (Plummer, 1978).

Enantiomeric resolution of amino acids is of great importance in the production of the amino acids. Thus, chiral analysis techniques have been developed to distinguish between and quantify D- and L-amino acids. Polarimetry is one such technique (Morin *et al.*, 1986a), but is generally not used as impurities, temperature and variation in solvent types all affect specific rotation and result in inaccurate determination of enantiomeric excess (Martens and Bhushan, 1989). Chromatographic methods including GC (gas chromatography), HPLC (high performance liquid chromatography), TLC (thin layer chromatography) and CE (capillary electrophoresis) have been reported (Gil Av *et al.*, 1966; Rogozhin and Davankov, 1971; Weinstein, 1984; Rogan *et al.*, 1994). The most recent HPLC and GC methods have utilised derivatization steps, where chiral stationary phases (CSP) and chiral mobile phases (CMP) form diastereomeric complexes with the different enantiomers. The differences in retention times of the molecules are attributed to the differences in stability of these diastereomeric complexes. In HPLC, a chiral copper (II) complex in the mobile phase is used with a chiral column. The disadvantages of these techniques include the expense and the requirement for derivatisation, which may in turn lead to racemisation (Martens and Bhushan, 1989). Chiral TLC has often been the

technique of choice (Lee *et al*., 1994) as it is more affordable and the organic solvents used are volatile and do not interfere with the detection reagents. Quantification of spots on TLC plates is possible using infra-red and densitometric scanners (Novak and Hercules, 1985 cited in Martens and Bhushan, 1989).

4. Molecular genetics of hydantoin-hydrolysing systems

4.1 Introduction

Molecular genetics has gained recognition and widespread application in the field of biotechnology as a tool for the development of biotechnological processes. The rapid advances in techniques and equipment have revolutionised the study of genetics. DNA technology has been used to isolate and characterise enzymes, and to manipulate the natural characteristics of enzymes for operation outside the optimal conditions. Furthermore, the use of *E. coli* has provided a convenient host for the production of recombinant heterologous protein (Baneyx, 1999).

The evolutionary process has resulted in a multitude of organisms adapted to specific environmental niches, thus resulting in an abundant pool of enzymes available for screening for desired properties (Kim *et al*., 2000b). The natural occurrence of hydantoin-hydrolysing microbes provides a wide assortment of hydantoin-hydrolysing enzymes that exhibit a variety of characteristics. However, the exploitation of these enzymes has remained largely unutilised. Evolution within a static environment has ensured that the abundant enzymes specifically function within a narrow range of substrate specificities (Kim *et al*., 2000b). Molecular techniques have been used for the overproduction of both hydantoinase and *N*-carbamoylase enzymes, and for the directed evolution to superior

enzymes (Syldatk *et al*., 1990a; Headon and Walsh, 1994; Chao *et al*., 1999a,b; Kim *et al*., 2000a,b).

4.2 Hydantoinase genes

The first hydantoin-hydrolysing genes were cloned from *Pseudomonas* sp. NS671 by Watabe and co-workers (Watabe *et al.*, 1992a,b), who cloned the hydantoinase, the *N*-carbamoylase, and the racemase from this organism. Following this, there has been several other hydantoinases cloned that exhibit both the D- and L- stereospecificities (Table 1). Many of these genes have been sequenced and modified. Some of the hydantoinase and *N*-carbamoylase genes have been fused to other proteins to facilitate purification and then immobilisation (Kim *et al*., 2000a; Pietszch *et al*., 2000). Directed evolution has resulted in the inversion of the enantioselectivity of a D-hydantoinase to an L-hydantoinase in *Arthrobacter* sp. DSM9771 (May *et al*., 2000).

4.2.1 D-Hydantoinase genes

A D-hydantoinase cloned from *Bacillu*s sp. LU1220A was patented (in 1987) by the German company, BASF, resulting in the four-fold increase in specific activity of the enzyme in comparison to the wild type (Jacob *et al*., 1987; Syldatk *et al*., 1990a). Subsequently, a further eleven D-hydantoinases have been cloned and sequenced (Table 1.2) and several patented for industrial application (Jacob *et al*., 1987; Neal *et al*., 1994, 1999; Burtscher *et al*., 1997; Grifantini *et al*., 1998).

Table 1.2 Hydantoinase enzymes cloned and expressed in *E.coli* (modified from Hartley, 2001)

Organism	Name	Protein (kDa)	Oligomeric Structure	Stereo-Selectivity	Reference
A.aurescens DSM 3747	*hyu* H	50	tetramer	L	Gross *et al*., 1987 Wilms *et al*., 1999 Pietszch *et al*., 2000
Arthrobacter sp. DSM 9771	hyu H	50	tetramer	D to L	May *et al*., 2000
B.stearothermophilus NS1122A	ORF(HN)	51.7		non	Mukohara *et al*., 1993; 1994
Pseudomonas sp. NS671	*hyu* A *hyu* B	76 66	tetramer	non	Watabe *et al*., 1992a,b,c
A.radiobacter NRRL B11291	ORF 2	50		D	Grifintini *et al*., 1998
Agrobacterium sp.				D	Neal *et al*., 1994
Bacillus sp. LU1220				D	Jacob *et al*., 1987
B.thermoglucosidasius *Thermus* sp.	D-HYD1 D-HYD2	nd nd	nd nd	D D	Keil *et al*., 1995 Burtscher *et al*., 1997
B.stearothermophilus SD-1	*dht*	52	dimer	D	Lee *et al*., 1996a,b; 1997; 1998
B.thermocatenulatus GH2	similar to SD-1	52	tetramer	D	Kim *et al*., 1997c
P.putida DSM84		60	tetramer	D	LaPointe *et al*., 1994 Chein *et al*., 1998
P.putida 7711-2	*dht*	52.5	tetramer	D	Chen and Tsai, 1997
P.putida CCRC 12857	*dht*	53.4	tetramer	D	Chein *et al*., 1998

4.2.1.1 D-hydantoinase genes from Pseudomonads

LaPointe and co-workers (1994) cloned and sequenced the D-hydantoinase gene from *Pseudomonas putida* DSM 84, and a similar sequence coding for sigma factor binding sites found upstream of the hydantoinase open reading frame (ORF), to the L-hydantoinase cloned from *Pseudomonas* sp. NS761, (Watabe *et al*., 1992a,c), was obtained. This suggested that regulation of the hydantoin-hydrolysing enzyme expression was in accordance with ntr-type catabolite repression (Merrik and Edwards, 1995). The gene from *Pseudomonas putida* DSM 84 showed three regions of high homology with other amidohydrolase enzymes but showed no homology with the non-specific

hydantoinase from *Pseudomona*s sp. NS671, and very low homology with the D-hydantoinase from thermophilic organisms (Jacob *et al*., 1987). This is consistent with the model proposed by May and co-workers (1998a,c) that places hydantoinases within the protein supergroup of amidohydrolases, related to ureases, but which subdivides them into a number of gene families (May *et al*., 1998a).

The *P. putida* DSM 84 D-hydantoinase gene was used as a probe to detect other hydantoinase genes from the total DNA of other bacteria. However, this was only successful for the detection of other Pseudomonad strains with type I rRNA (La Pointe *et al*., 1995). Further work resulted in the development of a probe based on the N-terminus of the gene, found to amplify a 122bp fragment in bacteria belonging to all *Pseudomonas* with D-hydantoinase activity and, under low stringency conditions, the genera of *Serratia, Arthrobacter, Agrobacterium* and *Cornybacterium* (La Pointe *et al*., 1995). Southern hybridisation to an *Eco* R1 gene bank from *Pseudomonas putida* 7711-2 was used by Chen and Tsai (1997), to isolated the D-hydantoinase gene (*dht*), by synthesising a cocktail of oligonucleotides to match the N-terminus of the D-hydantoinase.

P. putida CCRC 12857 and *P. putida* DSM 84 are identical and share significant homology to other reported D-hydantoinases, (Chien *et al*., 1998). However, differences were noted between these D-hydantoinases and that from *P. putida* 7711-2. The differences arose mainly in the C-terminus of the coding region (Chen and Tsai, 1997). The expression of the D-hydantoinase gene, (*dht*), from *P. putida* CCRC 12857, with a 6 histidine tag coupled to either the carboxy or amino terminus, did not affect the specific enzyme activity in *E. coli*. However, there was a twenty-fold increase in total activity when the gene was under control of the T5lac promoter and induced with 5 mM lactose, in

comparison to the parent strains (Chien *et al*., 1998). Immobilisation of these recombinant cells in sodium alginate resulted in the increase of the optimum temperature from 20 to 60 °C and increased the reusability of the immobilised biocatalyst. However, due to diffusion limitations of the alginate matrix, overall activity was lower than that of the free cells. Thus, a matrix with lower mass transfer resistance would be required for use in an immobilisation-based bioprocess (Chen *et al*., 1999).

4.2.1.2 D-Hydantoinases genes from Agrobacterium

The majority of research into D,L-5-hydantoin-hydrolysis for the formation of D-amino acids has been on the *A. tumefaciens* (*radiobacter*) NRRLB 11291. The enzymes from this organism are used in commercial production of D-*p*-hydroxyphenylglycine (Lee and Fan, 1999a; Lee *et al*., 1996a). The D-hydantoinase and the D-*N*-carbamoylase exhibit a high substrate specificity for D,L-5-*p*-hydroxyphenylhydantoin (Syldatk *et al*., 1992). However, this process needs refinement as the *N*-carbamoylase thermal and chemical stability is poor and the organism is susceptible to bacteriophage infection. High concentrations of an inducer are required to obtain high levels of enzyme activity (Grifantini *et al*., 1998).

To solve these problems, Grifantini and co-workers (Grifantini *et al*., 1998) isolated the *N*-carbamoylase by screening genomic libraries with oligonucleotides derived from the N-terminus of the D-*N*-carbamoylase from a *Comamonas* sp. (Ogawa *et al*., 1994a). Fortuitously, the D-hydantoinase gene was also isolated, approximately 100bp upstream from the *N*-carbamoylase gene. The rearrangement of the hydantoinase and *N*-carbamoylase ORF's in a polycistronic structure, placing the *N*-carbamoylase gene in front of the hydantoinase gene, allowed for a stable and constitutive expression of both enzymes that converted hydantoin to amino acid twice as efficiently as the wild type

Agrobacterium. This *E. coli* clone no longer required the addition of an inducer to obtain high levels of hydantoin-hydrolysing activity (Grifantini *et al*., 1998). Later, Chao and co-workers (Chao *et al*., 1999a,b) subcloned both genes into *E. coli* on two different plasmids. These clones were found to be stable for up to 70 generations and capable of converting 97 % D,L-5-*p*-hydroxyphenylhydantoin to its corresponding amino acid, within 8 hours, in comparison to the 20 % conversion by the wild type cells of *A. tumefaciens* (*radiobacter*) NRRLB 11291 (Grifantini *et al*., 1998). The recombinant cells were immobilised but also showed reduced activity due to mass transport limitation (Chao *et al*., 1999b), a phenomenon also shown with immobilised recombinant cells (of *P. putida* CCRC 12857) expressing D-hydantoinase (Chen *et al*., 1999).

4.2.2 L-Hydantoinase genes

Normally, the L-hydantoin-hydrolysing systems consist of a non-stereoselective hydantoinase enzyme coupled to an L-selective *N*-carbamoylase and possibly a racemase (Volkel and Wagner, 1995). However in the cases of *Arthrobacter aurescens* DSM 3747 and *Arthrobacter* sp. DSM 9771, the hydantoinase enzymes have been cloned, and these enzymes were L-selective (Pietzsch *et al*., 2000). These enzymes have been used in the investigation of improved enzyme purification, immobilisation and stabilisation using tag-technology. Specifically, fusion proteins of HyuH L-hydantoinase have been created for the following reasons:

- to simplify the purification of hydantoinases from *A. aurescens*
- to provide large amounts of hydantoinase for immobilisation and continuous miniplant production of amino acids

- to reduce the tendency for recombinant hydantoinase to form inclusion bodies (Pietzsch *et al*., 2000).

Three different fusion proteins were created with the HyuH hydantoinase. The fusion protein with the maltose binding protein (MalE-HyuH) resulted in a high yield of soluble protein (76 %) after separation by affinity chromatography. The other two fusion proteins, one with a chitin binding domain (HyuH-intein-CBD) and the other a histidine-tagged HyuH-His_6, were still obtained as inclusion bodies (Pietzsch *et al*., 2000). Even with the doubling of the size of the fusion protein the specific activity of the MalE-HyuH fusion protein remained the same as the wild type protein, (12.9 $U.mg^{-1}$ and 13 $U.mg^{-1}$). Thus, the fusion protein MalE-HyuH provided high yields of soluble recombinant hydantoinase in a single purification step, compared to a three-step procedure required for the hydantoinase from the wild type organism (Pietzsch *et al*., 2000).

4.2.3 Inversion of hydantoinase enantioselectivity through directed evolution

The technique of random mutagenesis was employed by May and co-workers (May *et al*., 2000) to alter the selectivity of the hydantoinase gene from *Arthrobacter* sp. DSM 9771. Error-prone PCR and saturation mutagenesis produced a library of 20000 clones. These were screened for altered hydantoinase enantioselectivity, by the conversion of D- or L-5(2-methylthioethyl)-hydantoin, and the use of cresol red as a pH indicator to detect the decrease in pH with the production of *N*-carbamyl-methionine. Results from selected mutant clones from the initial assays were confirmed using chiral HPLC, and a final evolved mutant hydantoinase, which was L-selective and exhibited a five-fold increase in activity, was produced (May *et al*., 2000). Amino acid sequencing results showed that a

single substitution of an isoleucine at position number 95 to a phenylalanine (I95F), was responsible for the hydantoinase enantioselectivity inversion.

4.3 *N*-carbamyl amino acid amidohydrolase genes

Like the isolated hydantoinase genes, all the *N*-carbamoylase genes which have been cloned and sequenced are from *Agrobacterium*, *Arthrobacter*, *Pseudomonas* and *Bacillus* species (Table 1.3).

Table 1.3 *N*-carbamoylase enzymes cloned and sequenced *E.coli* (modified from Hartley, 2001)

Organism	Name	Protein (kDa)	Oligomeric Structure	Stereo-Selectivity	Reference
A.aurescens DSM 3747	*hyu* C	44	dimer	L	Gross *et al*., 1987 Wilms *et al*., 1999 Pietszch *et al*., 2000
B.stearothermophilus NCIB8224	ama b	44	nd	L	Batisse *et al*., 1997
B.stearothermophilus NS1122A	ORF(NC)	44,278	dimer	L	Mukohara *et al*., 1993
Pseudomonas sp. NS671	*hyu* C	45	dimer	L	Watabe *et al*., 1992a,b
Pseudomonas KNK003A	DCase	38	dimer	D	Ikenaka *et al*., 1998b
A.radiobacter NRRL *A.radiobacter* B11291	ORF 1 *cau* A	34 34.247	dimer	D D	Grifintini *et al*., 1996, 1998 Buson *et al*., 1996
Agrobacterium sp.				D	Neal *et al*., 1994 Louwrier and Knowles, 1996
Agrobacterium sp. KNK712				D	Nanba *et al*., 1998a.b Ikenaka *et al*., 1998b, 1999

4.3.1 *N*-carbamyl-D-amino acid amidohydrolase genes from Pseudomonads

The thermotolerant *N*-carbamyl-D-amino acid amidohydrolase gene from *Pseudomonas* KNK003A was selected from a genomic library, cloned, sequenced and expressed in *E. coli* (Ikenaka *et al*., 1998a). This gene shared 62 % and 65 % homology with the *N*-carbamoylase from *A. radiobacter* NRRLB11291 and *Agrobacterium* sp. KNK712, respectively. The N-terminal region of these three genes showed eight different amino acids, whilst the C-terminal region of the *Pseudomonas* KNK003A gene showed the

highest amino acid homology (57 %) with the C-terminus of the gene of *Comamonas* sp. E222c (Ikenaka *et al*., 1998a). Alignment with ten known related amino acid sequences showed that only six amino acid residues were conserved, including cysteine (172), which appears to be related to enzyme activity as a replacement at this position with alanine resulted in loss of activity (Ikenaka *et al*., 1998a). The activity of this recombinant enzyme exhibited a 40-fold increase in activity, in comparison to the native strain. The recombinant enzyme was also 5-fold more stable after reuse (Ikenaka *et al*., 1998a).

4.3.2 *N*-carbamyl-D-amino acid amidohydrolase genes from Agrobacteria

As a result of these enzymes displaying an increased affinity to D,L-*N*-carbamylhydroxyphenylhydantoin, the remainder of the enzymes used in the industrial production of D-hydroxyphenylglycine all originate from the *Agrobacterium* spp. (Louwrier and Knowles, 1996). A British pharmaceutical company, Smithkline Beecham, holds the patent for the cloning of the *N*-carbamoylase gene from *A. tumefaciens* (*radiobacter*) NRRL B11291, and the cloning of the gene back into the parent strain, resulting in the over production of the *N*-carbamoylase protein (Neal *et al*., 1994, 1999).

A similar gene for the production of ammonium, using hydantoin as a substrate was also cloned from the genomic library of *A. tumefaciens* (*radiobacter*) NRRL B11291 (Grifantini *et al*., 1996). When placed under the control of the T7 RNA-dependent promotor and expressed in *E. col*i BL21, the *cau*A gene product reached 40 % of the total protein in the cell, and was found to be more stable than the native gene (Buson *et al*., 1996).

A fourth *N*-carbamoylase gene was isolated and sequenced from *Agrobacterium* sp. KNK712, by selection for clones using 5-methylhydantoin (Nanba *et al*., 1998a,b). The amino acid sequence was similar to that of the enzyme from *A. radiobacter* NRRL B11291, only differing by nine amino acid residues. Over-expression in *E. coli* resulted in a 5-fold increase in specific activity, compared to that reported for the *A. radiobacter* NRRL B11291 gene. This result was attributed to a more suitable host-vector system and better culture conditions of the recombinant *E. coli* (Nanba *et al*., 1998a,b).

Grifantini and co-workers (Grifantini *et al*., 1996) found five cystine residues in the *N*-carbamoylase amino acid sequence and analysed them by site directed mutagenesis and chemical derivatisation. The results showed that cys172 was crucial for enzyme activity. Site directed mutagenesis of the gene from *Agrobacterium* sp. NKN003A was used to produce an enzyme that exhibited greater thermo- and pH stability. Alteration of three amino acid residues, namely His57Tyr; Pro203Glu/Leu and Val236Ala, produced a more stable protein (Ikenaka *et al*., 1998c, 1999). The increased thermostability was postulated to have occurred because of the conformational changes caused by the mutations removing positive charges (Ikenaka *et al*., 1998b, 1999). The thermostability of the mutated protein increased by 5-10 °C and further immobilization of the mutated enzyme increased the enzyme half-life by two-fold (Nanba *et al*., 1998a).

4.3.3 *N*-carbamyl-L-amino acid amidohydrolase genes from Pseudomanads

The L-*N*-carbamoylase from *Pseudomonas* sp. NS671 is a homodimer of 45.7 kDa and was designated *Hyu*C (Watabe *et al*., 1992a). This gene shares some sequence homology with the *hyu* A and *hyu* B genes of this strain, which encode a non-specific hydantoinase enzyme. It is proposed that they evolved from a common ancestor by a gene duplication

event (Watabe *et al*., 1992a). The *Pseudomonas hyu* C gene has been expressed in *E. coli* and the purified protein was shown to be strictly L-specific with a broad substrate range. The presence of adenosine triphosphate (ATP) and sulphydryl compounds inhibited the recombinant enzyme. This was also the case with the wild type enzyme (Ishikawa *et al*., 1997). The only other reported *N*-carbamoylase from a *Pseudomonas* strain is D-stereoselective (Ikenaka *et al*., 1998b).

4.4 Hydantoin racemase genes

A racemase gene (*hyu* E) which is capable of racemising either the D- or L- enantiomer of a racemic 5-substituted hydantoin was isolated from *Pseudomonas* sp. NS671 (Watabe *et al*., 1992b). This protein was found to be 32.1 kDa in size, and was hexameric in structure (Watabe *et al*., 1992b). The recombinant racemase was inactivated by 5-isopropylhydantoin but could be protected by the presence of divalent sulphur-containing compounds (Watabe *et al*., 1992c). A further racemase gene (*hyu*R) was cloned and used in conjunction with an engineered L-hydantoinase to produce L-amino acids (May *et al*., 2000).

4.5 Fusion proteins of hydantoinase and *N*-carbamoylase

One alternative solution to the limitations of naturally occurring biocatalysts is the construction of active bifunctional proteins. A bifunctional fusion protein comprising a D-hydantoinase/D-*N*-carbamoylase enzyme was constructed (Kim *et al*., 2000a). A maltose binding protein was initially bound to the N-terminus of the D-hydantoinase to ensure no loss of activity occurred. Once activity was established an end-to-end fusion of the D-

hydantoinase gene from either the *B. stearothermophilus* SD-1 or *B. thermocatenulatus* GH2 and the *N*-carbamoylase gene from *A. radiobacter* NRRL B11291 was performed. The latter construct, CAB-HYD1 showed superior bifunctional activity compared to the earliest construct CAB-HYD. The activity of CAB-HYD1 was also superior to the activity of the two enzymes expressed separately in *E. coli* and equal to the activity of the two enzymes co-expressed on separate plasmids in *E. coli* (Kim *et al*., 2000a).

4.6 Protein modifications

In studies of protein extracts, the structure and function may be altered by temperature, redox effects, radiation, solvent types, metal ions all causing various modifications and alterations in structure and function. Enzyme-antibody complexes have been known to stabilise proteins. When polyclonal antibodies were raised against L-hydantoinase and racemase enzymes from *Arthrobacter aurescens* DSM 3747, a three-fold stabilisation of the L-hydantoinase, and two-fold stabilisation of the racemase resulted (Shami *et al.*, 1989 cited in Siemann *et al*., 1994). The racemase activity remained at 75 % of its initial activity after 76 hours, while the relevant controls, including L-hydantoinase incubated with antiracemase or phosphate buffered saline, showed a decline in activity after 1.5 hours. The enzyme-antibody complexes could be immobilised on a Sepharose 4B support, and enzyme activity was detected after 17 cycles of column flushing with substrate.

5. Isolation and screening for microbial hydantoinase producers

Morin and co-workers (1986a) reported the use of an indirect overlay assay method for the identification of hydantoinase-producing organisms, where inducer-supplemented plates were inoculated, then overlaid with agar containing the substrate of interest, for example hydantoin. The plates were incubated and the presence of the hydantoinase enzyme detected by the development of a yellow colour within 5-10 seconds, when the colonies were covered with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde). This "spot-test" was further developed by Morin *et al.*, (1987) using dihydrouracil as a screening substrate. The detection of false positives was suggested to be due to the non-specificity of the Ehrlich's reagent.

The "spot test" was modified by Chien and Hsu (1996) by using microtitre-plates containing a hydantoin-supplemented medium into which cells were inoculated and incubated for 18 hours. The cells were then washed with dihydrouracil in buffer and incubated for an additional 30 minutes. Hydantoinase activity was detected using 5 % *p*-dimethylaminobenzaldehyde added to the wells. The "spot test" of Morin *et al.*, (1986a) was used in conjunction with the same strains, as a comparison, and detection of two additional D-hydantoinase strains was achieved using the microtitre plate assay. 18 hours was required to obtain a result in the microtitre plate assay, whereas the "spot test" required two days, but the yellowish colour of the agar medium compromised the sensitivity of the "spot test". In addition, colour development in colonies within close proximity to each other led to interference, so that "false positives" were detected. However, this microtitre plate assay proved to be economical with respect to time and the sensitivity far greater than the "spot test".

A number of molecular techniques have been developed to further improve the sensitivity of screening methods for the detection of hydantoin-hydrolysing organisms. Polyclonal antibodies raised from *Arthrobacter* were used by Siemann *et al*. (1993a) to detect L-hydantoinase, racemase, and *N*-L-carbamyl amino acid amidohydrolase. Colonies were transferred onto cellulose membranes, lysed, washed, and used in an enzyme-linked immunosorbant assay (ELISA) test. Hydantoinase-producing colonies could be identified by an antibody-antigen reaction. Western-blot analysis was incorporated to estimate amounts of internal cellular enzyme.

A DNA probe was developed from the most conserved region of a known D-hydantoinase gene sequence from *P. putida* DSM 84 (La Pointe *et al*., 1994). Using this technique, rapid detection of the gene coding for this protein was made possible by DNA and colony hybridisation.

6. Characterisation of enzyme activities

6.1 Hydantoinase activity

Several authors have identified hydantoinase enzymes from different sources, and reported on activity in resting or whole cells, crude enzyme extracts and as purified enzymes.

P. fluorescens DSM 84 was cultured and the hydantoinase enzyme was isolated and purified (Morin *et al*., 1986b). The enzyme was reported to be a tetramer of similarly sized subunits. A 30 % conversion to *N*-carbamyl-D-valine was observed when using isopropylhydantoin as a substrate, and further chemical hydrolysis to D-valine was achieved. Metal ion requirement studies showed Fe^{2+} and Mn^{2+} to have a stimulatory

effect on conversion by the purified hydantoinase, while Cu^{2+} ions led to an inhibitory effect. Sun (1983), showed the same effect for a *P. flourescens* strain DSM84 (Morin *et al*., 1986b). *p*-Hydroxymercuribenzoate was shown to inhibit activity of this enzyme, suggesting sulfhydryl group participation in the reaction. Temperature and pH optima were determined using dihydrouracil as substrate, and the optimum temperature for activity was determined to be 45 °C, while stability was retained up to 55 °C. The pH range of activity was found to be 5.5 to 9.5, with an optimum at pH 9. Takahashi *et al*. (1978) report a similar temperature optimum, but less activity evident at alkaline pH for a different *P. putida* strain IFO 12996. Several substrates were tested and dihydrouracil was found to be the preferred substrate, with a K_M value of $1.1x10^{-2}$ M for this enzyme. A similarity to dihydropyrimidinase (EC 3.5.2.2) was proposed by Morin *et al*., (1986b) based on substrate preference and K_m value.

Runser and Meyer (1993) purified the *Agrobacterium* sp. IP I-671 hydantoinase enzyme to homogeneity. The molecular mass and oligomeric structure data reported for this enzyme were similar to that reported by Takahashi *et al*. (1978), and Morin *et al*. (1986b) for *P. striata* and *P. fluorescens* respectively. The activity was inhibited by thiol-blocking agents. The absence of catalytic hydrolysis of dihydropyrimidines by the enzyme from these strains suggested that they differed from dihydropyrimidinase (EC 3.5.2.2).

Syldatk *et al*. (1987) reported that the hydantoinase from *Arthrobacter* sp. BH20 had a wide substrate specificity and was inducible by D-, L- or D,L-indolylmethylhydantoin. Stereoselectivity was not demonstrated when using resting cells. Mn^{2+} and Co^{2+} ions enhanced enzyme activity, while Cu^{2+} and Zn^{2+} ions were inhibitors. This was comparable with the results on metal ion requirement obtained by Morin *et al*. (1986b). Substrate

selectivity, stereoselectivity, induction and metallodependence results suggested that this enzyme was dissimilar to the dihydropyrimidinase (EC 3.5.2.2) from Takahashi *et al*. (1978).

A hydantoinase from *Agrobacterium* sp. IP I-671 was partially purified and studied by Runser and Ohleyer (1990). The substrate specificity showed strict D-specificity for hydantoins substituted at the 5-position by an aromatic group. Di-substituted hydantoins containing a free acid group at position 5 were not hydrolysed, making this enzyme dissimilar from dihydropyrimidinase. Enhancement of the activity in the presence of Ni^{2+} ions was observed. Enzyme activity was retained up to 70 °C, while the pH optimum was found to be 10. The high temperature and pH stability shown were demonstrated to increase substrate solubility and spontaneous chemical racemisation at alkaline pH.

A *Pseudomonas stutzeri* strain ATCC 17588 was used as the source for partial purification of a dihydropyrimidinase enzyme (Xu and West, 1994). The reported data obtained were compared to those obtained for the dihydropyrimidinase from *Clostridium uracilicum* (Campbell, 1958 cited in Xu and West, 1994). The substrate preferences of this enzyme were in the order: hydantoin>dihydrothymine>dihydrouracil. The pH range for hydantoinase activity was determined to be between pH 7.5 and 9, and the temperature range for activity was between 25 and 70 °C, with an optimum at 45 °C. The presence of Cu^{2+} and Zn^{2+} ions inhibited the hydantoinase activity whilst Mg^{2+} ions enhanced enzyme activity to a lesser degree. The dihydropyrimidinase enzyme from *C. uracilicum* was different from that of *P. stutzeri* in that the former did not hydrolyse dihydrothymine or hydantoin. However, their pH optima were similar, but the temperature of activity for the

Clostridium hydantoinase was much lower, at 30 – 35 °C. Both the Pseudomonad and Clostridial enzyme activities were activated by magnesium ions.

Durham and Weber (1995) used cell-free extracts of *A. tumefaciens* 47C to study its hydantoinase activity. The enzyme displayed a broad specificity over a wide temperature range, with an optimum at 70 °C. A broad pH range for activity was observed, with an optimum between 8 and 10.5. 5,6-Dihydrouracil was the preferred substrate, with benzylhydantoin being the least hydrolysed. In contrast, the reverse was found for *Agrobacterium* IP I-671 (Runser and Ohleyer, 1990). The D-specificity for D,L-phenylhydantoin of *A. tumefaciens* was shown to be the same as that reported by Runser and Ohleyer (1990), for *Agrobacterium* sp. IP I-671. Metal ions showed little effect on hydantoinase activity, and addition of EDTA failed to indicate metal dependence. This was different from the data previously reported for other hydantoinases. However, the stability under high alkaline and temperature conditions was similar to that reported by Runser and Ohleyer (1990), for *Agrobacterium* sp. IP I-671.

Gokhale *et al*., (1996) reported for the first time the use of resting cells for the production of D-*N*-carbamylphenylglycine by an hydantoinase enzyme. The *Pseudomonas desmolyticum* NCIM 2112 strain showed optimum activity at pH 9.5, and a temperature optimum at 30 °C. Enzyme activity under alkaline conditions had also been shown for other hydantoinases (Runser and Ohleyer, 1990; Xu and West, 1994 and Durham and Weber, 1995).

6.2 The *N*-carbamylaminoacid amidohydrolase (*N*-carbamoylase)

In 1993, Ogawa and coworkers purified the *N*-carbamyl-D-amino acid amidohydrolase from a cell-free extract of *Comamonas* sp. E222C (Ogawa *et al*., 1993). This enzyme showed stereoselectivity for the D-isomers of substrates with hydrophobic groups, for example, *N*-carbamyl-D-phenylalanine, -D,L-methionine, -D,L-norleucine, and -D,L-tryptophan. The metal ions, Fe^{2+}, Fe^{3+}, Al^{2+}, Sn^{2+} and Pb^{2+} enhanced the activity of the enzyme to a small degree. Sulfhydryl reagents inhibited the enzyme, which is similar to hydantoinases (Morin *et al*., 1986b; Runser and Meyer, 1993 and Ogawa *et al*., 1997). Enzyme stability was maintained over pH 7 - 9, and below 40 °C. However, activity was optimum at pH 8-9, with maximum activity measured at 40 °C.

Ogawa *et al*., (1995c) also purified and characterised *N*-carbamoylase from *Alcaligenes xylosoxidans*. This enzyme was homodimeric and L-specific for a variety of substrates with *N*-carbamyl-L-valine being the preferred substrate. Addition of Mn^{2+}, Co^{2+} and Ni^{2+} ions increased enzyme activity considerably. Sulfhydryl reagents and sodium propionate, (a β-ureidopropionase inhibitor), significantly inhibited amidohydrolase activity. This suggested a similarity to the amidohydrolase of *Comamonas* sp. (Ogawa *et al*., 1993) and that both enzymes are possibly related to β-ureidopropionase (EC 3.5.1.6). The enzyme from *Blastobacter* sp. (Ogawa *et al*., 1994, cited in Ogawa *et al*., 1995a) and *Comamonas* sp. (Ogawa *et al*., 1993) had a similar N-terminal sequences. The pH range for activity was between pH 6 and 9.5, with an optimum being at pH 8 - 8.3. The optimum temperature for activity was reported as 30 °C, which is comparable to that of *Comamonas* sp. (Ogawa *et al*., 1993).

A purified form of the *N*-carbamoylase (*hyu*C product) from *Pseudomonas* strain NS671 was studied (Ishikawa *et al*., 1996) via the plasmid, pDST38, which is a derivative of pUC18, containing the gene for the enzyme. This was expressed in *E. coli* JM103 and then purified. The homodimeric enzyme required Mn^{2+}, Co^{2+} or Ni^{2+} ions for activity. This finding was in concurrence with that reported for the same enzyme in *A. xylosoxidans* (Ogawa *et al*., 1995c). The pH and temperature optima for this enzyme activity were determined to be pH 7.5 and 40 °C respectively, which was comparable the amidohydrolase of *Comamonas* sp. (Ogawa *et al*., 1993). The enzyme from this organism showed broad substrate specificity, with preferential hydrolysis of L-isomers. It was also reported that addition of ATP and *p*-mercuribenzoate inhibited enzyme activity.

Louwrier and Knowles (1996) purified a carbamylase enzyme from an *Agrobacterium sp*. This homodimeric enzyme displayed strict D-specificity and *N*-carbamyl-D-methionine was the preferred substrate. Aliphatic and aromatic *N*-carbamyl amino acids substrates were also hydrolysed. This enzyme activity was measured from pH 5.8 - 9, with an optimum at pH 7.3 - 7.4. Its temperature range for activity was from 25 - 80 °C, with an optimum at 70 °C. Metal ions had no effect on the activity. Possible sulfhydryl group involvement was demonstrated by irreversible inhibition of enzyme activity when enzyme was incubated with iodoacetic acid and iodocetamide, both thiol reagents. This inhibition was observed even in the presence of excess substrate as a protective agent.

Dependence on reducing agents has been reported. The absence of DTT (dithiothreitol) resulted in inhibition of the enzyme, and the authors suggested that rapid oxidation of sulphydryl groups by oxygen was the cause of inhibition. Ammonium sulphate, and ammonium chloride were both inhibitory, possibly by a product inhibition mechanism.

This has also been reported by Hartley *et al.* (1998) for another *Agrobacterium tumefaciens* where the ammonia produced as a by-product of the reaction inhibited the enzyme activity. Louwrier and Knowles (1996) further proposed that their enzyme was novel, as it differed from the ureidopropionase of *C. uracilicum* (Campbell, 1960 cited in Louwrier and Knowles, 1996), with respect to lower temperature range for activity, and sensitivity to ureidopropionase inhibitors.

6.3 Bacteria containing both hydantoinase and *N*-carbamoylase

Some bacterial strains possess both hydantoinase and the *N*-carbamylase enzymes, such that the complete conversion of substrates through to the corresponding amino acid is possible, (e.g., *Agrobacterium radiobacter* NRRL B11291 (Olivieri *et al*., 1981). Whole cells of this organism showed broad substrate specificity, with D,L-phenylhydantoin being the preferd substrate for hydantoinase activity. The optimal activity was at pH 9, and the conversion of the intermediate (*N*-carbamoylase activity) was observed over the pH range 7.5 - 9. The optimum temperatures for the hydantoinase and *N*-carbamoylase enzymes were found to be 60 °C and 50 °C, respectively. Ammonia and sodium chloride were observed to inhibit both enzymes, but the inhibition was more pronounced for the *N*-carbamoylase enzyme under alkaline conditions. Using *N*-carbamyl-D-phenylglycine as a substrate, toluene-treated or disrupted cells gave more D-phenylglycine production than intact cells. Limited permeability of the cells to the *N*-carbamoyl derivatives was suggested as a possible explanation for this observation. Using D,L-5-phenylhydantoin, the opposite effect was observed; the intact cells produced more amino acid than the toluene-treated or disrupted cells.

Arthrobacter sp. DSM 3747, isolated by Syldatk *et al*., (1990b), was capable of converting D,L-5-(3-indolylmethyl)hydantoin (D,L-5-IMH) to L-tryptophan. The enzyme system was shown to be inducible by D,L-5-IMH supplementation to the growth medium. This induction effect had previously been shown in the genera *Arthrobacter* and *Flavobacterium* (Syldatk *et al*., 1987; Yokozeki *et al*., 1987c). This organism also displayed a dependence on Mn^{2+} ions for the synthesis of the hydrolysing enzymes, as had also been observed for the hydantoinase enzyme of *A. aurescens* BH20 (Syldatk *et al*., 1987). Ammonium sulphate was reported to be a suitable nitrogen source for enzyme induction, whereas for the *Agrobacterium* sp. of Louwrier and Knowles (1996), this was not the case. Mn^{2+} ions and D,L-5-IMH were found to effect an increase in cell growth and enzyme specific activity, provided they were not added at the beginning of culture, but rather, 8-10 hours into fermentation. Maximum substrate conversion was achieved by a high cell mass (obtained under Mn^{2+}-free conditions). D,L-5-(3-indolylmethyl)-3-*N*-methylhydantoin, a non-metabolisable analogue of D,L-5-IMH, was used as an inducer at the onset of cultivation, and was shown to increase the enzyme specific activity, but had no effect on biomass yield. This resulted in the possibility of an enzyme production rationale involving supplementation with Mn^{2+} and D,L-5-IMH after 8 - 10 hours and inducer-free cultivation as a means of simultaneously obtaining rapid cell growth and higher specific activity.

Kim and Kim (1994) developed an alternative way of increasing enzyme efficiency in the production of D-*p*-hydroxyphenylglycine from hydroxyphenylhydantoin by *Agrobacterium* sp. I-671, where both enzymes were present in the organism. Ammonium ions, which are produced as a by-product of the reaction, were found to be inhibitors of the *N*-carbamoylase enzyme. The efficient removal of these ions by the use of a re-usable

ammonium ion adsorbent, AD 300 NS, drove the reaction in the direction of amino acid synthesis, and the yield of D-hydroxyphenylglycine increased from 50 to 98 %.

Only the *Bacillus* and *Pseudomonas* genera have been previously reported as being capable of conversion of D,L-5-(2-methylthioethyl)hydantoin to L-methionine. Wagner *et al*. (1996), evaluated *Arthrobacter* sp. DSM 9771 for its capability of effecting this conversion. The use of glucose and *N*-carbamyl-L-methionine (L-CAM), as the sole carbon and nitrogen sources respectively, resulted in the selection of a mutant of the parent strain, *Arthrobacter* DSM 7330, which had the ability to constitutively express hydantoin-hydrolysing enzymes when grown in the presence of these two compounds. The preferred inducer, D,L-5-indolylmethyl-3-methylhydantoin (N3-IMH) had previously been found to inhibit growth of the parent strain. Unlike the mutant, the parent strain produced little of the enzymes of interest. The mutant also showed higher hydantoinase activity on D,L-5-(2-methylthioethyl)hydantoin and higher growth rates even in the absence of inducer. However, the *N*-carbamoylase activity of the mutant was lower than that of the parent strain. In the parent strain *Arthrobacter* sp. DSM 7330, the hydantoinase enzyme was previously showed to be non-stereospecific, affording both D- and L-isomers of the *N*-carbamyl amino acid, (*N*-carbamyl methionine), whereas the *N*-carbamoylase enzyme of this strain was specific for L-CAM (Völkel and Wagner, 1995, cited in Wagner *et al*., 1996). The rate-limiting step of the reaction was thought to be the racemisation of D-CAM, and subsequent enzymatic conversion to L-methionine. This racemisation was thought to occur when most of the L-CAM had been converted to L-methionine. A subsequent shift in the *N*-carbamoylase pH optimum from 8.0 to 7.5 over a 2 hour incubation time, allowed the enzymatic conversion of D-CAM to L-CAM, three times faster than at pH 8.0 (Wagner *et al*., 1996).

A chemical conversion of D,L-5-(*p*-trimethylsilylphenylmethyl)hydantoin to *N*-carbamyl-D-*p*-trimethylsilylphenylalanine has been reported by Tsuji *et al*. (1997). The larger atomic radius and electronegativity of silicon as compared to carbon, and its hydrophobicity, could confer novel properties to penicillins and cephalosporins (Colvin, 1981, cited in Tsuji *et al*., 1997). An enzymatic conversion was not possible due to the presence of the bulky trimethylsilyl moiety. A partially purified *N*-carbamyl-D-amino acid amidohydrolase from *Blastobacter* A17p-4 was capable of converting the *N*-carbamyl-D-*p*-trimethylsilylphenylalanine to D-*p*-trimethylsilylphenylalanine. A pH of 8.0 was optimal for the amidohydrolase activity, and high optical purity of the product was observed.

Ishikawa *et al*. (1997), proposed a mechanism for the complete conversion of D,L-substituted hydantoins to L-amino acids by three enzymes; the hydantoinase, a racemase and an *N*-carbamoylase, using the enzymes of *Pseudomonas* sp. strain NS671. This proposed mechanism distinguishes this hydantoinase from all others reported, in that it cannot be classed as strictly D-specific, strictly L-specific, or non-specific. The L-form of D- and L-5-(2-methylthioethyl)hydantoin was hydrolysed preferentially in comparison to the D-form, and the D-form of the substrate was hydrolysed after nearly complete hydrolysis of the L-form. When the D-form of the substrate was used, both D- and L-forms of the intermediate (*N*-carbamyl-methionine) were detected. The authors concluded that the L-specific hydantoinase could hydrolyse D-hydantoins, although spontaneous racemisation of the D- to the L-form was suspected.

6.4 Thermostability of hydantoin-hydrolysing enzymes

There is considerable diversity amongst the mesophilic hydantoin-hydrolysing organisms and enzymes, with respect to functional properties such as pH and temperature ranges and optima, and substrate and stereospecificities as described in previous sections. Physiological differences also exist in terms of the inducer requirements and inhibitor effects in the different organisms, and further differences are found in the structural characteristics such as subunit organisation and molecular size. However, there is an important similarity in a large number of these enzymes in that they exhibit maximum enzyme activity at temperatures above 50 °C (Takahashi *et al*., 1978; Olivieri *et al*., 1981; Morin *et al*., 1986b; Runser and Ohleyer, 1990; Durham and Weber, 1995 and Louwrier and Knowles, 1996).

There are advantages in employing thermostable enzymes in bioprocesses where the reactions can then be carried out at elevated temperatures. At elevated temperatures the solubility of many reactants increases, and mass transfer within the reactor can increase. There is also a general reduction in medium viscosity, which alleviates some diffusion problems associated with cooler liquids. Volatile products, (that may be toxic or inhibitory to the cells or enzymes), may also be removed by evaporation (Paravaresh *et al*., 1990).

Proteins from thermophiles generally have better stability profiles than those of their mesophile counterparts which may be attributed to their subunit interaction, secondary or supersecondary structures, domain packing, and stabilisation effects by close association with carbohydrates and nucleic acids (Jaenicke, 1996). Trinkl and co-workers (1994)

further proposed that domain boundaries and extensions at the "carboxy" or "amino" termini were also involved in improved protein stability.

The first reported isolation of a thermostable D-hydantoinase was by Lee *et al*. (1994), who isolated a D-hydantoinase from a thermophilic *Bacillus* species. The enzyme exhibited thermostability, up to 70 °C, with a half life of 30 mins. This enzyme displayed strict stereoselectivity for D-hydantoins, and a broad pH range from 6.5 to 10.5, with an optimum at 8.0. The enzyme was purified to homogeneity by Lee *et al*. (1995). It was non-inducible, similar to the enzyme from *Pseudomonas fluorescens* reported by Morin *et al*. (1986b). Hydantoin was the preferred substrate and substituted hydantoins, particularly those with aromatic or aliphatic substituents, were poorly hydrolyzed. The addition of Mn^{2+} ions was required for enzyme activity. The enzyme activity was consistent over nine successive batch operations at 55 °C. *N*-carbamylase activity was not detected, and this was attributed to its instability at elevated temperatures.

With the incentive of increased aqueous solubility of D,L-5-hydroxyphenylhydantoin at temperatures above 55 °C, and hence potential efficient conversion of this substrate, Lee *et al*. (1996a) cloned the gene for the D-hydantoinase from *B. stearothermophilus* SD-1, into *E. coli* DH5α. The gene displayed higher expression under control of its own promoter compared to that of an *E. coli* promoter. Hydantoinase specific activity in the recombinant *E. coli* was 30 times greater than that of the wild type *B. stearothermophilus* SD-1. In the recombinant *E. coli* cell-free extracts, temperature and pH optima were found to be 55 °C and pH 9.0, respectively, both of which compare favourably with the optima for the immobilised enzyme from the wild type (Lee *et al*., 1996a).

Kim *et al*., (1997c) reported the primary structure and sequence of the *B. stearothermophilus* SD-1 D-hydantoinase. This enzyme was compared to other D-hydantoinases at the molecular level, all of which showed variation in substrate- and stereospecificities. The *Pseudomonas* sp. DSM84, *B. stearothermophilus* sp. NS1122A (Ishikawa *et al*., 1994) and *B. stearothermophilus* sp. SD-1 hydantoinases, were shown to be closely related, by analysis of sequence homology. The C-terminal regions of the D-hydantoinase from *B. stearothermophilus* sp. NS1122A and *B. stearothermophilus* sp. SD-1 showed significant differences. The differences in substrate specificity and stereospecificity were suggested to be attributable to the differences in the C-termini, with the *B. stearothermophilus* sp. SD-1 D-hydantoinase being more hydrophilic, and the *B. stearothermophilus* sp. NS1122A more hydrophobic. However, as secondary structures were similar and active-site amino acid residues are conserved, an evolutionary relationship was suggested.

Ishikawa *et al*., (1994) and Mukohara *et al*., (1994), isolated a thermophilic *Bacillus sp*., designated *B. stearothermophilus* NS1122A. The optimum temperature and pH were 60 °C and pH 9.5, respectively, and the hydantoinase hydrolysed both D- and L- 5-substituted hydantoins, although the D- form was more readily hydrolysed than the L- form.

Mukohara and co-workers (1994) cloned and sequenced the hydantoinase gene of *B. stearothermophilus* into *E. coli* JM105, but expression of the recombinant hydantoinase resulted in an insoluble hydantoinase aggregate. The enzyme was found to be a homotetramer, and was readily inhibited by the sulfhydryl reagent, *p*-chloromercuribenzoate. The addition of Mn^{2+} and Co^{2+} ions stimulated activity in a pH-dependent manner, and this pH dependence was not evident in the absence of the metal

ions. The authors proposed that the hydrophobicity of the enzyme, suggested by hydropathy studies, possibly contributed to thermostability Kim *et al*., (1997c).

Two commercially available thermostable hydantoinases (Roche Chemicals), D-HYD-1 and D-HYD-2, were characterised by Keil *et al*. (1995). Both enzymes had a pH optimum of pH 8.0. The activity of D-HYD-2 increased ten-fold when temperature was raised from 37 to 80 °C, and both enzymes were stable up to 70 °C, but stability decreased rapidly at temperatures greater than 80 °C. This temperature was comparable to that reported by Runser and Ohleyer (1990) for *Agrobacterium* sp. IP-671. Both enzymes hydrolysed aromatic substituted hydantoins preferentially, and D-HYD-2 hydrolysed aliphatic substituted hydantoins more readily than D-HYD-1.

A thermostable D-hydantoinase from *Bascillus stearothermophilus* was purified from a recombinant *E. coli* strain by Lee *et al*., (1996c). The optimum temperature for enzyme activity was observed to be 60°C. As reported by Lee *et al*. (1996a), higher temperatures increased substrate solubility, and thus facilitated the conversion of D,L-5-hydroxyphenylhydantoin. The biocatalyst was reusable 8 times at 50 °C, compared to the 9 times reported by Lee *et al*., (1996b). Lower operational stability of the biocatalyst was attributed to loss of enzyme by support fouling, validated by scanning electron microscopy.

A D-hydantoinase from a mesophilic *Bacillus* sp. AR9 was shown to have thermostability and stability in highly alkaline conditions when using crude extracts of the enzyme (Sharma and Vohra, 1997). The hydantoinase was strictly D-specific, and unsubstituted hydantoin was the preferred substrate. This contrasts with the substrate specificity reported

by Lee *et al*. (1994) for *B. stearothermophilus* SD-1. The addition of Co^{2+} ions enhanced hydantoinase activity considerably. The pH optimum for enzyme activity was pH 9.5, which was higher than the hydantoinase previously reported for a *Pseudomonas* species (Morin *et al*., (1990) cited in Sharma and Vohra, 1997; Yamada *et al*., 1978 and Yokozeki *et al*., 1987). The half-life of the enzyme at 50 °C was in excess of 12 hours.

6.5 Enzymes in organic media

Biocatalytic reactions may be accomplished under non-aqueous or nearly anhydrous conditions and organic solvents provide an environment in which the water content may be very low. An organic medium is distinguishable from a biphasic system, where a mixture of aqueous and water-immiscible organic solvent (such as octane) separate into distinguishable layers, or from a system that contains water-miscible solvents, for example, methanol and acetone. One advantage of an organic system is that some enzymes show increased stability in such media. For example, chymotrypsin retained full activity after 6 months in anhydrous octane at 20 °C, whereas the half-life for the same enzyme in aqueous media was a few days (Zaks and Klibanov, 1988).

Organic systems confer a number of other possible advantages for use with enzymes. New catalytic reactions may be observed and/or regiospecificity and substrate specificity can be altered and controlled. Thermal stability may also be enhanced, and the reversal of the reactions is possible. Enzymes can readily be recovered from the organic medium as they are suspended, rather than dissolved. The solubility of non-polar substrates may be enhanced, and undesirable water-dependent side-reactions may be eliminated.

The course of the reaction may be altered by the organic solvent. For example, polar products remaining near the enzyme may not be extracted into a non-polar solvent, resulting in product inhibition. Solvents may also interact with the substrates of the reaction (Kzandjian *et al*., 1986, cited in Dordick, 1989).

Enhancement of the operational stability of biocatalysts due to the presence of organic solvents has been investigated. Success has been achieved by enzyme modification, the use of additives and suitable support material. Modification of enzymes may involve coupling to compounds that have both hydrophobic and hydrophilic regions. An advantage of this modification is that limitations of mass-transfer of substrates and products between solvent and biocatalyst are eradicated.

6.6 Hydantoinases in organic media

Kim and Kim (1993) employed water-miscible organic solvents for use in hydantoinase reactions. Whole cells of the bacterial strain KBEL 101 were immobilised in a polyacrylamide gel matrix. The immobilised biocatalyst was used for the conversion of D,L-*p*-hydroxyphenylhydantoin to D-*p*-hydroxyphenylglycine in a medium containing 5 % (v/v) dimethylsulfoxide (DMSO). Increased conversion (99%) was observed with this solvent in comparison to other solvents due to the higher solubility of D,L-*p*-hydroxyphenylhydantoin in the organic-aqueous phase compared to aqueous systems. The rate of conversion declined after four successive batch operations, and the authors concluded that although conversion was satisfactory, it would be necessary to improve the operational stability of the immobilised biocatalyst.

7. Development of a biotransformation process

7.1 Immobilised and free-cell bicatalysts

The immobilisation of microbial whole cells and/or isolated enzymes to perform biotransformations is an important area in the field of biotechnology (Ramakrishna and Prakasham, 1999 and Venkatsubramian, 1980). Immobilisation localises the biocatalyst in a specific phase that can interact with a bulk phase containing the substrate for the biotransformation, while remaining separate from it. The biocatalyst support is usually a water insoluble, high molecular weight polymer, which restricts the movement of the biocatalyst, but is inert and normally takes no part in the chemical reaction (Tramper, 1985).

There are a number of advantages to using immobilised biocatalysts as opposed to soluble systems, including:

- more convenient handling
- easy separation of biocatalyst from product
- possible reuse of the biocatalyst
- possible enhancement of the thermostability, durability and activity of enzymes (which is particularly important when using expensive enzymes or continuous processes).

These advantages of using immobilised biocatalysts can potentially increase the economic viability of a process, especially if it is to be conducted on a large scale (Tischer and Kasche, 1999; Ramakrishna and Prakasham, 1999; Gekas, 1986).

Prior to immobilisation, certain factors must be considered (Marshall and Woodley, 1995) one of the most important being the form in which the biocatalyst is immobilised, *i.e.* as isolated enzymes or as whole cells. Whole cells are generally used in cases where the biotransformation is a multi-step process or the enzyme is unstable (Marshall and Woodley, 1995). Using whole cells as a biocatalyst, however, does introduce the possibility of unwanted and often uncontrollable side reactions occurring, or channelling or utilisation of the substrate and/or products into alternative metabolic pathways. Both of these situations lead to a decrease in overall yield of product (Tischer and Kasche, 1999). Further downstream processing (DSP) may also be required to release the products from the cells, increasing operational costs. Further costs may be incurred by the necessity of supplying growth medium and correct physical and physiological requirements for the cells, in addition to the substrate for the reaction.

The procedure of immobilisation and the matrix of the support used are also an important consideration. Cells may be immobilised by a variety of methods, such as support binding or entrapment, depending on the characteristics of the biocatalyst and the biotransformation system (Laskin, 1985; Scott, 1995; Ryu *et al*., 1997). Support binding involves interaction between the biocatalyst and the support via ionic or non-covalent forces. Although easy and simple, non-covalent immobilisation is relatively weak and desorption and subsequent loss of the biocatalyst is often experienced. A more attractive option for immobilisation is to entrap the biocatalyst within a matrix, such as a gel or membrane, leading to a stable, resistant and efficient biocatalyst. Novel immobilisation techniques have also been developed, such as the use of enzymes enclosed in reverse micelles, allowing for application in organic systems. The enzyme is held in the inner, polar region of the reverse micelle, while the outer layer is in contact with the organic bulk

solvent of the reaction containing the substrate, and the enzyme-catalysed reaction occurs at the interface of the polar and organic regions.

The choice of immobilisation procedure is not limited only by the biocatalyst characteristics, but also by factors such as the reactivity of the matrix, the reagents used in the reaction and the cost of the matrix (Tischer and Kasche, 1999). A variety of supports, including gels, membranes, powders and polyurethane foams, have been reported (Laskin, 1985; Scott, 1995; Ryu *et al.*, 1997; Gekas, 1986; Tramper, 1985).

The development of methods to stabilise hydantoin-hydrolysing enzymes for use biocatalytic reactions at elevated temperatures, including immobilisation, have often been stimulated by the fact that *N*-carbamoylases are frequently unstable in solution (Ogawa *et al.*, 1993, Ogawa *et al.*, 1994b; Ogawa *et al.*, 1995b).

There are several reports on the immobilisation of hydantoin-hydrolysing enzyme systems. Whole cells of *Pseudomonas putida* DSM 84 were immobilised in alginate beads and in hollow fibre cartridges (Chevalier *et al.*, 1989). The conversion rates of both continuous and batch systems were investigated under both immobilised and unimmobilised conditions and the cells in both systems were found to have similar activities. The effect of immobilisation on enzyme activity was also investigated. These results led to the development of a continuous system for the production of *N*-carbamyl-β-alanine, using an upflow, fluidised bed column. Plugging of the column did not occur by non-growing cells because of the use of a growth-limiting medium. Damage to the column bed occurred after 24 hours, due to swelling and bead disruption.

In a similar study, hollow fibre cartridges were found unsuitable for the production of amino acids due to membrane damage and leakage of the enzyme into the lumen of the membrane (Chevalier *et al*., 1989).

A *Pseudomonad* soil isolate was immobilised in polyacrylamide gel and used for the synthesis of D-*p*-hydroxyphenylglycine from DL-5-substituted hydantoin (Kim *et al*., 1994). The immobilised D-hydantoinase activity in whole cells displayed a half-life of about 50 hours. A carbon and nitrogen source was added to the reaction mixture to increase the operational stability; glycerol (0.1%, w:v) was found to be the most effective carbon source and activity of the immobilised whole cells was maintained for up to 7 days. Adding 0.1 % (w:v) yeast extract as a nitrogen source prolonged the half life of the immobilised whole cell D-hydantoinase to about 25 days.

8. Research Objectives

The primary objective of this study was the development of a biocatalytic process for the production of amino acids using an hydantoin-hydrolysing system. Our laboratory had previously isolated numerous local bacterial strains. Of these strains, four were selected for further study based on their ability to hydrolyse hydantoin and hydantoin derivatives to their corresponding amino acids, utilising an hydantoinase and an *N*-carbamoylase (Burton *et al.*, 1998). These strains were named RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR. The activity of the hydantoin-hydrolysing enzymes of these strains had been characterised, to a certain extent, in whole cells, for RU-KM3$_L$, RU-KM3$_S$, and RU-OR and in crude extracts for RU-KM1, RU-KM3$_l$ and RU-OR.

In the development of a biocatalytic process for the production of amino acids from hydantoins, it was considered valuable to have a biocatalyst capable of hydrolysing a broad range of hydantoin substrates, hence capable of producing a range of amino acids.

This study has focused initially on the utilisation of two strains, namely: RU-KM1 and RU-OR. Strain RU-KM1 was initially selected for its highly active hydantoinase, although it apparently lacked *N*-carbamoylase activity. Strain RU-OR displayed high *N*-carbamoylase activity. It was proposed that these two organisms could be utilised in conjunction with each another for the development of a biocatalytic process for the production of amino acids based on their combined hydantoin-hydrolysing activities.

After basic characterisation of both strains, the research was focused on the isolation, characterisation, and purification of the hydantoinase from RU-KM1.

On this basis, the objectives of this research project were:

- the isolation of organisms capable of hydrolysing hydantoin substrates.
- the development of a suitable assay procedure for the different enzyme activities.
- the characterisation of reaction conditions (temperature, pH and addition of metal ions) for these enzymes in whole cells and crude extracts, using the assay technique developed.
- the purification and characterisation of the hydantoinase from whole cells.
- scale-up of culture conditions for biomass production
- the development of a bioreactor system for the production of amino acids
- scale-up of the reactor system for the production of amino acids.

Chapter 2

2. Introduction

An established biocatalytic process may be based on already commercially available organisms or enzymes. Such a process would have started with a screening process for an organism that was capable of performing the desired reaction under conditions that were near the operating conditions of the proposed reaction (Figure 2.1). After isolation, the operating parameters and optimal reaction conditions for the biocatalyst must be determined (Schmid *et al.*, 2001). However, not all processes start this way; for some, isolation and screening needs to be conducted first in order to find the most suitable organism for the process to be developed around.

The selection and screening process is critical to the eventual economic success of a biocatalytic process. This chapter describes with the screening and selection of suitable microorganisms containing hydantoin-hydrolysing enzymes that exhibited potential for the use in a biocatalytic process.

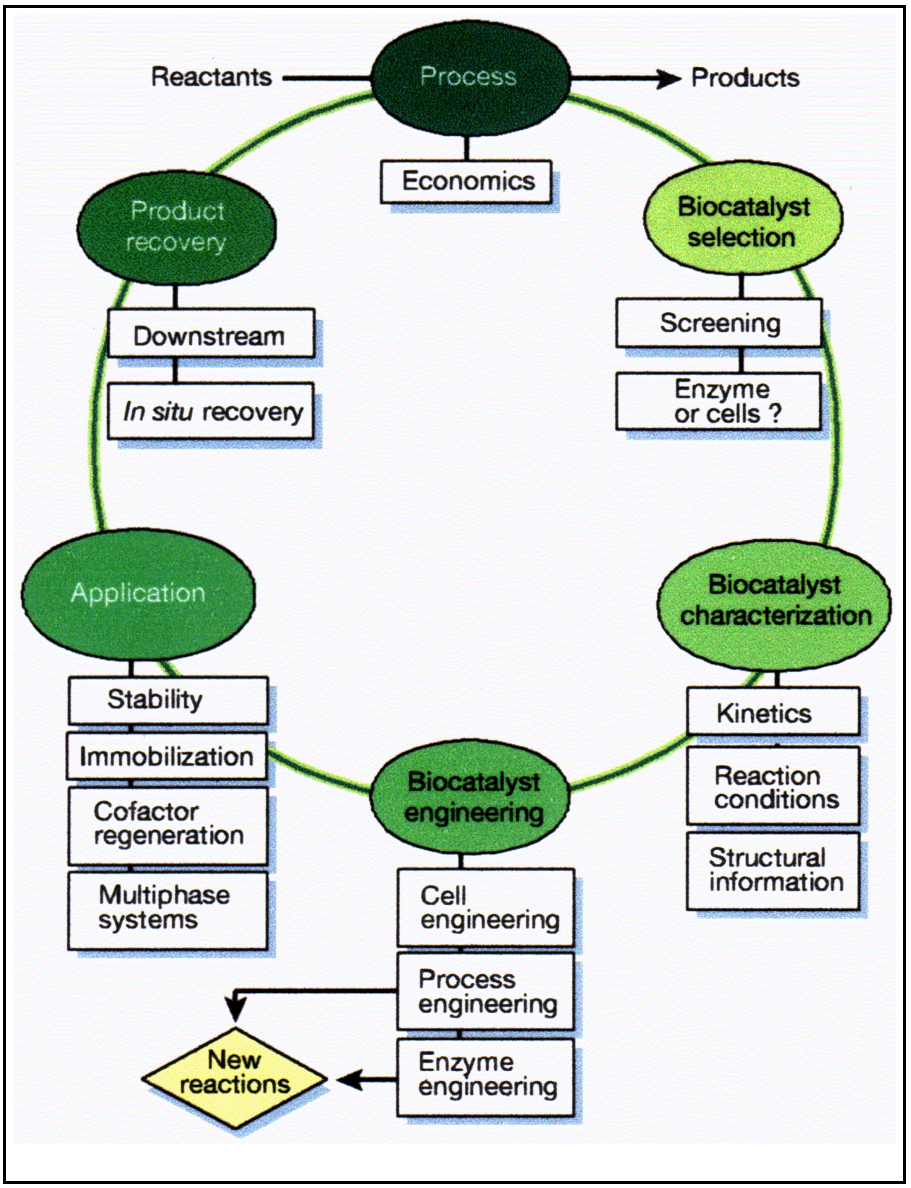


Figure 2.1: Cyclic Development of the biocatalyst for an industrial process (Schmid *et al.*, 2001) (Used with permission)

The selection criteria for isolating hydantoinase-producing organisms can be based on the utilisation of hydantoin or *N*-carbamylamino acids, as the sole nitrogen source (Syldatk *et al.*, 1986), and the use of Ehrlich's reagent to detect the production of the intermediate *N*-carbamylamino acid (Morin *et al.*, 1986, Yamada *et al.*, 1978). Thin layer chromatography has also been employed to examine the production of reaction products (Syldatk *et al.*, 1995).

A further aspect of a screening process involves the determination of the enantiomeric properties of the products (Schmidt, 2001). Measurement of optical purity classically involves the measurement of optical rotation and then comparison with optically pure enantiomers of the products. This technique is only appropriate for use when pure compounds are accessible. Hence, this technique is not suitable for the analysis of hydantoin hydrolysis reaction mixtures, as the optically active hydantoin, *N*-carbamyl amino acids and the amino acids in the reaction mixture would require prior separation and purification (Syldatk *et al.*, 1992). The use of chiral TLC and chiral HPLC with chiral supports or columns (Syldatk *et al.*, 1992), have provided more suitable methods for the enantiomeric analysis of the products of hydantoin hydrolysis (Morin *et al.*, 1987).

Once a promising organism has been isolated, standard metabolic and biochemical identification using simple test kits such as the Analytical Profile Index (API) test kit offers a method for preliminary identification of that organism. This identification provides information on the potential novelty of the organism. However, these techniques offer limited specificity and are often labour intensive (Brunk *et al.*, 1996). A more precise and highly specific molecular identification technique employs the use of prokaryote 16S rRNA genes as a method for identification. These genes have both highly conserved and more variable regions within the coding sequence (Avaniss-Aghaani *et al.*, 1996). The nucleotide sequence from the conserved regions of the gene allows facile polymerase chain reaction (PCR) amplification using universal primers. The sequence obtained from the divergent regions then offer distinctive characteristics that are used for identification of bacterial species (Avaniss-Aghaani *et al.*, 1996). There are many databases, such as the Ribosomal Database Project, of the different sequences, and these allow for the rapid and accurate identification of bacterial species (Maidak *et al.*, 2000).

This chapter describes the screening and isolation procedures for hydantoin-hydrolysing organisms and the eventual selection of two organisms for further study. Following initial preliminary characterisation of these two organisms, the selection of one of the strains was necessary to facilitate the complete evaluation of the organism in a biotransformation process.

2.1 Methods and Materials

Hydantoin, NCG, glycine and other reagents were purchased from Sigma-Aldrich Chemicals and were of analytical grade. 5-Methylhydantoin was chemically synthesized by the method of Bucherer-Bergs (Bucherer and Steiner, 1934).

2.1.2 Screening for hydantoin-hydrolysing organisms

In this study, sources for hydantoin-hydrolysing organisms included local gardens and the nearby Hogsback mountain range, 32°34.97”S; 26°57.17”E (Figure 2.2). A total of 206 organisms were isolated from these sites. These were assayed using Ehrlich’s reagent to detect the production of *N*-carbamylglycine from hydantoin. Of these, 146 exhibited hydantoin-hydrolysing activity. These isolates now form the Rhodes Hydantoinase Research Group strain collection.

Figure 2.2: Location of Hogsback Mountain Range, 1517m (32°34.97”S; 26°57.17”E) (adapted from Microsoft Corp and its suppliers; Expedia)

A 2 g soil sample was resuspended in 10mL sterile water and vortexed vigorously for 2 minutes. The soil particles were allowed to settle. The resulting supernatant (containing bacteria) was enriched overnight in a minimal medium (MM) containing hydantoin as a sole nitrogen source, with shaking (200 rpm) at 25 °C (Appendix A2). This enriched isolation culture was serially diluted and streaked to single colonies for further selection of hydantoin-hydrolysing activity by supplementation of medium A with: 20 $g.L^{-1}$ bacteriological agar and 1 % 5-methylhydantoin, *N*-carbamylglycine or, *N*-carbamylalanine as a sole nitrogen source, based on the plate overlay method developed by Morin *et al*. (1986a). Isolates were re-streaked to pure culture and incubated on agar plates containing the above components at 28 °C until growth appeared on the plates. Growth was examined visually and rated qualitatively on a scale of 1 to 10, with 1 representing slight growth and 10 representing rapid dense growth (Table 2.1). Resting cell biocatalytic reactions were performed on six of the isolates using an assay technique described below (Section 2.1.4).

2.1.3 Culture of microorganism

Unless otherwise stated, single colonies were picked and inoculated into 50 mL hydantoin minimal medium (HMM) broth supplemented with 1 % hydantoin as sole nitrogen source and 1 % glucose as the carbon source. This was incubated at 28 °C on an orbital shaker at 200 rpm for 24 hrs. This seed culture was used to inoculate the growth flasks.

A 2 L Ehrlenmeyer flask containing 400 mL HMM was inoculated with the seed culture to give an OD_{600} of 0.02 AU. This flask was incubated at 28 °C with shaking (200 rpm) for 40 h. The cells were harvested by centrifugation (6000 rpm x 10 min), and washed twice

in 0.1 M phosphate buffer pH 8.0 at 4 °C (KH_2PO_4 : K_2HPO_4). All experiments were conducted using cells prepared by this method unless otherwise stated.

To determine the growth kinetics of RU-OR and RU-KM1, 9 Ehrlenmeyer (500 mL) flasks containing either 100 mL HMM or 100 mL nutrient broth supplemented with 0.1 % hydantoin as an inducer were inoculated from the seed culture to give an OD_{600} of 0.02. These flasks were incubated at 28 °C shaking at 200 rpm, for 40 h. A single flask was removed at 5 h intervals and the cells harvested by centrifugation (6000 rpm x 10min), and washed twice in 0.1 M phosphate buffer pH 8.0 at 4 °C.

Dry cell weights were determined over the duration of the growth of the organism. Duplicate 2 mL samples were removed from the culture, and the cells harvested by centrifugation for 3 min at 13000 rpm in a bench-top microfuge in pre-weighed Eppendorf tubes. The supernatant was discarded and the pellet resuspended in 1 mL of 1 % HCl. The cells were again harvested by centrifugation and the supernatant discarded. The pellet was dried at 100 °C for 24 h and the mass of the pellet was calculated.

2.1.4 Development of biocatalytic assays

2.1.4.1 Whole cell (WC) reactions

The initial resting cell reactions were performed in the following standardised way in terms of the amount of cells added to each reaction vessel. The cell pellet was obtained by centrifugation (7000 rpm in a Beckman JA14 or JA21 rotor), and washed twice with potassium phosphate buffer (0.1 M, pH 8.0). After washing, the cells were resuspended in the same buffer to yield a final concentration of 40 $mg.mL^{-1}$. This was calculated as follows:

$$wcm = m_2 - m_1 \quad \text{.....(1)}$$

and

$$V_b = \frac{wcm}{40} \quad \text{.....(2)}$$

Where:

wcm - wet cell mass (mg)
m_2 - mass of centrifuge tube and pellet (mg)
m_1 - mass of dry centrifuge tube (mg)
V_b - volume of buffer to add to harvested cells (mL)

Equal volumes of the cell suspension and substrate (100 mM hydantoin, 5-methylhydantoin, 60 mM *p*-hydroxymethylhydantoin or 50 mM *N*-carbamylamino acid) were added together in a reaction vessel and incubated at 40 °C at 200 rpm, giving a final concentration of 20 $mg.mL^{-1}$ of cells per total reaction. The reaction was maintained under these conditions for 6 h, after which the solution was clarified by centrifugation and the supernatant analysed colorimetrically with Ehrlich's reagent for detection of the *N*-carbamyl intermediate and ninhydrin for detection of the amino acid (Section 2.1.5.1). Data shown are the mean (±SEM) values of 3 separate triplicate determinations.

2.1.4.2 Crude extract (CE) preparation

Cell suspensions (40 $mg.mL^{-1}$) were prepared according to the protocol described in the previous section 2.1.3.1, were disrupted by passage through a French press (Yeda-press, (LINCA Lamon Instrumentations Co., Ltd. Tel-Aviv) at 15 MPa, 4 °C, at a flow rate of 10 $mL.min^{-1}$. Aliquots (10 mL) were removed after each pass through the press, and protein concentrations determined by the method of Bradford (1976). The resulting extract was termed the crude extract (CE). Crude extract (2.5 mL) was added to an equal volume of

substrate solution and incubated at 40 °C for 6 hours. The supernatant was assayed for the formation of the intermediate NCG and the amino acid glycine (Section 2.1.5.1).

After optimisation for the production of CE using the French press, all subsequent CE preparations were routinely carried out using a single pass through the French press. The resulting extract was frozen using liquid nitrogen and dried under vacuum at –40 °C. For assay purposes, the CE was resuspended (0.1 $g.mL^{-1}$) in 0.1 M potassium phosphate buffer, pH 8.0, unless otherwise stated.

2.1.5 The assay system

The detection and quantification of the products of hydantoin hydrolysis were achieved by the adaptation of two colorimetric assays, namely the Ehrlichs assay for the detection of the *N*-carbamylamino acid intermediate and the ninhydrin assay for the final amino acid. Activity is defined as amount of product (µmol) produced from the substrate per hour per mL reaction containing 20 $mg.mL^{-1}$ biocatalyst.

2.1.5.1 Method for the detection and quantification of *N*-carbamylamino acids

Ehrlich's assay for the production of *N*-carbamylamino acids

This assay involves the reaction of Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in 6 N HCl) with an *N*-carbamyl group. This reaction yields a yellow product with a maximum absorbance detectable at 420 nm, and can be compared to that of known standards. This assay method was based on that reported by Yamada *et al.*, (1978).

The reaction mixtures from the biocatalytic reactions and the controls were clarified by centrifugation at 13 000 rpm for 3 minutes in a microfuge. The supernatant (1 mL) was

aliquoted into test tubes containing 0.5 mL of 12 % trichloroacetic acid, and vortexed briefly. Ehrlich's reagent (0.5 mL) was added to the solution and the mixture vortexed vigorously. The solution was further diluted by the addition of 3 mL of dH_2O, vortexed and allowed to stand for 20 minutes. The absorbance of the samples was read at 420 nm on a UV-Visible spectrophotometer (Shimadzu UV-VIS120) and the concentration of the *N*-carbamylamino acid produced was calculated using a standard curve (0-50 mM) prepared using *N*-carbamyl glycine (NCG) as a standard (Appendix A4).

2.1.5.2 Method for the detection and quantification of amino acids

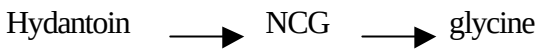
Ninhydrin assay for the quantification of amino acids

This colorimetric assay involved the reaction of any amino group present in the reaction mixture with ninhydrin reagent (Plummer, 1987) (Appendix A4).

A suitable dilution (1:20 or 1:50) of the sample was made with 0.1 M potassium phosphate buffer, pH 8.0, to a final volume of 1 mL, in acid washed test tubes. The ninhydrin reagent (1 mL) was added, the mixture was vortexed and heated in closed tubes in a boiling water bath for 15 min. The tubes were allowed to cool to room temperature and diluted with 3 mL of 50 % ethanol. The solution was vortexed and allowed to stand for a further 10 min. The absorbances of the unknowns were determined at 570 nm, and compared to those of known standard concentrations (0 - 0.5 mM) of glycine in 0.1 M poshate buffer pH 8.0. The concentration was calculated using this standard curve (Appendix A4).

2.1.6 Calculation of enzyme activities

In order to calculate the efficiency of the biocatalytic reaction, two reactions need to be considered; firstly the conversion of the hydantoin to the intermediate NCG, and secondly the conversion of the hydantoin to glycine.



Thus, the measurement of the **TOTAL** activity of the system is the combination of the NCG produced from the hydantoin as a starting substrate, and the subsequent conversion of the NCG to glycine. Hence total hydantoinase activity can be defined as:

Total activity = [NCG] + [Gly] $\mu mol.h^{-1}.mL^{-1}$……...……(1)

This is hydantoinase activity because any NCG which is converted to glycine still originally came from hydantoin, so ALL the conversion from hydantoin makes up the hydantoinase activity when using hydantoin as a substrate.

To calculate the activity of the *N*-carbamoylase (equation 2), the hydantoin starting substrate (equation 1) is replaced with the intermediate as the substrate, and the concentration of the NCG to the glycine is measured.

Hence, the individual activities can be defined as follows:

N-carbamoylase = [gly] $\mu mol.h^{-1}.mL^{-1}$…….……..(2)

Hydantoinase = [NCG] + [Gly] $\mu mol.h^{-1}.mL^{-1}$……..….……(3)

For the purposes of this work, hydantoinase activity is defined as the sum of the total product produced (µmol) per hour per mL reaction containing 20 $mg.mL^{-1}$ biocatalyst.

2.2 Results and Discussion

The initial search for suitable organisms was conducted using soil samples from the surrounding gardens and local countryside. Organisms were selected for their ability to grow when utilising hydantoin or *N*-carbamylamino acids as the sole nitrogen source (MacLeod, 1994; Gardner, 1995). A total of 146 isolates, showing hydantoin-hydrolysing activities were obtained. Five isolates were selected for further evaluation of their ability to utilise hydantoin as a sole nitrogen source.

2.2.1 Screening for hydantoin-hydrolysing organisms

From the 5 isolates, 2 mesophilic, hydantoin-hydrolysing strains displayed better growth on hydantoin and its derivatives, and thus potential greater potential for use as biocatalysts (Table 2.1). Strain RU-KM1 was isolated from a local garden soil and strain RU-OR was isolated from Hogsback. They were later identified respectively as a *Pseudomonas sp,* and an *Agrobacterium tumefaciens* strain, by 16S rRNA sequence analysis (Hartley *et al.,* 1998).

Table 2.1: **Growth of isolates utilising different compounds as a sole nitrogen source (1=poor to 10=good)**

Strain	Hydantoin	NCG	Me-Hydantoin	NCA
RU-KM1	7	7	3	3
RU-OR	6	8	5	5
RU-KM3	5	5	7	5
RU-OG	3	3.5	3	3
RU-1L	4	7	3	3

The use of plates incorporating hydantoins or *N*-carbamylamino acids as the sole nitrogen source, and cell growth as an indicator of hydantoin-hydrolysing activity, yields only a qualitative data. Selection of these organisms was based on the growth rates of the organisms as a result of their ability to convert hydantoin to the intermediate *N*-carbamyl amino acid (NCAA), and or NCAA to the corresponding amino acid. The results are subjective and can therefore only be used as an indication of the ability of the organism to hydrolyse the different substrates for use as a sole nitrogen source. The results give little indication of the level of activity of the enzymes present in the micoorganism.

Quantitative methods for the determination of the *N*-carbamylamino acid and the amino acid permitting hydantoinase and *N*-carbamoylase activities to be determined were adapted from existing techniques reported in the literature (Yamada *et. al.*, 1978).

Resting cells were assayed for the two enzyme activities. The resting cells and the crude extract were prepared as described above (Section 2.1.4.). After reaction with the substrate, product concentrations were determined (Table 2.2) using the colorimetric assays (Sections 2.1.5.1 and 2.1.5.2). The determination of the products using the colorimetric assays represents the results of the mean (±SEM) of 3 separate triplicate determinations. Table 2.2 shows typical hydantoin-hydrolysing activities obtained for 5 different mesophilic organisms that were isolated.

Table 2.2: Hydantoinase and *N*-carbamoylase activity of different strains using hydantoin as a substrate in a 1 hr reaction

Strain	Hydantoinase ($\mu mol.h^{-1}.mL^{-1}$)	NCAAH ($\mu mol.h^{-1}.mL^{-1}$)	Total Activity ($\mu mol.h^{-1}.mL^{-1}$)
RU-KM1	13.0 (±0.6)	5.7 (±1.2)	18.7
RU-OR	1.9 (±0.6)	9.6 (±1.0)	11.5
RU-KM3	15.7 (±1.4)	2.0 (±1.5)	17.7
RU-OG	3.5 (±0.01)	1.2 (±0.7)	4.7
RU-1L	2.3 (±0.9)	1.6 (±1.0)	3.9

From these results, RU-KM1 showed the highest total hydantoinase activity as measured by the addition of the NCG and the glycine produced (using Ehrlich's reagent and ninhydrin reagent respectively). Strain RU-KM3 also showed high hydantoinase activity, but the *N*-carbamoylase activity was lower than that observed in RU-KM1. Strain RU-OR yielded the greatest amount of glycine from these experiments, indicating a highly active *N*-carbamoylase enzyme. On the basis of these results it was decided to further characterise only 2 strains, *viz*. RU-KM1 and RU-OR. It was proposed that if a process were to be developed, a two-stage hydrolysis of the hydantoin would be used. The initial stage might utilise the hydantoinase from RU-KM1 to produce the NCG. This NCG would be further hydrolysed by the *N*-carbamylase from strain RU-OR, liberating the amino acid.

2.2.3 Cell disruption by French-pressing

To determine if there was a substrate / product mass transfer limitation in measuring intracellular enzyme activity, disruption of the cells was investigated. The possibility of mass transfer being a problem was considered important especially when utilising the

more highly substituted hydantoins, such as the butylhydantoins that contain bulky side chains. If the low activities were as a result of poor transport of the substrate into the cell or of poor export of the products out of the cell, this would result in apparent low activities being measured. The disruption of cell membranes might lead to hydrolysis of a wider substrate range, due to the increased access of the substrate to the enzymes. The effect of the disruption of the cells was quantified in terms of the changes in protein content and enzyme activity (Table 2.3). Specific activity (U) was defined as: 1Unit = 1 µmol product mg^{-1} protein min^{-1}.

Table 2.3 shows the results of the optimisation of the French-press method to disrupt cell walls of the two organisms. In the case of RU-KM1, there was more than a 2-fold increase in hydantoinase specific activity of the crude extract obtained after a single pass through the press. The protein concentration increased with subsequent passes through the press, but the enzyme activity did not increase significantly. Thus there was a decrease in calculated specific activity with every subsequent pass through the press.

Strain RU-OR showed the same increase in protein concentration with each subsequent pass through the press (Table 2.3). However, the hydantoinase specific activity droped after a singe pass through the press. This may be as a result of the presence of proteases or as a result of denaturation of the protein due to mechanical stress or heat.

Table 2.3: The changes in hydantoinase activity of strain RU-KM1 and RU-OR with increasing number of passes through the French press

Number of Passes Through Press	Strain	Total hydantoinas activity. ($\mu mol.mL^{-1}$)	Protein conc. ($\mu g.mL^{-1}$)	Specific Activity ($\mu mol.min^{-1}.mg^{-1}$)
0	RU-KM1 RU-OR	0.9 (±0.02) 0.9 (±0.47)	176.25 (±10.8) 164.0 (±6.7)	0.17 0.18
1	RU-KM1 RU-OR	26.8 (±2.65) 1.6 (±0.9)	217.1 (±22.4) 358.4 (±18.5)	2.06 0.07
2	RU-KM1 RU-OR	28.3 (±1.54) 2.0 (±0.75)	276.4 (±9.45) 425.6 (±43.1)	1.71 0.08
3	RU-KM1 RU-OR	31.3 (±3.45) 1.3 (±0.86)	275.0 (±16.7) 531.07 (±12.4)	1.90 0.04
4	RU-KM1 RU-OR	26.2 (±2.14) 3.0 (±0.01)	304.3 (±6.87) 564.2 (±18.3)	1.43 0.09

2.2.4 Culture of strains RU-KM1 and RU-OR

Flask culture experiments were used to determine the growth characteristics of these two organisms. A chemically defined growth medium using 1 % hydantoin as a sole nitrogen source and 1 % glucose as a carbon source was compared to a complete medium supplemented with 1 % hydantoin. The growth was monitored by determining the OD_{600} at 5 hourly internals (Figure 2.3). Samples were removed from flasks for protein determination (Figure 2.4) and enzyme activity (Figure 2.5).

Figure 2.3 shows the results of the growth as determined by a change in the absorbance of the culture. In complete medium, (Figure 2.3A) RU-KM1 reached stationary phase by approximately 10 hours. The culture had a maximum growth rate (μ_{max}) of 0.3 h^{-1} at 5 hours. Growth in this medium resulted in a dry cell weight of 3.5 $g.L^{-1}$. RU-OR reached

stationary phase after 15 hours and had a maximum growth rate of 0.18 h^{-1} (Figure 2.3B). This organism yielded 2.9 $g.L^{-1}$ DCW when cultured in this medium.

When the microorganisms were grown in a defined medium, HMM, slower growth kinetics were observed. RU-KM1 reached stationary phase after 15 hours and had a maximum growth rate of 0.3 h^{-1}. A dry cell weight of 2.2 $g.L^{-1}$ was obtained. Strain RU-OR entered stationary phase after 20 h and showed a maximum growth rate of 0.14 h^{-1}. This organism yielded a dry cell weight of 2.1 $g.L^{-1}$ when grown on the minimal medium. In both cases the lower biomass yield (c.f. complete medium) was attributed to hydantoin being a poor nitrogen source in comparison to the yeast and meat extract contained in nutrient broth. From these results it was clear that the rich medium resulted in faster growth in both cases, with the advantage of an increase in biomass yield. Flasks were removed and the cells disrupted by a single pass through the French press at a pressure of 15 MPa. The protein concentration was calculated by comparison to known standards (Figure. 2.4).

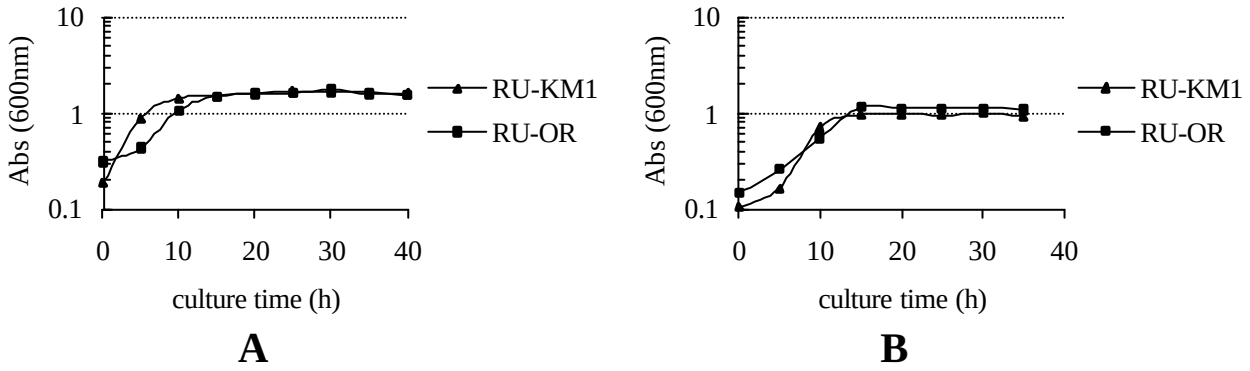
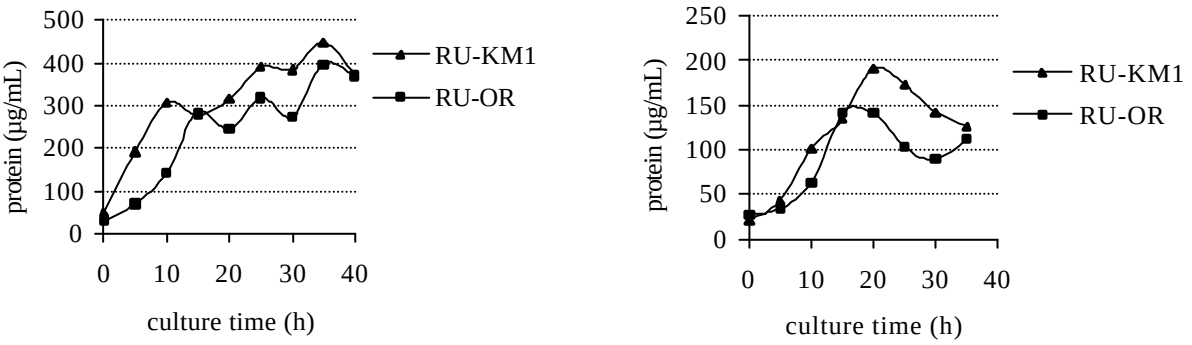


Figure 2.3: Growth curves of RU-KM1 and RU-OR in a complete medium (A) and in hydantoin minimal medium (B)

Flasks were removed and the cells disrupted by a single pass through the French press at a pressure of 15 MPa.

The protein concentration, measured over the duration of growth phase of both organisms (Figure 2.4), was greater in the cultures in nutrient broth supplemented with hydantoin. This is expected due to the presence of the better nitrogen source supplied in the nutrient broth. A maximum protein concentration of 468 $\mu g.mL^{-1}$ was reached after 30 h for RU-KM1 compared with 398 $\mu g.mL^{-1}$ after 35 h for RU-OR.

Protein concentrations for the organisms did not reach more than 200 $\mu g.mL^{-1}$ when the organisms were cultured in the defined medium. This was expected as hydantoin is a poor nitrogen source in comparison to that of the nutrient broth. RU-KM1 reached a final protein concentration of 190 $\mu g.mL^{-1}$ after 20 h and RU-OR had a protein concentration of 141 $\mu g.mL^{-1}$ after 20 h.



A **B**

Figure 2.4: Changes in protein concentration of RU-KM1 and RU-OR in a complete medium (A) and in hydantoin minimal medium (B), over a 40 h batch culture

Enzyme activity was determined at 5 h intervals during the growth of the organisms. The cells were harvested by centrifugation and prepared for a resting cell reaction (Section 2.15). The cells were used for a biocatalytic assay in the presence of 50 mM hydantoin as a substrate.

When RU-KM1 was grown in a complete medium (Figure 2.5) the hydantoinase activity peaked at 25 h with 5 $\mu mol.h^{-1}.mL^{-1}$ NCG and 3.1 $\mu mol.h^{-1}.mL^{-1}$ glycine being produced by 20 $mg.mL^{-1}$ cells during a 1 h reaction. There was a gradual increase in hydantoin-hydrolysing activity after the organism entered stationary phase after 5 hours of growth (Figure 2.3 A). This was probably due to the initial nitrogen becoming limiting, and hydantoin being utilised as an alternative nitrogen source. Subsequent to this a decline in hydantoinase activity after 25 h was observed, this is probably as a result of the organism being well into stationary phase and having exhausted all its nitrogen supplies.

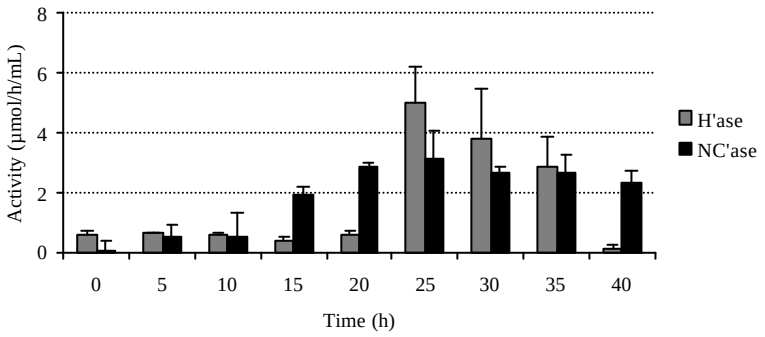


Figure 2.5: Changes in hydantoin-hydrolysing activity of strain RU-KM1 over a 40 h batch culture in a complete medium

Strain RU-OR had higher *N*-carbamoylase activity than strain RU-KM1, with the greatest activity observed after 25-30 h growth, over 10 h into stationary phase, where the nitrogen supplied in the growth medium was depleted and the hydantoin was utilised as a nitrogen source.

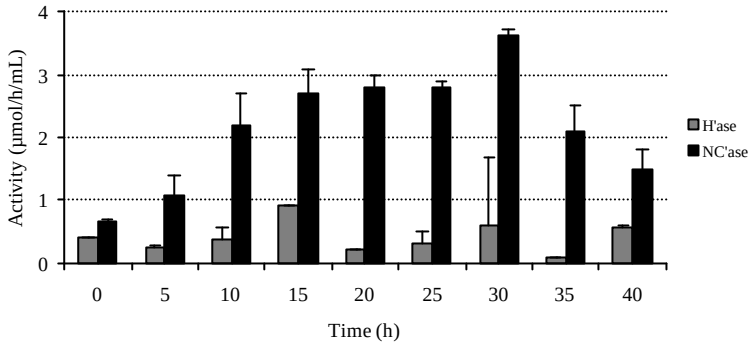


Figure 2.6: Changes in hydantoin hydrolysing activity of strain RU-OR over a 40 h batch culture in a complete medium

A parallel experiment, culturing RU-KM1 and RU-OR in minimal medium using hydantoin as a sole nitrogen source was performed (Figure 2.7).

Figure 2.7A shows the hydantoin-hydrolysing activity of RU-KM1 when cultured in hydantoin minimal medium. Since the hydantoin is the only nitrogen source, hydantoin-hydrolysing activity was detected from the onset of the growth phase. This was expected as the organism needed to produce the hydantoinase enzymes to liberate nitrogen from the hydantoin for cellular growth. A similar trend was observed for strain RU-OR (Figure 2.7B) where the hydantoin-hydrolysing activity was observed from the onset of growth. However, in RU-OR the *N*-carbamoylase activity was geater than the hydantoinase activity under these growth conditions.

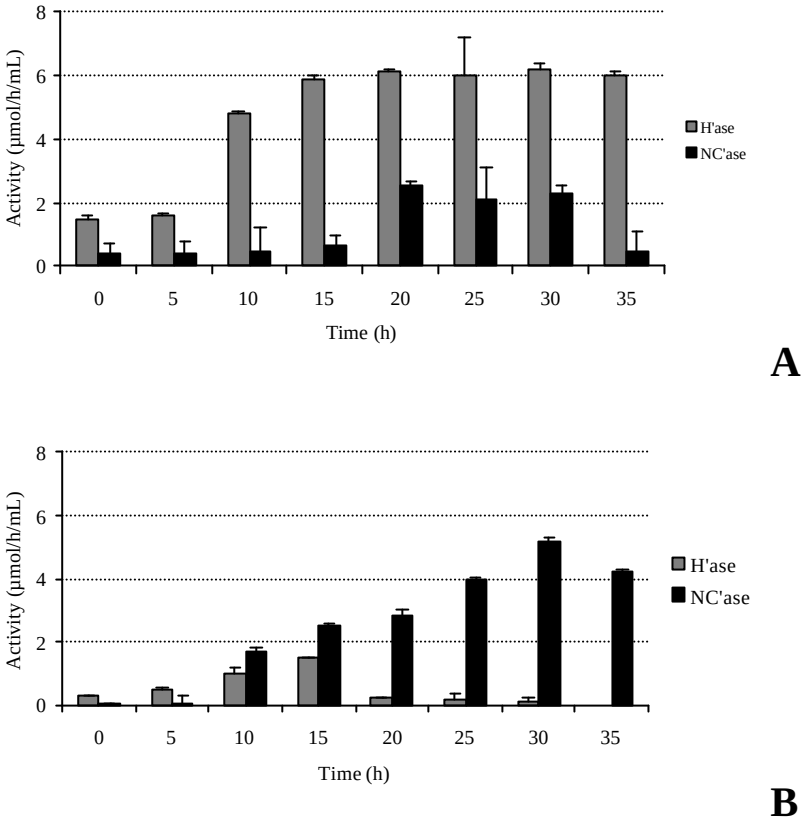


Figure 2.7: Changes in hydantoin hydrolysing activity of strains RU-KM1, (A), and RU-OR, (B), over a 35 h batch culture in a defined medium

2.4 Conclusions

Most of the hydantoin-hydrolysing bacteria isolated to date were obtained from soil samples, using an enrichment technique similar to the method used in this work, and employing hydantoin as the sole nitrogen source (Syldatk *et al.*, 1992). In our laboratory several local organisms were isolated, which showed differing abilities to utilise various hydantoins as substrates. Of the two organisms investigated in this study, strain RU-KM1 (identified as a *Pseudomonad*) showed greater activity in assays of cell extract, with hydantoin as a substrate yielding NCG. Strain RU-OR (identified as *Agrobacterium*) showed high *N*-carbamoylase activity, which resulted in conversion of intermediate NCAA to amino acids, (and hence low concentrations of NCAA was detected in the assays).

The disruption of the cells by passage through a French-press was optimised, resulting in increased hydantoin-hydrolysing activity, when compared to that of intact cells. The disruption of the cell walls reduce any mass transfer limitations imposed by the cell wall, resulting in more effective contact between substrate and active enzyme, and hence the higher product yields observed. These results are consistent with results reported for enzyme activities of other *Agrobacterium* and *Pseudomonas* species (Durham and Webber, 1995; Möller *et al*., 1988; Runser *et al*., 1990)

Both organisms showed typical growth kinetics when cultured in liquid medium. They exhibited a short lag phase followed by exponential growth and finally a stationary phase. Growth in hydantoin minimal medium lasted approximately 20 hours and growth in a complete medium was faster with the organism reaching stationary phase in about 15

hours. The change in protein concentration in cell extracts followed a similar trend to the increase in cell concentration in the culture medium.

The combination of the two microorganisms RU-KM1 and RU-OR was recognised to have potential for utilisation in a bioprocess. The rationale for the selection of these two organisms was that the first enzymatic reaction, the ring-opening hydrolysis of the hydantoin, could be carried out by the hydantoinase from the RU-KM1, which exhibited high hydantoinase activity but lower *N*-carbamoylase activity. The *N*-carbamylamino acid product would be hydrolysed by the *N*-carbamoylase from the second organism, RU-OR, which exhibited better *N*-carbamoylase activity. Thus these two organisms were selected for further enzyme characterisation, and optimisation of reaction conditions to yield the greatest production of NCG and glycine.

Chapter 3

3. Introduction

3.1 General Introduction

Hydantoinases show varied substrate selectivity and stereoselectivity, depending on the source of the enzyme (Syldatk *et al*., 1990a). These enzyme systems can be also considered multifunctional, as the structure of the amino acid that is produced depends on the side chain of the hydantoin derivative used.

This chapter describes an investigation into the substrate specificity of the two previously selected mesophilic hydantoinase-producing bacteria, *viz*., RU-KM1 and RU-OR. An objective of this investigation was to determine the capacity of these strains to hydrolyse the various hydantoin substrates, to elucidate which substrate was most effectively converted by which strain, and hence which strain would be the suitable candidate for production of a particular amino acid. Thus, various 5-monosubstituted hydantoin substrates were synthesised for use as substrates. The conditions used for the biocatalytic reactions of the two strains with the different substrates were based on the results presented in Chapter 2.

The investigation was carried out on both of the strains, comparing their activities using different substrates, and the relative activity of the whole cell and crude extracts of each strains. These strains might exhibit different activities with hydantoin derivatives with varying side chains indicating differing suitability for applications in production of specific amino acids.

3.2 Materials and Methods

3.2.1 Chemicals

Hydantoin and W1 were purchased from Sigma-Aldrich. Acetaldehyde was purchased from Fluka Chemika, and the other aldehydes were purchased from Sigma-Aldrich. Ammonium carbonate ($(NH_4)_2CO_3$) was purchased from Merck N.T. Laboratory Supplies (Pty) Ltd. (S.A.) and potassium cyanide (KCN) was obtained from Saarchem (S.A.). For the synthesis of *p*-hydroxyphenylhydantoin, glyoxylic acid was obtained from Sigma-Aldrich, and urea and phenol were purchased from Merck Laboratory Supplies (Pty) Ltd. Complete® Protease Cocktail was purchased from Boehringer. All chemicals used were of analytical grade.

3.2.2 Synthesis of 5-monosubstituted hydantoin substrates

3.2.2.1 Synthesis of aliphatic 5-monosubstituted hydantoins

The Bucherer-Bergs method was employed in the synthesis of the different hydantoins (Bucherer and Steiner, 1934). 90 mmol of the appropriate aldehyde was dissolved in 100 mL 50 % absolute ethanol. To this solution 18.2 g $(NH_4)_2CO_3$ and 5.2 g KCN was added. The reaction mixture was then stirred under reflux at 60 °C for 2 hours. The mixture was immediately concentrated to 2/3 volume, using a rotary evaporator (Büchi Rotavapor, Labotech) and water bath at 60 °C. The liquid was then cooled on ice, and acidified to pH 2.0 with 50 % HCl. The mixture was frozen in liquid nitrogen and dried on a freeze drier. The resulting powder was dissolved in 100 mL absolute ethanol and any residue obtained was removed by filtration through a Buchner funnel. The ethanol was removed from the filtrate by evaporation on a rotary evaporator at 60 °C. The thick oil resulting from this step was left to crystallise at 4 °C for 48 h. Any liquid present was removed by washing

the crystals with diethyl ether in a Buchner funnel, and the residue left to dry in a vacuum dessicator. ^{1H}NMR was used to determine the purity of the synthesised products.

3.2.2.2 Synthesis of *p*-hydroxyphenylhydantoin

For the synthesis of *p*-hydroxyphenylhydantoin (*p*-HPH), water (16.9 g), 32 % hydrochloric acid (15.4 g), phenol (4.3 g), and urea (5.6 g) were mixed together in a round bottom flask and heated to 90 °C, with constant stirring (Ohashi *et al.*, 1981). Aqueous glyoxylic acid (6.01g, 8.1 mmol) was added drop-wise over 10 h, while the temperature was maintained at 90 °C, under reflux conditions. After about 4 h, a dense white precipitate was observed. After the reaction time was complete, the mixture was left to cool to 4 °C overnight. The precipitate (product) formed was filtered, washed with water, and dried in a vacuum dessicator. The purity of the synthesised substrate was determined by ^{1H}NMR.

3.2.3 Culture conditions for RU-KM1 and RU-OR

Seed cultures were prepared by inoculating the bacterial strains (RU-KM1 and RU-OR) from minimal medium agar plates containing hydantoin as the sole nitrogen source, into 100 mL liquid hydantoin minimal medium (Appendix A2) in 500 mL Erlenmeyer flasks. These were incubated at 28 °C for 3 - 4 d with shaking at 200 rpm on an orbital shaker. Bacterial growth was monitored every 24 h by measuring the change in optical density (600 nm) using a Shimadzu UV-Visible spectrophotometer. The seed cultures were used to inoculate nutrient broth (200 mL) supplemented with 0.1 % hydantoin to give an initial cell optical density (600 nm) of 0.02. These were incubated at 28 °C, with shaking at 200 rpm, until stationary phase was reached.

3.2.4 Resting cell reactions with 5-monosubstituted hydantoin substrates

Cells were harvested by centrifugation at 7000 g for 10 minutes at 4 °C, washed with 0.1 M phosphate buffer (pH 8.0), and the cell pellet recovered by centrifugation at 8000 g. The cell pellet was resuspended in the same buffer at a concentration of 0.1 $g.mL^{-1}$. Biocatalytic reaction mixtures were prepared by the addition of the hydantoin stock (50 mM) substrates (1 mL) and the cell solution (1 mL) to yield a final substrate concentration of 25 mM in a total reaction volume of 2.0 mL.

Due to the poor solubility of *p*-HPH, this substrate was prepared at a stock concentration of 30 mM, and thus the final reaction mixtures contained 15 mM substrate at the start of the reaction. Reactions were carried out for 6 h at 40 °C with shaking at 200 rpm. Hydrolysis of the different substrates by the bacterial cells was monitored by analysis of the supernatant, using Ehrlich's reagent for *N*-carbamylamino acid detection, and ninhydrin reagent for amino acid detection (Chapter 2).

3.2.5 Hydrolysis of synthesised hydantoins of RU-KM1 and RU-OR crude extracts

The crude extract was prepared by passing cells through a French-press at 15 MPa, at a rate of one $drop.s^{-1}$ (Section 2.1.4.2). This procedure was carried out at 4 °C. Biocatalytic reactions were conducted using 1.0 mL of the respective crude extract preparations with substrates, at a final concentration of 25 mM for the aliphatic substrates and 15 mM for *p*-HPH, in a total volume of 2.0 mL. Reaction conditions and product detection were described above (Chapter 2).

3.2.6 Inducer requirements of RU-KM1 cells

A seed culture of strain RU-KM1 was grown to an optical density of 0.85 and used to inoculate nutrient broth (200 mL) (to yield an initial optical density of 0.02), supplemented with the following concentrations of different inducers: 0.01, 0.1 and 1 % hydantoin, 5,5-dihydrouracil, 5,5-dimethylhydantoin and 5-thiouracil. The cells were allowed to grow until they reached stationary phase and all had an absorbance reading of 1. The cultures were harvested and resuspended in 0.1 M cold potassium phosphate buffer, pH 8.0. Cells were disrupted using a French-press and 1 mL portions of the extract were incubated with equal volumes of 100 mM hydantoin for 1 h at 40 °C.

3.2.7 The effect of substrate concentration on the hydantoin-hydrolysing enzymes RU-KM1 crude extract

A solution containing 0.1 $g.mL^{-1}$ crude extract was prepared in potassium phosphate buffer (0.1 M; pH 8.0). 1 mL aliquots of this solution were transferred to reaction bottles. A stock solution of hydantoin (1.2 M) was aliquoted into each of the bottles to give final substrate concentrations of 5, 12.5, 25, 50, 75, 100, 250, 500 and 600 mM, in a total reaction volume of 2 mL. The biocatalytic reactions were carried out at 40 °C for 1 h, shaking at 200 rpm. The concentration of *N*-carbamylglycine and glycine in the supernatants was analysed after the samples were microfuged at 13 000 g for 3 min (Section 2.1.4).

3.2.8 The effect of pH on the hydantoin hydrolysing activity of RU-KM1 and RU-OR crude extract

Cells were harvested as above and washed twice with 0.05 M cold potassium phosphate buffer at neutral pH. After the final wash, a cell suspension was prepared (0.1 $g.mL^{-1}$) in each of the desired buffers at the desired pH. The cells were disrupted in the French press and 1 mL aliquots of the extract were immediately incubated with 1mL of 100 mM hydantoin in each of the following buffers: 0.1 M citric acid-trisodium citrate buffer (pH 4-5), potassium phosphate buffer (pH 6 - 8) or Tris-HCl buffer (pH 7 - 9) or sodium hydrogen carbonate-sodium carbonate buffer (pH 9 - 11). Incubation was for 1 h at 40 °C. Assays for product formation were carried out according to the normal protocol (Section 2.1.4).

3.2.9 The effect of temperature on the hydantoin-hydrolysing activity of RU-KM1 and RU-OR crude extract.

1 mL samples of crude extract were prepared according to the standard procedure (Section 2.1.4.2) for the preparation of crude extract, and incubated with an equal volume of 100 mM hydantoin. Incubation was carried out for 1 hour at 30, 40, 50, 60 and 70 °C. Product formation was determined colorimetrically as before (Section 2.1.4).

3.2.10 The effect of separating cell debris and cell extracts, on the hydantoin-hydrolysing activity of RU-KM1 and RU-OR

A 200 mL HMM culture was grown to late-exponential phase, and the cells were harvested by centrifugation, washed twice in 0.1 M phosphate buffer (pH 8.0) buffer, and resuspended in the same buffer to yield a 0.1 $g.mL^{-1}$ cell suspension. The culture

supernatant was treated with 50 % acetone and the precipitate was collected by centrifugation at 13 000 x g for 20 min, then dissolved in 5 mL of the same buffer. 1 mL aliquots of this solution were transferred to reaction bottles.

The crude extract was prepared by passing cells through a French-press at 15 MPa, at a rate of 10 $mL.min^{-1}$, at 4 °C. A sample of the disrupted cell extract removed and 1 mL aliquots were prepared for reaction with substrate. The remaining extract was clarified at 4 °C by centrifugation at 13 000 g for 20 min to remove the cellular debris. The resulting supernatant was removed and aliquoted into 1 mL samples for reaction with substrate. The pellet was resuspended in the same volume 0.1 M phosphate buffer (pH 8.0) buffer and aliquoted into 1 mL samples for reaction with substrate.

Stock solutions of hydantoin (100 mM) and NCG (50 mM) were aliquoted into each of the reaction bottles to give final substrate concentrations of 50 mM and 25 mM respectively, in a total reaction volume of 2 mL.

3.2.11 Lysozyme treatment of cells to produce protoplasts

Protoplasts were produced by resuspending the washed cells in the same buffer containing 1.0 $mg.mL^{-1}$ lysozyme, and incubated at 37 °C for 2.0 h. After incubation, the cells were centrifuged at 13000 g for 30 min at room temperature, to yield a supernatant (periplasmic fraction) and a protoplast pellet. The protoplasts were lysed by resuspending in cold 0.1 M phosphate buffer (pH 8.0). The lysate was centrifuged at 13000 g for 20 min to give a supernatant (cytosolic fraction) and a pellet (membrane fraction). All liquid samples were lyophilised and the pellet was resuspended in 20 mL ddH_2O and lyophilised to concentrate

the proteins. All samples were tested for hydantoinase activity at a concentration of 0.1 $g.mL^{-1}$ of the lyophilised powder.

3.2.12 The effect of detergent W-1 on the hydantoin-hydrolysing enzymes in RU-KM1 crude extract

An extract solution (Section 2.1.4.2) with a concentration of 0.1 $g.mL^{-1}$ powder was prepared. The solution was aliquoted into reaction bottles (1 mL per reaction). A 4 % (w/v) stock solution of W-1 was added to each of the bottles to give final W-1 concentrations of 0.1, 0.5, and 1 % (w/v). A 0 % control was also prepared. All reactions and controls were prepared in triplicate. The reaction bottles were incubated on ice for 10 min, after which the substrate (1 mL of 50 mM 5-methylhydantoin) was added. The reaction bottles were incubated at 40 °C for 1 h, shaking at 200 rpm. The samples were then microfuged, and production of *N*-carbamylglycine and alanine in the supernatant was analysed (Section 2.1.4).

3.2.13 The effect of selected additives for the hydantoin-hydrolysing activity of RU-KM1 and RU-OR crude extracts

A stationary phase seed culture (200 mL) of RU-KM1 was inoculated into nutrient broth supplemented with 0.1% hydantoin to give an initial OD_{600} of 0.02. Cells were harvested at early stationary phase by centrifugation and resuspended in 0.1 M cold potassium phosphate buffer, pH 8.0. The cells were disrupted by a single pass through a French-press. The effect of the different additive compounds was determined by dissolving them in the same buffer containing the substrate. A single stock solution was made for each of the components to be tested, and diluted to the correct concentration prior to the addition

of the substrate. Aliquots (1 mL) of the extract were incubated with an equal volume of 100 mM hydantoin for 1 h in the presence of the additives: 1 mM DTT, 2 $mg.mL^{-1}$ Complete® Protease Cocktail or a range (0-15 mM) of ATP concentrations. The reaction was carried out at 40 °C for 1 h with shaking at 200 rpm. Samples were microfuged for 3 min and the supernatant used for product analysis. Product concentration (NCG and glycine) was quantified colorimetrically (Section 2.1.4).

3.2.14 The effect of metal ions on the hydantoin hydrolysing activity of RU-KM1

RU-KM1 was cultured until early stationary phase in HMM supplemented with 1 % hydantoin and harvested by centrifugation. Cells were then disrupted by a single pass through the French press, and the extract obtained was dialysed against 0.1 M potassium phosphate buffer, pH 8.0, containing 2.5 mM EDTA for 1.5 h, at 4 °C, with buffer changes every 30 min. 1 mL aliquots were transferred to reaction bottles. Stock solutions of different metal sulphates (Fe^{2+}, Cu^{2+}, Co^{2+}, and Mn^{2+}) were added to the reaction bottles containing extract solutions to give final concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 4 and 5 mM. The volumes were adjusted to 1 mL using the same buffer. Each reaction was carried out in triplicate. The substrate, 1 mL of 100 mM hydantoin, was added to each and the reaction bottles were incubated at 40 °C for 1 h, shaking at 200 rpm. Controls containing the crude extract solution and metal sulphate solutions without the substrate were prepared. The samples were microfuged after reaction and product (*N*-carbamylglycine and glycine) concentrations in the supernatants were analysed. Protein concentration in the crude extract was determined by the method of Bradford (Bradford, 1973). Specific activity was calculated as the concentration of product (µmol.) per µg of protein, per min.

3.2.15 Biocatalytic activity of RU-KM1 whole cells and crude extract using optimal and sub-optimal conditions

A 100 mL HMM culture, grown to late-exponential phase, was inoculated into two 1 L Erlenmeyer flasks containing nutrient broth (200 mL) to give an initial absorbance at 600 nm of 0.02. The flasks were supplemented with 0.1 % hydantoin as an inducer. The cells were grown for 18 h at 25 °C with shaking (200 rpm). The cells in each of the flasks were harvested by centrifugation, washed twice in 0.1 M potassium phosphate buffer pH 8.0, and finally resuspended in the same buffer to yield a 0.1 $g.mL^{-1}$ cell suspension. Aliquots (1 mL) of the cell suspension were removed and added to an equal volume of hydantoin (100 mM) and the mixture was incubated at 40 °C for 3 h. This sample was named "whole cell sub-optimal" (WCSO). Further aliquots were removed and reacted at 50 °C with 100 mM hydantoin. This sample was termed the whole cell semi-optimised (WCO).

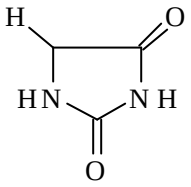
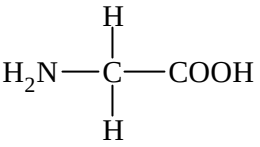
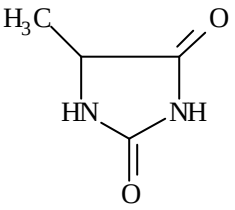
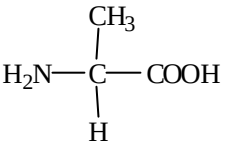
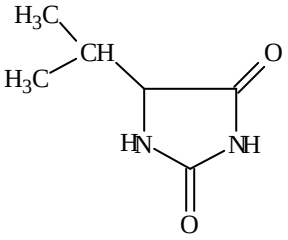
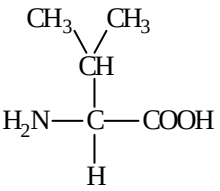
Further aliquots were removed and incubated for 2 hours at 37 °C with lysozyme (1 $mg.mL^{-1}$). The membrane fraction from the lysed cells was collected and reacted with hydantoin (100 mM) for 1 hour at 40 °C. This fraction was termed the "crude extract sub-optimal" (CESO). A further aliquot was incubated in the presence of lysozyme (1 $mg.mL^{-1}$), DTT (1 mM), Complete ® protease inhibitor (2 $mg.mL^{-1}$) and the surfactant W1 (0.1 %) for 2 h. The membrane fractions were collected by centrifugation and added to an equal volume of hydantoin (100 mM), protease inhibitor (2 $mg.mL^{-1}$), ATP (0.5 mM) and $MnSO_4$ (4 mM). This was termed the "crude extract semi-optimised fraction" (CESOF). All samples were centrifuged and analysed for the production of NCG and glycine (Section 2.1.4).

3.3 Results and Discussion

3.3.1 Synthesis of 5-monosubstituted hydantoin substrates

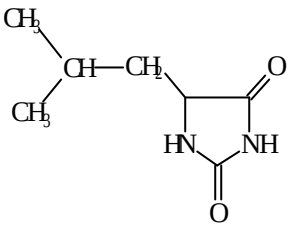
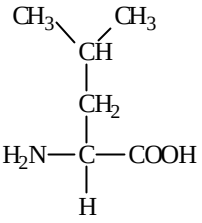
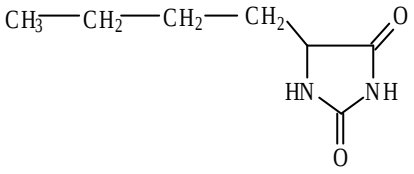
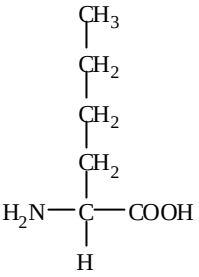
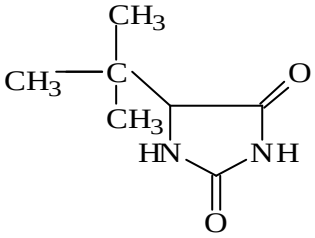
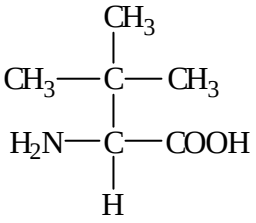
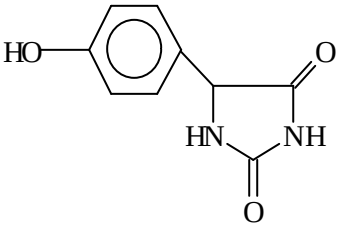
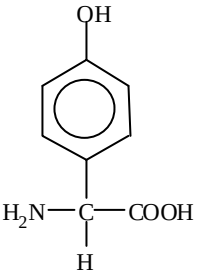
The structures of the synthesised hydantoin derivatives, and the structures of the amino acids resulting from their hydrolysis by hydantoinase and *N*-carbamoylase enzymes, are shown in Table 3.1.

Table 3.1: 5-monosubstituted hydantoin substrates synthesised, yields obtained, and amino acid products obtained by hydantoinase reaction

Chemical structure	Yield (%)	Amino acid
 Hydantoin	n/a*	 Glycine
 5-Methylhydantoin	21.5	 Alanine
 5-Isopropylhydantoin	19.4	 Valine

* obtained commercially

Table 3.1: (contd.) 5-monosubstituted hydantoin substrates synthesised, yields obtained, and amino acid products obtained by hydantoinase reaction

Chemical structure	Yield (%)	Amino acid
 5-*Iso*butylhyantoin	25.0	 Leucine
 5-*n*-Butylhydantoin	27.5	 Norleucine
 5-*tert*-Butylhydantoin	43.5	 *tert*-Leucine
 *p*-HPH	21.1	 *p*-HPG

(Key: *p*- HPH = *p*-Hydroxyphenylhydantoin; *p*-HPG = *p*-Hydroxyphenylglycine)

The purity of the synthesised substrates was confirmed by ^{1H}NMR (Appendix A5). Although the yields were low, the optimisation of the substrate synthesis was not an objective of this study, and the products were just used for further investigation.

3.3.2 Hydrolysis of synthesised hydantoin substrates

The substrate selectivity and specificity of the whole cells and crude extracts of RU-KM1 and RU-OR was investigated using the synthesised substrates.

3.3.2.1 Hydrolysis by RU-KM1 whole cells and crude extracts

The hydrolysis of hydantoin and 5-monosubstituted hydantoin substrates by RU-KM1 whole cells and RU-KM1 crude extract was investigated. Whole cells were cultured as previously described (Section 2.1.4) and reacted different substrates. The reactions were carried out at a final substrate concentration of 25 mM in a total reaction volume of 2.0 mL for all the hydantoins except the *p*-HPH, where the final reaction mixture contained 15 mM substrate. Hydrolysis of the different substrates by the bacterial cells was monitored by analysis of the supernatant, for *N*-carbamylamino acid and amino acid production (Chapter 2). The results are shown in Table 3.2 and Table 3.3 with the substrates listed in order of decreasing total conversion (%). The tables also show percentages of the intermediate that was converted to the amino acids. This indicates the selectivities of the *N*-carbamoylase with repect to their ability to produce the amino acid from the more substituted intermediate. Total conversions of both activities are also tabulated.

Table 3.2: Hydrolysis of hydantoin substrates by RU-KM1 whole cells

Hydantoin Substrate	Final conc* NCAA (µmol.mL^{-1})	Final conc* Amino acid (µmol.mL^{-1})	NCAA converted (%)	Total conversion (%)
5-Isopropyl-	10.9 (±0.02)	3.3 (±0.01)	23.4	57.0
5-*n*-Butyl-	9.5 (±0.03)	1.5 (±0.1)	13.7	44.1
5-Methyl-	7.6 (±0.2)	0.6 (±0.56)	7.6	33.1
5-*tert*-Butyl-	4.1 (±0.6)	3.5 (±0.27)	46.5	30.5
Hydantoin	4.7 (±0.12)	2.1 (±0.12)	31.1	27.3
p-HPH	2.5 (±0.68)	0.9 (±0.3)	26.8	22.9
5-Isobutyl-	2.3 (±0.2)	0.4 (±0.23)	15.1	10.8

This data represents the mean (SEM) of triplicate determinations, (standard errors)
* After 6 h reaction

Table 3.3: Hydrolysis of hydantoin substrates by RU-KM1 crude extract

Hydantoin substrate	Final conc* NCAA (µmol.mL^{-1})	Final conc* Amino acid (µmol.mL^{-1})	NCAA converted (%)	Total conversion (%)
p-HPH	6.7 (±0.3)	1.2 (±0.01)	14.8	52.5
5-Isopropyl-	10.4 (±0.02)	1.3 (±0.01)	11.34	45.6
5-*n*-Butyl-	9.5 (±0.27)	0.1 (±0.01)	1.1	38.4
5-Methyl-	7.9 (±0.02)	0.7 (±0.23)	7.7	34.2
5-*tert*-Butyl-	5.4 (±0.87)	1.0 (±0.01)	16.2	25.7
Hydantoin-	3.4 (±0.01)	2.2 (±0.02)	39.4	22.5
5-isobutyl-	3.1(±0.09)	1.1 (±0.12)	26.6	16.7

This data represents the mean (SEM) of triplicate determinations, (standard errors)
* After 6 h reaction

Table 3.4: Comparison of total hydantoin substrate conversions by RU-KM1 whole cells and crude extract

Hydantoin substrate	Total conversion (%) by whole cells	Total conversion (%) by the crude extract
Hydantoin	27.3	22.5
Isopropyl-	57.0	45.6
n-Butyl-	44.1	38.4
tert-Butyl-	30.5	25.7
Methyl-	33.1	34.2
p-HPH	22.9	52.5
Isobutyl-	10.8	16.7

RU-KM1 whole cells and crude extract demonstrated different substrate selectivity patterns. The substrate hydrolysed to the greatest extent by whole cells was 5-isopropylhydantoin, with a total hydantoin conversion of 57,. %, whereas the preferred substrate for the crude extract was *p*-HPH, with a total conversion of 52.5 %. However, the least hydrolysed substrate in both cases was the 5-isobutylhydantoin, with whole cells and crude extract achieving 10.8 % and 16.7 % conversion of this hydantoin, respectively. The results also indicated that generally, the whole cells showed higher hydantoin hydrolysing activity than the crude extract. Thus in the cases of hydantoin, isopropylhydantoin, *n*-butylhydantoin, and *tert*-butylhydantoin, the whole cell reactions achieved higher total conversion yields than the crude extract reactions (Table 3.4). This may be due to the disruption of conformational or biochemical stability of the enzymes, or some other effect such as product inhibition, oxidation, or changes in chemical conditions when the cells are disrupted by French-pressing. Similar results were obtained for the hydrolysis of methylhydantoin by whole cells and crude extract, where yields were 33.1 % and 34.2 % respectively.

The crude extract reactions gave higher hydrolysis yields than the whole cells with only two of the seven hydantoin substrates tested, namely iso-butylhydantoin and *p*-hydroxyphenylhydantoin. The reason for this could be related to the relative difficulty encountered with the solubility of these substrates and or their steric size. Disruption of cell membranes could have resulted in more ready access of these substrates to the enzymes, and hence the higher yields observed with the crude extract.

Higher NCAA conversion rates were also observed with whole cells than with the crude extract. This is possibly related to or inactivation, oxidation, or other chemical changes of the *N*-carbamoylase enzyme after French-pressing. Whole cells and crude extract of RU-KM1 achieved 31.1 % and 39.4 % conversion of *N*-carbamylglycine, respectively. By contrast, *N*-carbamyl-*tert*-leucine was hydrolysed to a greater extent by RU-KM1 whole cells (46.5 % conversion as compared with crude extract which had a yield of 16.2 % conversion).

These results suggest, overall, that RU-KM1 whole cells would be a better biocatalyst than the crude extract, for the production of various amino acids from 5-monosubstituted hydantoin substrates, and that the biocatalyst would be most suitable for production of glycine (from hydantoin) and valine (from 5-isopropylhydantoin). these results would need to be confirmed using purified enzyme to determine the true kinetics of the enzymes before any definite conclusion could be made on the substrate preferences.

3.3.2.2 Hydrolysis by RU-OR whole cells and crude extract

Whole cells were cultured as previously described (Section 2.1.4) and reacted with stock solutions of the different substrates, at an initial substrate concentration of 25 mM, in a

total reaction volume of 2.0 mL for all the hydantoins except the *p*-HPH, where reaction mixtures contained 15 mM substrate at the start of the reaction. Hydrolysis of the different substrates by the bacterial cells was monitored by analysis of the supernatant, for *N*-carbamylamino acid and amino acid production (Section 2.1.4). The results of the investigation of the substrate selectivity of RU-OR are illustrated in the following tables (Tables 3.6 and 3.7). The substrates are listed in the order of decreasing total conversion.

Table 3.5: Hydrolysis of hydantoin substrates by RU-OR whole cells

Hydantoin substrate	**Final conc* NCAA ($\mu mol.mL^{-1}$)**	**Final conc* Amino acid ($\mu mol.mL^{-1}$)**	**NCAA converted (%)**	**Total conversion (%)**
5-Methyl-	9.7 (±0.2)	2.7 (±0.01)	21.6	49.5
5-Isopropyl-	9.6 (±0.32)	1.4 (±0.32)	13.0	43.9
5-Isobutyl-	7.3 (±0.01)	1.5 (±0.39)	16.0	35.0
5-*tert*-Butyl-	6.8 (±0.45)	0.5 (±0.05)	6.9	29.0
5-*n*-Butyl-	4.5 (±0.85)	0.2 (±0.01)	4.7	18.8
p-Hydroxyphenyl-	1.5 (±0.24)	0.8 (±0.01)	35.1	15.0
Hydantoin	0.8 (±0.14)	1.7 (±0.12)	67.6	10.0

This data represents the mean (SEM) of triplicate determinations, (standard errors)
* After 6 h reaction

Table 3.6: Hydrolysis of hydantoin substrates by RU-OR crude extract

Hydantoin substrate	Final conc* NCAA (µmol.mL^{-1})	Final conc* Amino acid (µmol.mL^{-1})	NCAA converted (%)	Total conversion (%)
5-Isopropyl-	11.4 (±0.01)	1.5 (±0.41)	11.7	51.5
p-Hydroxyphenyl-	6.3 (±0.11)	1.0 (±0.4)	13.4	48.3
5-Isobutyl-	7.6 (±0.03)	3.0 (±0.8)	28.4	42.5
5-Methyl-	6.8 (±0.4)	2.7 (±0.41)	28.8	38.0
5-*tert*-Butyl-	9.1 (±0.3)	0.4 (±0.2)	3.8	38.0
Hydantoin	2.8 (±0.01)	3.1 (±0.25)	53.0	23.6
5-*n*-Butyl-	4.2 (±0.02)	0.1 (±0.41)	3.2	17.3

This data represents the mean (SEM) of triplicate determinations, (standard errors)
* After 6 h reaction

Table 3.7: Comparison of total substrate conversions by RU-OR whole cells and crude extract

Hydantoin substrate	Total conversion (%) by whole cells	Total conversion (%) by the crude extract
Hydantoin	10.0	23.6
Isopropyl-	43.9	51.5
n-Butyl-	18.8	17.3
tert-Butyl-	29.0	38.0
Methyl-	49.5	38.0
p-Hydroxyphenyl-	15.0	48.3
Isobutyl-	35.0	42.5

Different substrate selectivity patterns were observed with whole cells and crude extract of RU-OR (Tables 3.5 and 3.6). The preferred substrate for whole cells was 5-methylhydantoin, where a 49.5 % conversion was observed after 6 hour reaction time.

However, the results from the crude extract reactions indicated that the preferential substrate in this case was the 5-isopropylhydantoin, where a 51.5 % conversion was observed. The crude extract from RU-OR hydrolysed the aromatic 5-substituted hydantoin, *p*-HPH to a greater extent than whole cells, where the biocatalytic conversions were 48.3 % and 15.0 %, respectively. This sugests that the disruption of cell walls and membranes resulted in some improvement of the accessibility of substrate to the enzyme and hence an increase in the conversion yields of substrates. This is further supported by the observation that the crude extract reactions resulted in higher conversion yields than whole cells, with 5 of the tested substrates, namely hydantoin, 5-methyl-, 5-isobutyl-, 5-tert-butyl, and *p*-HPH (Table 3.7). By contrast the whole cells and crude extract hydrolysed the 5-*n*-butylhydantoin to similar extents, with conversion yields of 18.8 % and 17.3 %, respectively.

Since appreciable levels of hydrolysis of 5-isopropyl- (51.5 %), *p*-hydroxyphenyl- (48.3 %), and 5-isobutylhydantoin (42.5 %) were achieved using RU-OR crude extract, it may be suggested that this biocatalyst has potential for application in the production of the amino acids valine (from isopropylhydantoin), *p*-hydroxyphenylglycine (from *p*- HPH), and leucine (from isobutylhydantoin), all of which have valuable industrial applications. In contrast, RU-OR whole cell reactions gave high levels of methyl- and isopropylhydantoin conversion, and thus have potential application in the synthesis of alanine and valine. Alanine is used in the synthesis of enalapril, which is an ACE inhibitor.

3.3.3 Inducer requirements for expression of hydantoin-hydrolysing enzymes in RU-KM1 cells

The production of high levels of hydantoin-hydrolysing enzymes within cells would contribute to high yields of the final product of the biotransformation. Production of the enzymes is controlled at a genetic level, and the induction of the genes involved in the enzyme production would important for the overall success of the process; thus the use of an inducer during the culture of an organism may be necessary. The enzyme activity in RU-KM1 whole cells was investigated to determine whether an inducer was required, and whether any such inducers might be toxic to the cells at the concentrations required (Figure 3.1). Similar work was carried out by other researchers for RU-OR.

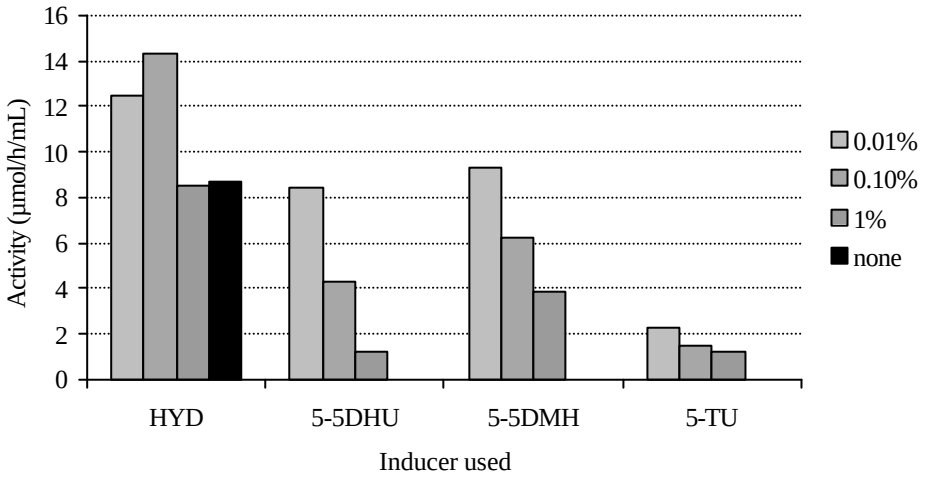


Figure 3.1: The effect of different concentrations of hydantoin (Hyd), 5-5-dihydrouracil (5-5DHU), 5-5-dimethylhydantoin (5-5DMH) and 5-thiouracil (5-TU) used as inducers for the hydantoin-hydrolysing activity of RU-KM1 whole cells

Figure 3.1 shows colorimetric assay data produced from the hydrolysis of hydantoin to NCG and glycine, which represents the total hydantoinase activity. This data shows that a lowering of hydantoin-hydrolysing activities occurred with an increase in inducer concentration above 0.1 % for all inducers tested. However, when hydantoin was used as an inducer, there was an increase in hydantoinase activity from 8.6 $\mu mol.h^{-1}.mL^{-1}$ in the

control to 12.5 and 14.3 µmol.h^{-1}.mL^{-1} in the 0.01 % and the 0.1 % reactions respectively. The use of 5-5DHU and 5-5-dimethylhydantoin had no effects at a concentration of 0.01% but became toxic at the higher concentrations, with a significant reduction in hydantoinase activity. 5-TU inhibited the activity at all concentrations.

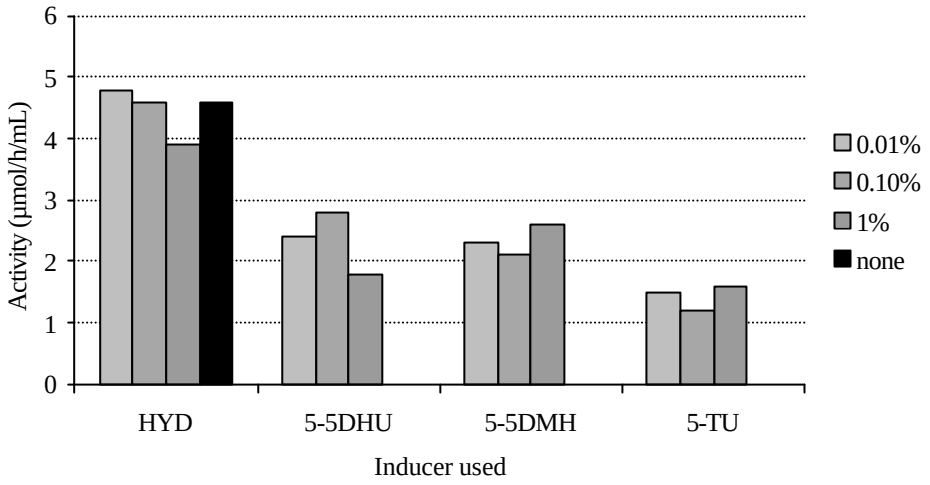


Figure 3.2: The effect of different concentrations of hydantoin (Hyd), 5-5-dihydrouracil (5-5DHU), 5-5-dimethylhydantoin (5-5DMH) and 5-thiouracil (5-TU) used as inducers on the *N*-carbamoylase activity of RU-KM1 whole cells

Figure 3.2 shows the carbamoylase assay activity that was measured after the cells were induced with different concentrations of: hydantoin (Hyd), 5-5-dihydrouracil (5-5DHU), 5-5dimethylhydantoin (5-5DMH) and 5-thiouracil (5-TU). The use of hydantoin as an inducer seemed to have no effect on the *N*-carbamoylase activity of RU-KM1, but there was a slight reduction in activity when 1 % hydantoin was used as an inducer. The 5-5DHU, 5-5DMH and the 5-TU all significantly reduced the *N*-carbamoylase yield, with the greatest effect being in the reaction using 5-TU induced cells. Thus the use of an inducer for RU-KM1 is only important for the induction of the hydantoiase enzyme. The inducers had no effect or inhibited *N*-carbamoylase activity. From a process perspective, this would be problematic if the process was based on the use of both this organism enzymes, however, as the proposed process would utilise only the hydantoinase from RU-

KM1, it would not affect the productivity of the process. The inducer requirements of RU-OR have been determined by other researchers, and the use of 5-TU was shown to increase the *N*-carbamoylase activity two fold (Hartley *et al*., 1998).

3.3.4 The effect of substrate concentration on the total hydantoin-hydrolysing activity of RU-KM1 crude extract

To determine the optimal concentration of the substrate in the reactions, different concentrations of the substrate (0.1 to 1.2 M) were added to the reaction mixtures, and the formation of the products was measured.

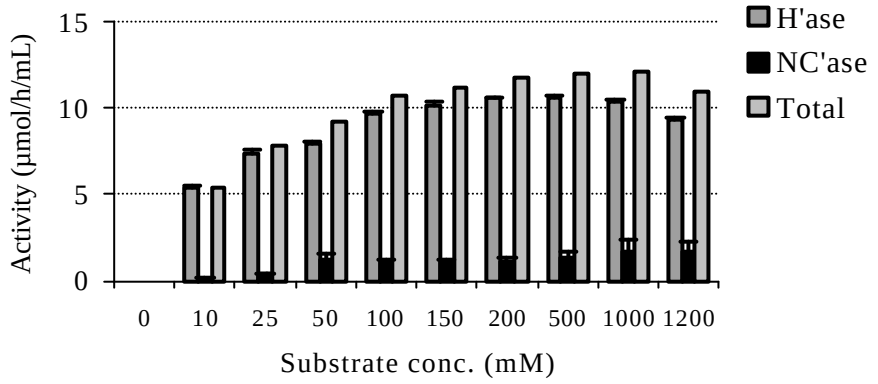


Figure 3.3: The effect of a range of the substrate concentrations on the total hydantoin-hydrolysing activity of RU-KM1 crude extract

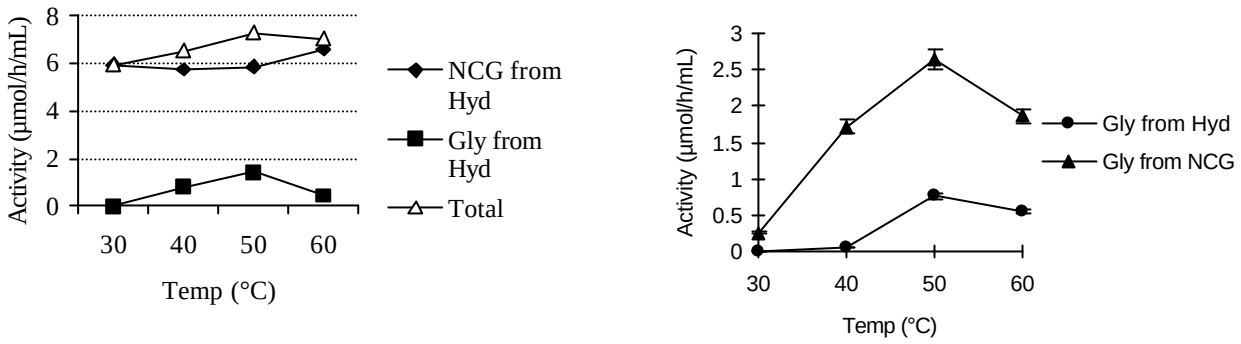
From Figure 3.3 it is evident that the higher concentrations (0.1 - 1.0 M), of substrate were all hydrolysed to a similar extent. There was no obvious substrate inhibition at these concentrations, until 1.2 M, where there was a small decrease in hydantoinase yield, which may be due to substrate inhibition, however without a more complete understanding of the reaction kinetics on a the purified protein, the type of inhibition is not clear. It is important to note that the hydantoin hydrolysis is comprised of a two-stage reaction, the conversion

of the hydantoin to the NCG, and then the conversion of the NCG to glycine. Thus we must consider the *N*-carbamoylase reaction in conjunction with hydantoinase yields. Figure 3.3 shows that there was no significant increase in the carbamoylase activity over the substrate concentration range above 50 mM.

3.3.6 The effects of different temperatures and pH, on the hydantoin-hydrolysing activity of RU-KM1 and RU-OR whole cells

The operational temperature profile of a biocatalyst is important to determine in the development of a process as higher temperatures result in better solubilities and faster reactions rates. The temperature optima of the hydantoin-hydrolysing enzymes of RU-KM1 and RU-OR were determined by incubating the biocatalyst and the substrate (hydantoin) at different temperatures, and assaying for the production of NCG and glycine. For strain RU-KM1, the hydantoinase activity (converting hydantoin to NCG and Gly) was constant over a temperature range of 30 to 60 °C (Figure 3.4a). In the production of glycine from hydantoin, the *N*-carbamoylase of RU-KM1 was also active over a wide temperature range, *viz.*, 40-60 °C. In further experiments, activity ceased at temperatures above 70 °C.

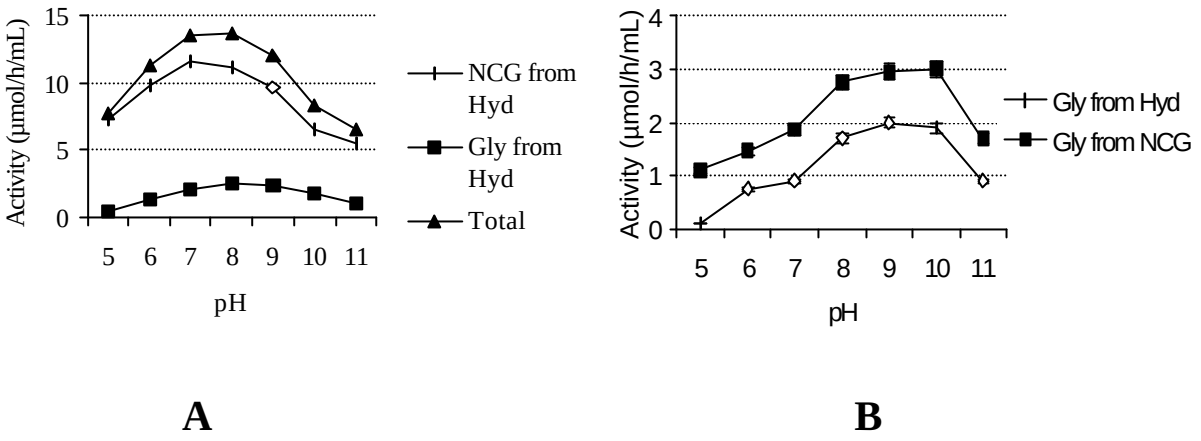
In the case of reactions using the extract of strain RU-OR, low yields were measured at temperatures below 50 °C but at temperatures above 50 °C, higher glycine production than RU-KM1 was observed (Figure 3.4b). The use of NCG, rather than hydantoin, as the enzyme substrate, resulted in higher production of glycine at lower temperatures. This microorganism was originally selected based on its high *N*-carbamoylase activity (Section 2.2.1). The results of this work indicate that the use of this organism as a biocatalyst for the conversion of the NCG to glycine is enhanced by an increase in temperature.



A **B**

Figure 3.4: The effect of temperature on hydantoinase and *N*-carbamoylase activity of RU-KM1 (A) and RU-OR (B) cell extracts

In experiments to determine pH optima, the conversion of hydantoin to NCG by RU-KM1 was found to be optimal between pH 7.0 and 8.0. Glycine production from both hydantoin and from NCG was optimal between pH 7.0 and 8.0 (Figure 3.5a). Glycine production using RU-OR extracts was enhanced under more alkaline conditions and optimal production was achieved at around pH 10.0 (Figure 3.5b). This result suggests a higher pH optima for the *N*-carbamylaminoacid amidohydrolase of RU-OR than that of the hydantoinase from this organism, as shown for bacterial hydantoin-hydrolysing enzymes (Takahashi *et al.*, 1979; Olivieri *et al.*, 1981; Syldatk *et al.*, 1990a; Gokhale *et al.*, 1996).



A **B**

Figure 3.5: The effect of pH on hydantoinase and *N*-carbamoylase activity in RU-KM1 (A) and RU-OR (B) crude extracts

3.3.7 Cellular location of RU-KM1 hydantoin-hydrolysing enzymes

3.3.7.1 Crude Extracts

The location of the hydantoin-hydrolysing enzymes in strains RU-KM1 and RU-OR was unknown. Literature reports suggest that the enzymes to be cytoplasmic (Abendroth *et al.*, 2000a,b; Louwrier and Knowles, 1996, 1997) and not associated with the membrane fractions of the disrupted cells. Lee e*t al*. (2001) reported that the differences in the membrane permeability of intact and disrupted cells played a significant role in the production of the final amino acid. This result was based on the membrane being less permeable to the intermediate in cells with damaged cell walls. In this study comparison of biocatalytic assays carried out in the presence and absence of cell debris, after a single pass through a French-press, showed wide variation in bioconversion yields (Table 3.8).

Table 3.8: Fractionation of hydantoinase and *N*-carbamoylase activities of extracts of RU-KM1 and RU-OR cell extracts

Strain	Product	Product concentration ($\mu mol.h^{-1}.mL^{-1}$)		
		With cell debris	Without cell debris	Resuspended pellet
RU-KM1	NCG Glycine	13.8 (±0.01) 4.3 (±0.1)	3.7 (±0.1) 1.1 (±0.1)	16.3 (±0.1) 1.6 (±0.3)
RU-OR	NCG Glycine	2.1 (±0.05) 8.7 (±0.3)	2.4 (±0.2) 2.4 (±0.2)	2.1 (±0.04) 2.0 (±0.4)

This data represents the mean (SEM) of triplicate determinations, (standard errors)
After a 1 h reaction

When crude cell extracts were added to substrate solutions, hydantoin conversion yields of 13.8 $\mu mol.h^{-1}.mL^{-1}$ and 2.1 $\mu mol.h^{-1}.mL^{-1}$ were observed for RU-KM1 and RU-OR respectively. When cell debris was removed from the RU-KM1 extract by centrifugation, the bioconversion yields were significantly lower (3.7 $\mu mol.h^{-1}.mL^{-1}$). In contrast the NCG detected in RU-OR showed a small increase to 2.4 $\mu mol.h^{-1}.mL^{-1}$ (Table 3.8). Resuspension of the RU-KM1 pellet in buffer resulted in higher hydantoinase titre (16.3 $\mu mol.h^{-1}.mL^{-1}$) and lower *N*-carbamoylase activity (1.6 $\mu mol.h^{-1}.mL^{-1}$). The latter may be due to the fact that there would be little NCG produced, to be converted to glycine, or that the *N*-carbamoylase enzyme is unstable, as reported by others (Hartley *et al*., 1998; Louwrier and Knowles, 1996). Thus it appears that either the RU-KM1 hydantoinase enzyme or some co-factor that is essential for activity is associated with the cellular debris, whilst the *N*-carbamoylase of RU-KM1 did not appear to be predominantly associated with the cellular debris. This is evident from the similar activities (1.1 $\mu mol.h^{-1}.mL^{-1}$ and 1.6 $\mu mol.h^{-1}.mL^{-1}$) assayed in the fraction without cell debris, and the resuspended pellet. The low *N*-carbamoylase activity in the fraction without cell debris can be attributed to the removal of the hydantoinase enzyme and thus the decrease in the production of the NCG,

which is the substrate for glycine production. When the RU-KM1 pellet was resuspended, a small amount of *N*-carbamoylase activity was detected (1.6 $\mu mol.h^{-1}.mL^{-1}$). This is to be expected as most of the *N*-carbamoylase was removed in the soluble fraction. This was further supported by the fact that the production of glycine from NCG showed no significant decrease in glycine production when the cellular debris was removed (2.3 $\mu mol.h^{-1}.mL^{-1}$ to 2.1 $\mu mol.h^{-1}.mL^{-1}$) respectively (Table 3.9).

Table 3.9: The production of glycine from NCG by cellular fractions of RU-KM1 and RU-OR crude extract

Strain	Glycine concentration ($\mu mol.h^{-1}.mL^{-1}$)		
	With cell debris	**Without cell debris**	**Resuspended pellet**
RU-KM1	2.3 (0.002)	2.1 (±0.3)	1.1 (±0.3)
RU-OR	8.7 (0.12)	7.4 (± 0.01)	2.0 (±0.1)

This data represents the mean (SEM) of triplicate determinations, (standard errors)
After a 1 h reaction

RU-OR showed relatively high amounts of glycine production (8.7 $\mu mol.h^{-1}.mL^{-1}$) when the cell debris was included in the reaction. Low amounts of NCG were detected in the reaction when hydantoin was used as a substrate. This is to be expected, since RU-OR was selected on the basis of its high *N*-carbamoylase activity. In the absence of the RU-OR pellet, there were small amounts of product (NCG and Gly) detected from the hydantoin-hydrolysis (2.4 $\mu mol.h^{-1}.mL^{-1}$). This is due to the fact that there would be no NCG produced by the hydantoinase to act as a substrate for the *N*-carbamoylase. With the resuspension of the RU-OR pellet, there were still only small amounts (2.1 and 2.0 $\mu mol.h^{-1}.mL^{-1}$) of activity detected, again suggesting instability of the enzyme. Thus, it

appears that the hydantoinase of RU-OR might be associated with membrane fraction of the cell. Table 3.9 shows *N*-carbamoylase activity of 7.4 µmol.h^{-1}.mL^{-1} in the fraction without cell debris (cellular supernatant), and 8.7 µmol.h^{-1}.mL^{-1} in the crude extract. These results indicate that the *N*-carbamoylase is probably not associated with the cellular debris but with the cytoplasm.

3.3.7.2 Protoplasted cells

To further expand on the results described above (Section 3.3.7.1), RU-KM1 cells were protoplasted by incubating the cells in the presence of lysozyme (Kirby, R. pers comm.). The protoplasts were lysed by resuspending in 20 mL cold 0.1 M phosphate buffer (pH 8.0). The lysate was centrifuged at 13000 g for 20 min to give a supernatant (cytosolic fraction) and a pellet (membrane fraction). This would differentiate the different regions of the cell, and allow better determination of the location of the different enzymes. The different fractions were collected and assayed for hydantoinase and *N*-carbamoylase activity (Tables 3.10 and 3.11).

Table 3.10: Hydantoin-hydrolysing activity of RU-KM1 fractions using hydantoin as a substrate

Fraction	Hydantoinase (µmol.h^{-1}.mL^{-1})	NCAAH (µmol.h^{-1}.mL^{-1})
Crude extract	10.3 (±0.2)	2.6 (±0.06)
Periplasmic	0.5 (±0.01)	1.3 (±0.01)
Cytosolic	1.8 (±0.04)	1.9 (±0.02)
Membrane	17.7 (±0.09)	2.3 (±0.03)

This data represents the mean (SEM) of triplicate determinations, (standard errors)
After a 1 hour reaction

From these results (Table 3.10), it is evident that the majority of the hydantoinase activity (17.7 µmol.mL^{-1}) was present in the membrane fraction with only a small amount of *N*-carbamoylase activity (2.3 µmol.mL^{-1}) present in this fraction. The lack of hydantoinase and *N*-carbamoylase activity in the periplasmic and cytosolic fractions supports the suggestion that the hydantoinase activity of RU-KM1 is membrane-associated. The lack of *N*-carbamoylase activity in these two fractions was expected, under these conditions, as hydantoin was used as a substrate, so no NCG was produced for conversion to glycine. The positive control (crude extract) displayed both hydantoinase and *N*-carbamoylase activity. When NCG was used as a substrate (Table 3.11), a small amount (1.3 µmol.h^{-1}.mL^{-1}) of glycine was produced by the membrane fraction. Significant activity (3.4 µmol.h^{-1}.mL^{-1} and 6.1 µmol.h^{-1}.mL^{-1}) was detected in the periplasmic and cytosolic fractions respectively. This again supports the previous findings that the *N*-carbamoylase is not membrane-associated.

Table 3.11: *N*-carbamoylase activity of RU-KM1 fractions using NCG as a substrate

Fraction	**Gly (µmol.mL^{-1})**
Crude extract	4.7 (±0.01)
Periplasmic	3.4 (±0.1)
Cytosolic	6.1 (±0.6)
Membrane	1.3 (±0.1)

This data represents the mean (SEM) of triplicate determinations, (standard errors) after a 1 hr reaction

3.3.8 The effect of detergents for membrane solubilisation

Surfactants were added to membrane fractions to investigate the release of enzymes from cell membranes. When Triton X-100 and Triton X-114 were added to the cell extracts, and assays were carried out over 30 mins, the yields of NCG and glycine were unchanged (data not shown). However, when the surfactant polyoxyethylene ether W-1 was used, increased NCG production was observed in reactions using extracts of RU-KM1 (Table 3.12). This suggests that the hydantoinase from RU-KM1 was released from the membranes in an active form or the enzyme was activated by the detergent. However, the amounts of glycine produced from NCG were lower when compared to previous investigations (Table 3.10). Thus the observed increase in NCG concentration is likely to be a combination of an inhibition of the *N*-carbamoylase activity, and release of the membrane-bound hydantoinase (Table 3.12). When NCG was used as a substrate there was a general decrease in activity in the presence of 1 % W-1 as compared to its absence. In the case of RU-OR extract, it is likely that the increase in hydantoinase activity was also due to the release or activation of the hydantoinase enzyme, and inhibition of the *N*-carbamylamino acid amidohydrolase.

Table 3.12: The effect of different concentrations of polyoxyethylene ether W-1 on hydantoinase and *N*-carbamoylase activities of RU-KM1 and RU-OR crude extract using 100 mM hydantoin as a substrate

[POE W-1]* (%)	RU-KM1 H'ase ($\mu mol.h^{-1}.mL^{-1}$)	RU-KM1 NCAAH ($\mu mol.h^{-1}.mL^{-1}$)	RU-OR H'ase ($\mu mol.h^{-1}.mL^{-1}$)	RU-OR NCAAH ($\mu mol.h^{-1}.mL^{-1}$)
0	10.5 (±0.001)	6.7 (±0.01)	0.0 (±0.02)	0.4 (±0.44)
0.1	16.9 (±0.15)	1.0 (±0.11)	9.3 (±0.3)	0.2 (±0.75)
0.25	16.5 (±0.05)	1.4 (±0.16)	9.7 (±0.87)	1.0 (±0.01)
0.5	16.0 (±0.15)	2.3 (±0.23)	10.4 (±0.56)	0.8 (±0.03)
1.0	15.8 (±0.24)	1.9 (±0.12)	11.1 (±0.35)	0.7 (±0.12)

This data represents the mean (SEM) of triplicate determinations, (standard errors) after a 6 h reaction
* POE W-1 : Polyoxyethylene ether W-1

Table 3.13: The effect of different concentrations of polyoxyethylene ether W-1 on *N*-carbamoylase activities of RU-KM1 and RU-OR using 25 mM NCG as a substrate

[POE W-1]* (%)	RU-KM1 NCAAH ($\mu mol.h^{-1}.mL^{-1}$)	RU-OR NCAAH ($\mu mol.h^{-1}.mL^{-1}$)
0	3.5 (±0.2)	6.4 (±0.3)
0.1	1.2 (±0.4)	2.3 (±0.4)
0.25	1.4 (±0.3)	1.4 (±0.02)
0.5	1.4 (±0.2)	1.9 (±0.03)
1.0	1.5 (±0.04)	2.1 (±0.04)

This data represents the mean (SEM) of triplicate determinations, (standard errors) after a 1 h reaction
* POE W-1 : Polyoxyethylene ether W-1

3.3.9 Addition of activity-promoting compounds

The effects of DTT, ATP and protease inhibitors were investigated, as a possible means of enhancing the enzyme activities in the cell extracts by inhibition of proteolysis and stabilisation of disulphide bridges, or in the case of ATP, to determine whether the enzymes were ATP dependent.

In the presence of 1 mM DTT, the hydantoinase and *N*-carbamoylase activities of RU-KM1 increased by 19.4 % (20.3 $\mu mol.h^{-1}.mL^{-1}$ to 24.3 $\mu mol.h^{-1}.mL^{-1}$) and 20.0 % (4.5 $\mu mol.h^{-1}.mL^{-1}$ to 5.4 $\mu mol.h^{-1}.mL^{-1}$), respectively. In the case of RU-OR, the hydantoinase activity was unaffected by the addition of the DTT (5.8 $\mu mol.h^{-1}.mL^{-1}$ to 5.3 $\mu mol.h^{-1}.mL^{-1}$), and *N*-carbamylamino acid amidohydrolase activity was reduced by 51 % (7.0 $\mu mol.h^{-1}.mL^{-1}$ to 3.4 $\mu mol.h^{-1}.mL^{-1}$).

In the presence of a cocktail of protease inhibitors, the hydantoinase activity of RU-KM1 was higher by 42.6 % (20.3 $\mu mol.h^{-1}.mL^{-1}$ to 28.9 $\mu mol.h^{-1}.mL^{-1}$). The higher production of glycine was attributed to the increased amounts of NCG available for bioconversion and possible stabilisation of of the *N*-carbamoylase activity. The addition of protease inhibitors had no effect on the hydantoinase activity of RU-OR (Table 3.14).

Table 3.14: The effect of DTT and protease inhibitor on the hydantoinase and *N*-carbamoylase activities of RU-KM1 and RU-OR using 50 mM hydantoin as a substrate

Additive	RU-KM1		RU-OR	
	H'ase ($\mu mol.h^{-1}.mL^{-1}$)	NCAAH ($\mu mol.h^{-1}.mL^{-1}$)	H'ase ($\mu mol.h^{-1}.mL^{-1}$)	NCAAH ($\mu mol.h^{-1}.mL^{-1}$)
None	20.3 (±0.1)	4.5 (±0.2)	5.8 (±0.1)	7.0 (±0.1)
1 mM DTT	24.2 (±0.4)	5.4 (±0.5)	5.3 (±0.2)	3.4 (±0.1)
2 mg/mL Compl	28.9 (±0.2)	5.6 (±0.2)	5.7 (±0.1)	4.1 (±0.1)

This data represents the mean (SEM) of triplicate determinations, (standard errors) after 1 h reaction
DTT : dithiothreitol
Compl: Complete protease inhibitor ® (Roche)

There have been reports of ATP-dependent hydantoin hydrolysing enzymes (Kim *et al.*, 1987; Ogawa *et al.*, 1995b,c). Hydantoin-hydrolysing activity of both strains was therefore monitored at a range of ATP concentrations. An increase in hydantoinase activity of RU-KM1 extract was observed over the full range of concentrations used (Table 3.15), other than the highest concentration used (15 mM). Highest activity (8.4 $\mu mol.mL^{-1}$) was seen with the addition of 0.5 mM ATP, where there was a 44 % increase in activity. This trend was not evident in the production of glycine from the NCG. Together these data suggest that the RU-KM1 hydantoinase is ATP-dependent, although high concentrations (>10 mM) may be inhibitory. A similar result was reported by Ishikawa *et al.* (1993), who showed ATP-dependent hydantoin ring cleavage and *N*-carbamoylase inhibition at concentrations greater than 10 mM in *Pseudomonas* sp. NS671.

Table 3.15: The effect of different concentrations of ATP on the hydantoinase and *N*-carbamoylase activity of RU-KM1 and RU-OR using 100 mM hydantoin as a substrate

[ATP] (mM)	RU-KM1		RU-OR	
	H'ase ($\mu mol.h^{-1}.mL^{-1}$)	NCAAH ($\mu mol.h^{-1}.mL^{-1}$)	H'ase ($\mu mol.h^{-1}.mL^{-1}$)	NCAAH ($\mu mol.h^{-1}.mL^{-1}$)
0	5.8 (0.09)	2.3 (0.02)	n/d	2.5 (0.23)
0.1	7.8 (0.12)	2.5 (0.52)	n/d	2.5 (0.07)
0.5	8.4 (0.02)	2.5 (0.51)	n/d	2.4 (0.06)
1.0	8.0 (0.08)	2.6 (0.47)	n/d	2.5 (0.16)
5	7.6 (0.32)	2.7 (0.62)	n/d	2.1 (0.27)
10	6.9 (0.12)	2.1 (0.65)	n/d	0.9 (0.37)
15	5.2 (0.22)	2.2 (0.12)	n/d	1.4 (0.29)

This data represents the mean (SEM) of triplicate determinations, (standard errors) after a 1 h reaction
n/d = not determined

3.3.10 The effect of metal ions on the hydantoin-hydrolysing activity of RU-KM1 crude extracts

It has been reported that various metal ions may affect both hydantoinase and *N*-carbamoylase activities (Syldatk *et al*., 1987; May *et al*., 1998b), ranging from total inhibition to the significant activation. May *et al*. (1998b) showed that the hydantoinase of *Arthrobacter aurescens* DSM 3745 was a metalloenzyme containing a catalytically important zinc ion. Mukohara *et al*. (1994) showed similar results with *Bacillus stearothermophilus* NS1122A, where Mn^{2+} and Co^{2+} were important for activity in a purified extracts. In this study, the effects on specific activities of RU-KM1 enzymes in the presence of four different metal ions at concentrations ranging from 0 mM to 5 mM were investigated (Figure 3.6 and Figure 3.7).

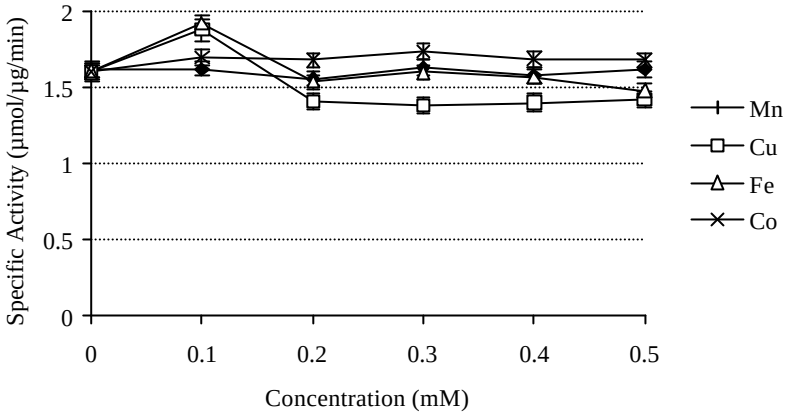


Figure 3.6: The effect of varying low concentrations (0 to 0.5 mM) of different metal ions, on the hydantoinase specific activity of RU-KM1

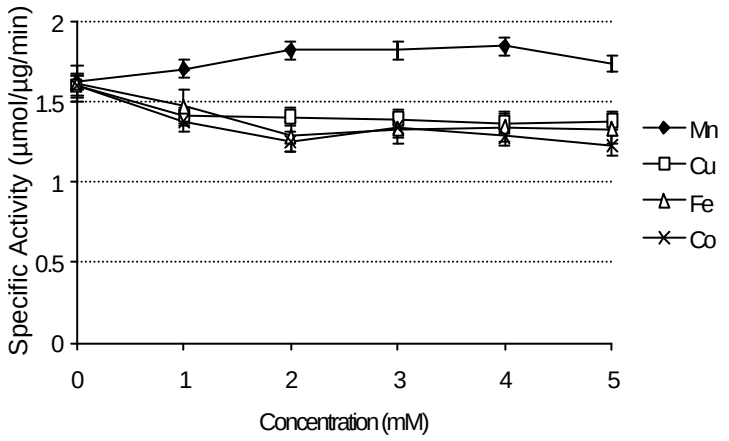


Figure 3.7: The effect of varying high concentrations (0 to 5 mM) of different metal ions, on the hydantoinase specific activity of RU-KM1

$MnSO_4$ appeared to have no effect on the specific activity of RU-KM1 hydantoinase at low concentrations, below 0.5 mM (Figure 3.6). However, at higher Mn^{2+} concentrations (1-4 mM) an increase in specific activity was observed, with the highest specific activity (1.8 $\mu mol.\mu g^{-1}.min^{-1}$) measured at 4 mM (Figure 3.7). In the presence of $FeSO_4$ and $CuSO_4$ at concentration of 0.1 mM, a higher specific activity was measured, whilst specific activity decreased with an increase in concentrations thereafter. At higher concentrations

(Figure 3.7) there was a small decrease in specific activity in all the metal ions except the Mn^{2+}.

3.3.11 Evaluation of optimal biocatalytic conditions for RU-KM1 whole cells and crude extract

The optimal reaction conditions determined in the previous experiments were combined in a single reaction to determine the total effect of these parameters on the hydantoin hydrolysing activity of RU-KM1.

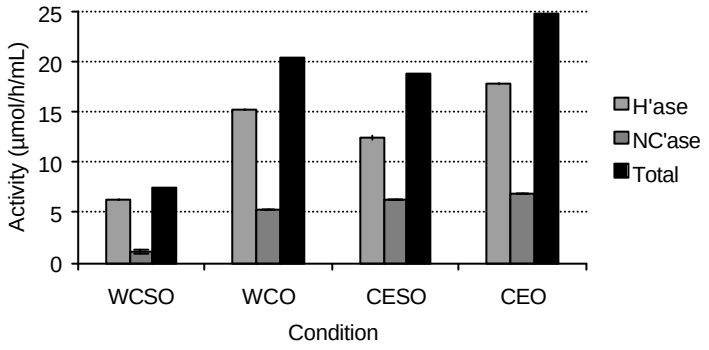


Figure 3.8: Comparison of the hydantoin-hydrolysing activities using whole cells and crude extract, under sub-optimal (WCSO), (CESO) and semi-optimised conditions (WCO), (CEO)

Figure 3.8 shows of the relative activities when whole cells and crude extracts were treated under semi-optimised and sub-optimal conditions (Section 3.2.15). The whole cells that were reacted under sub-optimal conditions showed a 14.8 % (7.4 $\mu mol.h^{-1}.mL^{-1}$) conversion of the substrate to NCG and glycine. In comparison the cells that were reacted under the semi-optimised conditions at 50 °C, showed a 41.6 % (20.5 $\mu mol.h^{-1}.mL^{-1}$) conversion over the 1 h assay period. The crude extract reacted under sub-optimal conditions yielded a 37.5 % (18.8 $\mu mol.h^{-1}.mL^{-1}$) conversion in 1 h. The crude extract that was incubated under the semi-optimised conditions, (addition of protease inhibitor (2

mg.ml^{-1}), ATP (0.5 mM)) and $MnSO_4$ (4 mM)) showed a 49.75% (24.9 µmol.h^{-1}.mL^{-1}) conversion over a 1 h assay period. Crude extract produced by bursting protoplasts of whole RU-KM1 cells and reacting them at 50 °C in the presence of Complete ® protease inhibitor (2 mg.mL^{-1}), ATP (0.5 mM) and $MnSO_4$ (4 mM) resulted in almost 50 % of the substrate being converted to NCG and glycine.

Thus, in the whole cell and the crude assays, under semi optimised conditions a 42 and 50 % improvement in activity was measured respectively. It appears that the extracts activity was better under these conditions, however from a process perspective it may be advantageous to use whole cells as there would be no need to add some of the additives for activity when using the whole cells as a biocatalyst.

3.4 Conclusions

This chapter describes the further optisation of the biocatalytic reactions through the addition of different compounds with the primary aim of enhancing hydantoin-hydrolysing activity of the two organisms under consideration.

5-monosubstituted hydantoin substrates were successfully synthesised by the method of Bucherer-Bergs, with product yields ranging from 19.4 % to 43.5 %. Products were shown to be free of impurities by analysis using ^{1H}NMR. Both crude extracts and whole cells of strains RU-KM1, and RU-OR were capable of hydrolysing all seven hydantoin substrates investigated, with differing degrees of conversion. Whole cells of RU-KM1 showed some selectivity for 5-isopropylhydantoin (57 % conversion), whilst the crude extract of this organism was the most effective in conversion of *p*-hydroxyphenylhydantoin (52 % conversion). Whole cells of strain RU-OR exhibited some selectivity for 5-methylhydantoin (49 % conversion) while the crude extract of this organism showed a 51.5 % conversion with 5-isopropylhydantoin. In both cases the differences in selectivities of the crude and whole cells is probably a result of mass transfer effects. Both strains were found to have relatively broad substrate selectivity. The hydantoins containing the more hydrophobic side chains resulted in better reaction yields. This may be due to the 5-C substituent interacting with other regions of the protein, resulting in better positioning of the hydantoin ring in the active site of the enzyme. This is further supported by the poor reaction yields observed with hydantoin as a substrate.

Different concentrations of hydantoin analogues were used to assess their ability to induce the hydantoin-hydrolysing activities in growing cultures. The most effective inducer was

hydantoin at a concentration of 0.1 %. Dihydrouracil, thiouracil and dimethylhydantoin, at concentrations greater than 0.01 % resulted in substantial loss of hydantoinase activity. *N*-carbamoylase activity was not affected by addition of hydantoin, while the addition of dihydrouracil, thiouracil and dimethylhydantoin to the culture medium resulted in a significant reduction. Thus for the induction of hydanoinase activity in RU-KM1, hydantoin was the best inducer for hydantoinase activity. These results are consistant with results reported for other Pseudomondas sp (Meyer and Runser, 1993; Watabe *et al*., 1992a,b,c).

In the investigation of the effect of substrate concentrations on hydantoinase activity in RU-KM1 crude extracts, concentrations between 0.1 and 1 M hydantoin were found to be the most optimal in terms of total hydantoinase activity.

RU-KM1 hydantoinase showed a broad operating temperature range (30 to 60 °C) would be feasible, and the highest hydantoinase activity was obtained at 60 °C. The *N*-carbamoylase of RU-KM1 also exhibited the same broad temperature profile, with an optimum between 50 and 60 °C. RU-KM1 hydantoinase was active between pH 5 and 10, with ioptimum activity at pH 7.0. The *N*-carbamoylase showed similarly broad pH range, but with an optimum between pH 8 and 9. These result are consistent with those reported for hydantoin-hydrolysing enzyme systems of other bacteria (Runser and Meyer 1993; Takahashi *et al.,* 1978; Morin *et al.,* 1986b)

The *N*-carbamoylase of RU-OR showed the same broad temperature profile, with its optimum at 60 °C. The pH profiles of hydantoinase activity in the two organisms also showed a broad operational range. The *N*-carbamoylase activity of RU-OR exhibited a

higher pH-activity optimum (pH 9 to 10). Theses results are consistent with reported results on other *Agrobacterium* sp (Durham and Webber, 1995; Möller *et al*., 1988; Runser *et al*., 1990).

To determine the location of the enzymes within the cells, cellular membranes were disrupted and sub-cellular fractions were assayed for hydantoinase and *N*-carbamoylase activity. No reports have given conclusive evidence of the location of the enzymes in any organism. The results from this study indicated that the hydantoinase of RU-KM1 was associated with the membranes, since the removal of membrane fractions from French-pressed and protoplasted cells resulted in an almost complete loss of activity. The resuspension of cellular debris and membranes from the burst protoplasts resulted in the recovery of the hydantoinase activity. The *N*-carbamoylase was not associated with the membranes of the cells, and was thus not affected by the removal of the cell debris. Activity was largely detected in the cytosolic fraction of the protoplasts. The differential localisation of the two enzymes may facilitate their further purification by removal of extraneous contaminating proteins from the preparation. Similar purification work is currently being conducted by I. Foster on the RU-OR microorganism.

Numerous surfactants were used in attempts to dissociate the hydantoinase from the cell membranes, but release of the enzyme was only successfully achieved after addition of the W-1 surfactant. This surfactant increased the yield of NCG due to the hydantoinase liberation or activation in both RU-KM1 and RU-OR extracts. However, it also resulted in the loss of the *N*-carbamoylase activity of both organisms. Thus this surfactant would only be useful for the liberation of the hydantoinase enzyme and would not be a feasible option in a process.

The addition of a cocktail of protease inhibitors increased the hydantoinase activity in RU-KM1, but had no effect on the activity in RU-OR extracts. The amino acid yield resulting from RU-KM1 *N*-carbamoylase activity was greater. This may be because of increased NCG produced, and available for conversion. The *N*-carbamoylase activity of RU-OR was unaffected by the addition of protease inhibitors.

The presence of the reducing agent DTT also enhanced the hydantoinase activity of RU-KM1, suggesting that the enzymes contain –SH groups that were protected from oxidation. The increase in glycine produced under these conditions can be attributed to the increased amounts of NCG available for conversion. Crude extracts of RU-OR showed no hydantoinase or *N*-carbamoylase activities in the presence of DTT.

The addition of ATP at concentrations below 1mM significantly enhanced RU-KM1 hydantoinase activity in crude. At higher concentrations, the hydantoinase activity declined in a concentration-dependent manner. ATP did not have a significant effect on the *N*-carbamoylase activity of RU-KM1. There was a small increase in glycine production at the lower concentrations (0.1 mM) of ATP. RU-OR was not affected by the presence of ATP.

The effects of addition of various metal ions were investigated over a range of concentrations from (0.1 to 5 mM). The metal ions, $MnSO_4$, $CuSO_4$, $FeSO_4$ and $CoSO_4$ displayed differing effects on the enzyme activities. The Mn^{2+} had an activating role in the hydantoinase activity of RU-KM1 at concentration between 1 and - 4 mM. At levels greater than 4 mM, there was a decrease in measured activity. The Fe^{2+} and the Cu^{2+} also

enhanced this activity, but only up to a concentration of 0.1 mM. At concentrations greater than this, the specific activity decreased. Similar results were shown by Sun (1983), and Morin *et al*. (1986b), who reported Fe^{2+} and Mn^{2+} resulting in a stimulatory effect on hydantoinase activity, and Cu^{2+} led to an inhibitory effect.

Both whole cells and crude extract of RU-KM1 showed a significant increase, 26.8 and 12.3 % respectively, when they were incubated under semi-optimal conditions involving addition of protease inhibitors, reducing agent and ATP, coupled to an increased reaction temperature (40 up to 50 °C) for the reaction.

The results discussed in this chapter have shown different requirements and possibilities for the use of RU-KM1 and RU-OR in a bioprocess to produce amino acids from hydantoins. Strain RU-KM1 showed good hydantoinase activity, and was capable of hydrolysing a broad set of hydantoins to differing degrees of conversion. The degree of conversion that was evident for this strain was different when whole cells or extract was used, with the more substituted hydantoins showing llower general conversions in the whole cells than in the extracts. This was probably as a result of reduced mass transfrer into the whole cells. Similar to other enzymes reported in literature, this organisms enzymes were inducible by hydantoins. The best inducer for this organism was hydantoin when used at a concentration of 0.1 %. This organism also showed a broad temperature and pH range, making it a good candidate for a process, as substrates show better solubilities at higher temperatures and reaction rates would be higher. The hydantoinase from RU-KM1 was shown to be associated with the membrane fraction of disrupted cells of this microorganism, this is a novel finding and has not been reported in the literature to date. The *N*-carbamoylase was shown to be cytoplasmic, which is consistent with the

literature. The optimisation of the biotransformation conditions resulted in 50 % improvement in yield over the unoptimised conditions. Again, there were difference in the whole cells and extracts in terms of product yields. These differences occurred in the additives that were needed to enhance activity or reduce the loss of activity. From a process perspective the economics of this would be the deciding factor for the use of crude extracts or whole cells.

Strain RU-OR has been well studied in our laboratories (Hartley *et al*, 1998; Hartley 2001, Burton *et al*., 1998). This organism has shown wide substrate selectivity, with differing degrees of conversion being shown between the whole cells and the extracts. The best inducer for this organism was 5-thiouracil, which resulted in a 2-fold inrease in acivity (Hartley, 2001; I. Foster pers comm.). Although strain RU-OR has shown a broad operating temperature and pH profile its optima were slightly different to that of RU-KM1, however there is significant overlap in the ranges that would allow them to be used successfully in a bioprocess together.

Chapter 4

4. Introduction

4.1 General Introduction

This chapter discusses the use of lysis methods, and evaluates specific techniques for possible further use in purification of the hydantoinase from RU-KM1. When evaluating the use of lysed cells or purified enzyme as biocatalysts in the development of an industrial process, it is important to consider that lysed cells have no growth requirements that viable cells do. However, the cost involved in purification may outweigh the advantages of having a purified protein or cell lysate. For this study it was necessary to investigate the potential of purification of the protein in order to make a comparison with intact cells for use as a biocatalyst in a process.

The preparation of lysates is one of the more critical stages in the purification of proteins from bacterial cells, this process influences the quantity of the desired protein, and its biological activity and function. Numerous variables determine the efficiency of a particular lysis method. The successful retention of biological activity of the protein may be influenced by: whether the cells are processed immediately or frozen, the choice of the buffering systems osmolarities, the presence of protease inhibitors, cellular resuspension densities, culture time, and the manner in which the cells are harvested (Neugebauer, 1990). There are typically three classical methods for the lysis of cell, viz, enzymatic lysis, mechanical lysis, and non-enzymatic or chemical lysis. Enzymatic lysis methods tend to reduce protein denaturation, are scale independent, and allow for some selectivity in the release of different cellular products. The lysis occurs through the digestion of the bacterial structural components (e.g., peptidoglycan cell wall) thereby exposing the inner

cytoplasmic membranes. The cytoplasmic membranes are then disrupted by detergents, osmotic pressure or mechanical action.

Mechanical lysis of cells has several advantages over enzymatic lysis. Firstly, it does not require the addition of chemicals; secondly, mechanical techniques are scalable to commercial large-scale production, and thirdly, strain differences are minimised. However, one major drawback is that the mechanical forces used lead to heat generation which must be carefully controlled to prevent protein denaturation. Futher more, foaming must also be controlled to reduce surface denaturation and oxidation processes. The release of DNA during mechanical lysis may also hinder purification and DNA may need to be removed.

Non-enzymatic or chemical lysis methods are usually used in conjunction with enzymatic or mechanical methods. These techniques involve the addition of permeabilising agents or cell wall synthesis inhibitors.

4.2 Materials and Methods

4.2.1 Chemicals

Hydantoin was purchased from Sigma-Aldrich. All other chemicals were purchased from local suppliers and were of analytical grade. Phospholipase A_2, Chymotrypsin, and Lyticase were purchased from Sigma-Aldrich.

4.2.2 Carbon source manipulation: effects on extracellular polysaccharides (EPS) production

Strain RU-KM1 stored on agar plates containing 10 g glucose, 40 g hydantoin, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 10 mL trace elements, 0.2 mM $MgCl_2$, 0.2 mM $CaCl_2$ per litre. A single colony was picked from the plate and inoculated into a 1 L Erlenmeyer flask containing 200 mL of the same medium. Cells were grown for 25 h at 25 °C with shaking (200 rpm). The cells were harvested by centrifugation (6000 rpm x 10 min), and washed twice in 0.1 M phosphate buffer pH 8.0 at 4 °C. The cells were resuspended in 200 mL of the same buffer. The cells were subcultured into 1 L Erlenmeyer flasks containing 40 g hydantoin, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 10 mL trace elements, 0.2 mM $MgCl_2$, 0.2 mM $CaCl_2$ and either 10 g glucose, or 10 g manitol per litre. The growth of the culture was followed by measuring optical density. The polysaccharide production was determined by precipitation following the addition of 2.5 x sample volume of cold propanol, ethanol or acetone to the sample. The sample was incubated 5 °C for 1 h and microfuged for 10 min. The total dry cell weight (DCW) of each of the different precipitation methods was determined. The amount of polysaccharide produced was calculated by the difference in DCW. The cells from each of the media were harvested, washed, and reacted with 100 mM hydantoin and 50 mM NCG to determine the hydantoin-hydrolysing activity of each of the cultures. The specific activity was defined as the amount (µmol) of product produced per µg of biomass per min.

4.2.3 The preparation of RU-KM1 extracts (for use in purification of RU-KM1 hydantoinase)

4.3.2.1 Enzymatic disruption of RU-KM1 cells

The use of lysozyme as an enzymatic treatment for the production of RU-KM1 lysate is shown in Section 3.2.11. Enzymes utilised in the disruption of RU-KM1 cells were: Phospholipase A_2, Phospholipase C, Chymotrypsin, Lysostaphin and Lyticase. A single colony of RU-KM1 was inoculated into 1 L Erlenmeyer flasks containing 200 mL of the following: 40 g hydantoin, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 10 mL trace elements, 0.2 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 10 g manitol per litre. The culture was incubated at 28 °C for 72 h with shaking (200 rpm). The cells were harvested and washed twice using 0.1 M phosphate buffer pH 8.0. The cells were resuspended (0.1 $g.mL^{-1}$) in the same buffer containing 4 $mg.mL^{-1}$ of the test enzymes, except lysozyme which was used at 1 $mg.mL^{-1}$. BSA (10 $mg.mL^{-1}$) was added to each of the solutions containing the enzymes as a competitive substrate for any proteases present. The solutions (1 mL) were incubated at 35 °C for 1 h with gentle mixing after which the cell debris was removed by centrifugation. 1 mL substrate solution (100 mM hydantoin or 50 mM NCG) was added to the supernatants and incubated at 40 °C for 4 h. The supernatants were assayed for the production of NCG and glycine (Section 2.1.4).

4.2.3.2 Mechanical disruption of RU-KM1 cells

Single colonies of RU-KM1 were inoculated into 1 L Erlenmeyer flasks containing 200 mL the following: 40 g hydantoin, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 10 mL trace elements, 0.2 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 10 g manitol per litre. Cultures were incubated at 28 °C for 72 h with shaking (200 rpm). The cells were harvested and washed twice using 0.1 M phosphate buffer pH 8.0. The cells were resuspended in the same buffer

to yield a final concentration of 0.1 g.mL^{-1}. The cell suspension was sonicated for 7 min with an on/off cycle time of 1 min on 1 min off. Aliquots (4 mL) were removed at 1 min intervals and centrifuged for 10 min to pellet the cell debris. Protein concentration was determined in the supernatant by the method of Bradford (1976). Hydantoinase and *N*-carbamoylase activity was determined in the pellet and the supernatant using 100 mM hydantoin and 50 mM NCG as substrates (Section 2.1.4).

4.2.3.3 Enzymatic disruption of RU-KM1 cells

Single colonies of RU-KM1 were inoculated into 1 L Erlenmeyer flasks containing 200 mL the following: 40 g hydantoin, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 10 mL trace elements, 0.2 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 10 g manitol per litre. Cultures were incubated at 28 °C for 72 h with shaking (200 rpm). The cells were harvested and washed twice using 0.1 M phosphate buffer pH 8.0. The cells were resuspended in the same buffer to yield a final concentration of 0.1 g.mL^{-1}. The cells were resuspended (0.1 g.mL^{-1}) in the same buffer containing lysozyme at 1 mg.mL^{-1} and BSA (1mg.mL^{-1}). Enzyme activities were measured using 100 mM hydantoin as substrate, for 1 h at 40 °C with shaking (Section 2.1.4).

4.2.4 Purification of RU-KM1 Hydantoinase

10 g French-pressed and freeze-dried cell powder was resuspended in 50 mL of 0.1 M phosphate buffer pH 8.0. This extract was sonicated for 3.5 min, at 4 °C, in 1min intervals to disrupt the cell membranes. The cell debris was removed by centrifugation at 15 000 rpm for 30 min. The resulting supernatant was used as the crude extract. The hydantoinase activity was determined using the standard assay procedure over a 1 h period (Section 2.1.4).

4.2.4.1 Ammonium sulphate precipitation

The crude extract (Section 4.2.4) was subjected to ammonium sulphate fractionation. Crystalline ammonium sulphate was added to the solution at 4 °C, dissolved completely by rapid stirring, and the mixture allowed to stand for 5 h before centrifugation at 12000 rpm for 20 min. The pellet was dissolved in 5 mL 50 mM phosphate buffer pH 8.0 and reacted with 100mM hydantoin for 6 h at 40 °C. Hydantoinase activity was determined using the standard procedure (Section 2.1.4). Pellets displaying hydantoinase activity were pooled and dialysed for 12 h against 3 changes of the same buffer.

4.2.4.2 Hydrophobic Interaction Chromatography (HIC)

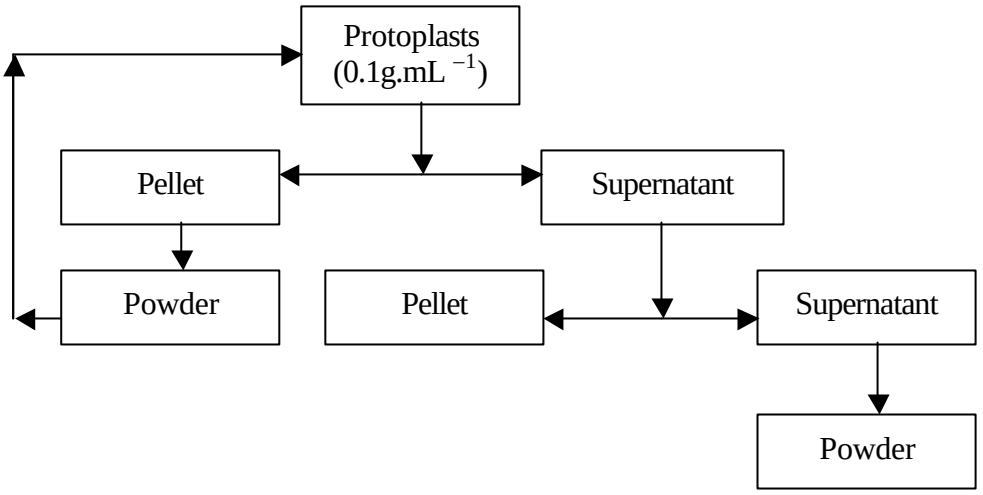
The pooled pellet (Section 4.2.4.1) was freeze-dried and used for chromatographic purification. Adapting the method of Burton and Kirchmann (1997) a Phenyl-Sepharose CL4B column, packed and equilibrated at room temperature with elution buffer (EB1), 0.05 M KH_2PO_4/KOH buffer, pH 8.0 containing 0.4 M ammonium sulphate. The freeze dried pellet was suspended in 50 % EB1:H_2O and loaded onto the column. In a typical experiment the column (2.2 x 12.9 cm) was eluted stepwise with elution buffer (EB1) dilutions: EB1, 60 mL EB1/H_2O, 4:1 40 mL; EB1/H_2O, 3:2, 40 mL; EB1/H_2O, 2:3 40 mL; EB1/H_2O, 1:4, 40 mL; ethylene glycol/water, 1:1, 40 mL; H_2O, 40 mL. Fractions (3 mL) were collected at a flow rate of 1.6 mL.min^{-1}. Fractions that contained active hydantoinase were pooled and dialysed against half strength elution buffer 2. This sample was freeze-dried and resuspended in 1 mL elution buffer 2 and applied to a Butyl-Toyapearl 650M column (2.2 x 12.9 cm) equilibrated with elution buffer 2 (EB2), 0.05 M KH_2PO_4/KOH buffer, pH 8.0 containing 1.0 M ammonium sulphate. The sample (1 mL) was applied to the column and eluted in a stepwise manner with dilutions of the elution buffer 2 (EB2): EB2, 60 mL EB2/H_2O, 4:1 40 mL; EB2/H_2O, 3:2, 40 mL; EB2/H_2O, 2:3 40 mL;

EB2/H_2O, 1:4, 40 mL; ethylene glycol/water, 1:1, 40 mL; H_2O, 40 mL. Fractions (3 mL) were collected at a flow rate of 1.6 $mL.min^{-1}$. Samples showing hydantoin-hydrolysing activity were pooled, dialysed against water and freeze-dried. These samples were used for SDS- PAGE analysis.

SDS-page analysis was performed on the crude extract and samples obtained from the ammonium sulphate fractionation step and the HIC purification step. The protein separations were performed according to the method of Judd (1990) in a Hoeffer electrophoresis apparatus. The proteins were separated in a 10 % acrylamide gel at 120 V for 3 h.

4.2.4.3 Acetone Precipitation

Protoplasts from RU-KM1 cells were produced according to the protocol previously described (Section 3.2.11). The protoplasts were resuspended in cold 0.1 M potassium phosphate buffer pH 8.0. This suspension was sonicated for 3.5 min using a 1min on/off cycle. The suspension was then clarified by centrifugation at 13000 g for 20 min. The pellet was freeze-dried and the cycle repeated. The proteins in the supernatant were precipitated by the drop-wise addition of ice-cold acetone to the suspension. The solution was stirred continuously at 4 °C throughout the procedure. The mixture was clarified by centrifugation at 13 000 g for 20 min. The resulting pellet was freeze-dried and stored. The supernatant was freeze-dried and stored until enough sufficient quantities of all the samples had been produced. Hydantoinase and *N*-carbamoylase activities were determined at all stages of the procedure. Scheme 4.1 illustrates the procedure for the production of solubilised RU-KM1 hydantoinase.



Scheme 4.1: Procedure for the acetone precipitation of RU-KM1 hydantoinase from protoplasts of RU-KM1

4.3 Results and Discussion

4.3.1 Carbon source and the production of extracellular polysaccharide (EPS)

It is commonly reported that the availability of a good carbon source in a bacterial culture often results in the production of extracellular polysaccharide (EPS) which surrounds the cell in the form of a capsule, and can be utilised when the carbon source becomes depleted. In the purification of the enzymes from these cells, the polysaccharide becomes problematic as it inhibits the release of the membrane-bound proteins and the disrupted membranes themselves, and may make the lysis of the cells difficult. Different carbon sources were investigated in order to minimise the production of polysaccharide. When RU-KM1 was using mannitol (A) as a carbon source, very little pollysacharide was evident when compared to cells grown using glucose as acarbon source (B) (Plate 1). Carbon sources were compared with respect to their effect on the amount of polysaccharide production, culture duration and enzyme activity in RU-KM1 cultures.

(A) (B)

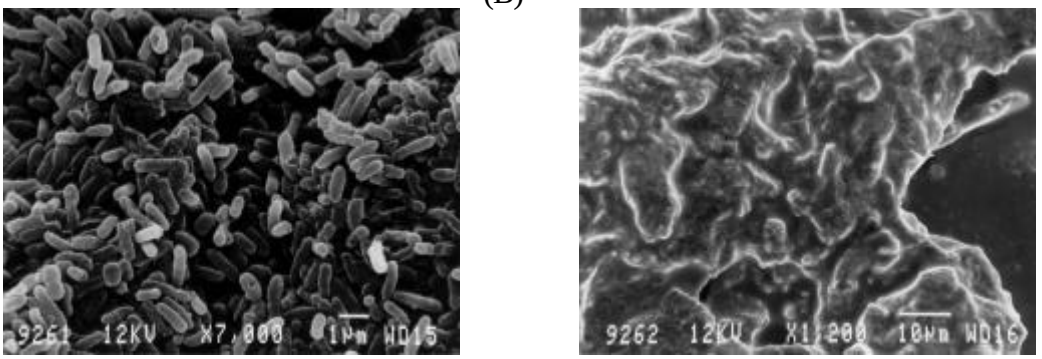


Plate 1: RU-KM1 cells grown in 1 % mannitol (A) until the onset of stationary phase (72hrs) and RU-KM1 cells grown in 1 % glucose (B) until the onset of stationary phase (25h)

The quantification of the polysaccharides and proteins was determined by precipitation with cold organic solvents and then measurment of the change in DCW of the solvent

treated culture and untreated culture (Figure 4.1). The efficacy of different organic solvents in precipitating the polysaccharides and proteins produced in the two different media was investigated using 3 different solvents.

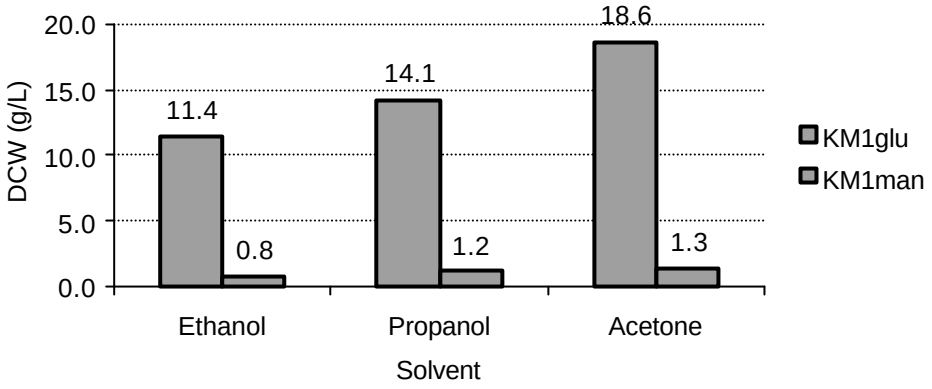


Figure 4.1: Differences in polysaccharide production by RU-KM1 grown on a 1 % glucose medium (KM1glu) and a 1 % mannitol medium (KM1man)

The cold acetone precipitation was the best solvent for the precipitation of the polysaccharide (Figure 4.1). This figure also shows the differences in polysaccharide production by RU-KM1 when it was cultured in hydantoin minimal medium with 1 % glucose (KM1glu) and 1 % (KM1man) mannitol as a carbon source. When the cells were in the glucose medium, up to 18.6 $g.L^{-1}$ polysaccharides and proteins was produced, as compared to 1.3 $g.L^{-1}$ that was produced when RU-KM1 was grown in the mannitol medium. Table 4.1 shows the biomass of the each of the cultures and also the enzyme activity that was detected in the reaction of each with 100 mM hydantoin as substrate.

Table 4.1: Biomass yields and hydantoin-hydrolysing activity of RU-KM1 when grown in minimal medium with 1 % glucose and 1 % mannitol as a carbon source

Carbon source	DCW ($g.L^{-1}$)	Time to stationary phase (h)	Hydantoinase ($\mu mol.h^{-1}.mL^{-1}$)	NCAAH ($\mu mol.h^{-1}.mL^{-1}$)	Specific Activity ($\mu mol.min^{-1}.mg^{-1}$)
Glucose	7.1	25	6.4 (±0.2)	3.2 (±0.3)	0.063
Mannitol	5.6	72	4.9 (±0.09)	3.6 (±0.09)	0.076

This data represents the mean (SEM) of triplicate after a 1 h reaction

From Table 4.1 it can be seen that there was a decrease of biomass yield (5.6 $g.L^{-1}$) when the cells were cultured in the minimal medium with mannitol as the carbon source, in comparison to the same organism grown on glucose as the carbon source (7.1 $g.L^{-1}$) source. An increase in the duration of the growth phase also occurred when the poor carbon source (mannitol) was used. However there was a small increase in specific activity when the poorer carbon source was utilised, possibly as a result of there being less polysaccharide to retard the movement of the substrates and products into and out of the cells.

4.3.2 The preparation of RU-KM1 extracts

4.3.2.1 Enzymatic disruption of RU-KM1 cell walls for release of cellular proteins

Bacterial cell walls confer stability and protect the contents of the cell. In order to allow purification of intracellular proteins the cell wall must be disrupted. Different enzymes are capable of selectively digesting the different constituents of the cell wall, thereby resulting in disrupted cells.

The changes in the hydantoinase and *N*-carbamoylase activities by the addition of the different enzymes used are shown in Figure 4.2. As expected, the Macerase had no effect on the cell wall of the organism. This enzyme was incorporated into the experiment as a negative control (digests the cell walls of plants, and specifically the pectin layer associated with plant tissue), and no increase in hydantoin-hydrolysing activity was observed (Figure 4.2). The other enzymes all contain bacterial cell wall degrading activities and therefore might digest the bacterial cell wall and the release the hydantoin-hydrolysing activity of RU-KM1.

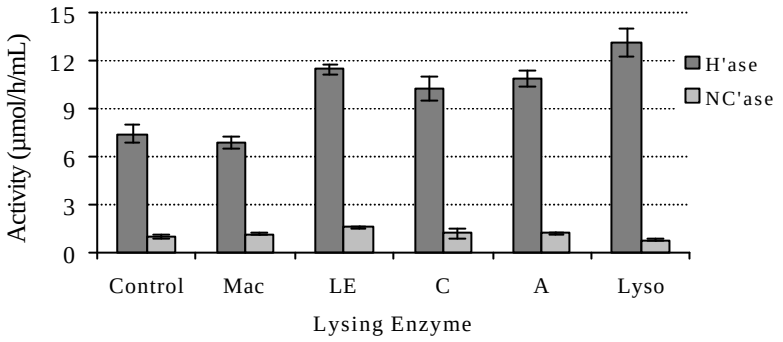


Figure 4.2: The changes in hydantoinase and *N*-carbamoylase activity in RU-KM1 cells that have been disrupted with 4 mg.mL^{-1} Macerase (Mac), Lyticase enzyme (LE), Chymotrypsin (C), Phospholipase A_2 (A) and 1 mg.mL^{-1} of lysozyme (lyso)

All the other enzymes added resulted in an increase in hydantoin-hydrolysing activity due to their hydrolytic activity. The addition of lysozyme (which hydrolyses *N*-acetylmuramide linkages), was the best enzyme used for the digestion of the cell wall, resulting in a near doubling of the hydantoinase activity of the sample. The addition of the other enzymes also resulted in an increase in the hydantoin-hydrolysing activity of the cells of between 2 and 4 µmol.h^{-1}.mL^{-1}. Thus, these enzymes can be used for the disruption of cells for purification of cellular proteins.

4.3.2.2 Mechanical disruption of RU-KM1 cell walls

Mechanical disruption of bacterial cell walls, as a result of a physical force (pressure, high frequency sound waves or abrasion) being applied to the cell suspension, causes the cell wall to disintegrate. However, these methods lead to the generation of heat which may cause protein denaturation, and careful control of the process is necessary to minimise heating during mechanical lysis. Figure 4.3 and 4.4 show the changes in hydantoinase and *N*-carbamoylase activity in sonicated cells of RU-KM1 respectively. The hydantoinase activity (Figure 4.3) in the pellet was initially high (6.3 µmol.h^{-1}.mL^{-1}), and was reduced as the sonication period progressed. In parallel, there was an increase in the hydantoinase activity detected in the supernatant of the samples, due to the fact the cells were disrupted by the sonication and the hydantoinase had been successfully released into the supernatant.

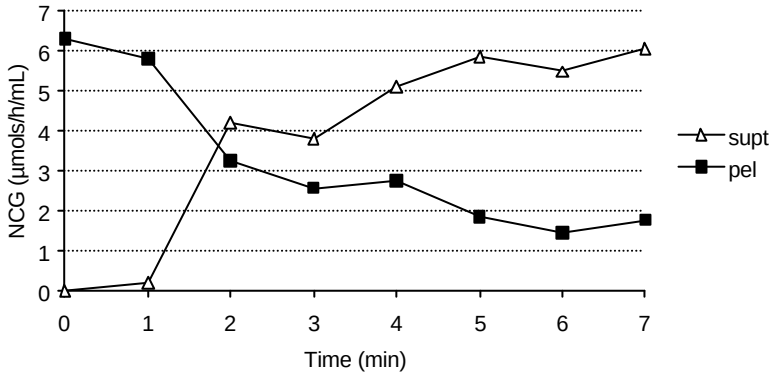


Figure 4.3: Changes in hydantoinase activity in cells of RU-KM1 sonicated over a 7 min period. Cells were subjected to 1 min cycles of sonication and the changes in activity in the pellet (pel) and the supernatant (supt) were determined

Figure 4.4 shows the changes in the *N*-carbamoylase activity in the sonicated samples over the 7 min experiment period. There was a gradual increase in activity up to 2 min of sonication in both the supernatant and the pellet. Thus, the initial increase in activity was as a result of the gradual disruption of the cell wall and the liberation of the *N*-

carbamoylase into the supernatant. After 2 min of sonication the measured activity remained constant in the supernatant and the pellet, but the activity of the pellet started to drop after 4 min. This was accompanied by a gradual increase in activity in the supernatant. The differences in the rate of decrease of activity in the pellet and the rate of increase in the supernatant may be due to protein denaturation due to localised heat generation around the tip of the sonicator, but *N*-carbamoylase enzymes are also known to be unstable (Ogawa *et al*., 1995b,d).

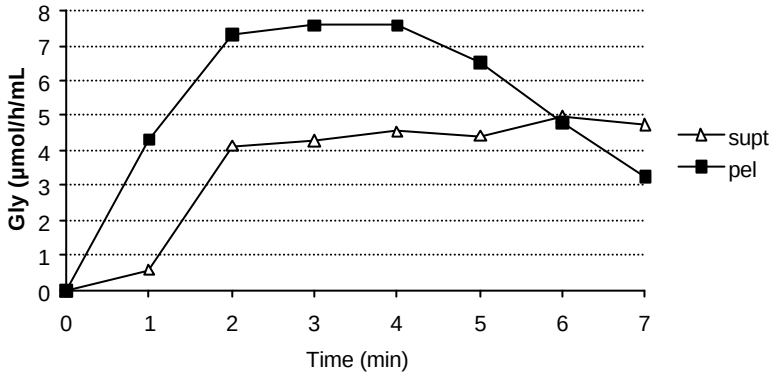


Figure 4.4: Changes in *N*-carbamoylase activity in sonicated cells of RU-KM1 over a 7 min period. Cells were subjected to 1 min cycles of sonication and the changes in activity in the pellet (pel) and the supernatant (supt) were determined

Figure 4.5 indicates the changes in total activity, which is the sum of the hydantoinase and the *N*-carbamoylase activities. This data was useful in determining the best possible length of time to sonicate the cells, which was shown by a loss of activity in the pellet, coupled to the highestactivity measured in the supernatant. This can be seen to be between 3 and 4 minutes. The combined activity in the supernatant reaches the same level as the initial activity measured in the pellet at 4 minutes.

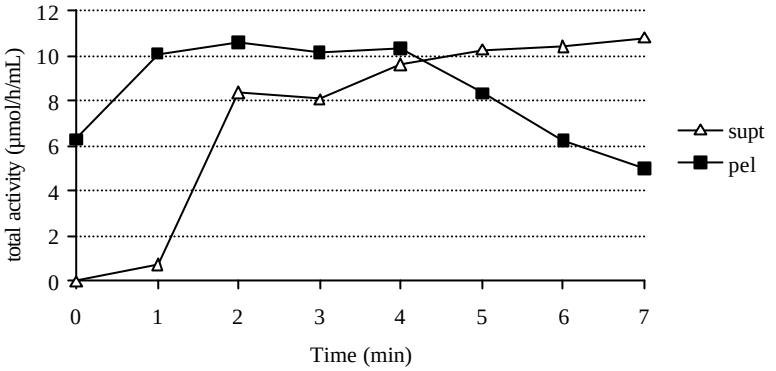


Figure 4.5: Changes in total activity in cells of RU-KM1 sonicated over a 7 min period

Protein concentration and specific activities were calculated for each sample taken (Table 4.2). There was a rapid increase in protein concentration in the supernatant as the sonication time increased, as a result of the disruption of the cells. The highest specific activity of the supernatant was measured between 2 and 4 min of sonication, and thereafter the specific activity declined with increasing sonication. As expected, the pellet showed the opposite result with the initial specific activity being high and a reduction in specific activity as more protein was released from the cells.

Table 4.2: Changes in protein concentration and specific activity of RU-KM1 hydantoinase with sonication

	Protein concentration ($mg.mL^{-1}$)		**Specific activity ($\mu mol.min^{-1}.mg^{-1}$)**	
Time	**Supernatant**	**Pellet**	**Supernatant**	**Pellet**
0	0.12 (±0.04)	0.67 (±0.01)	ND	1.54×10^{-3}
1	0.33 (±0.003)	1.10 (±0.03)	3.08×10^{-4}	1.53×10^{-3}
2	0.99 (±0.01)	1.45 (±0.003)	1.37×10^{-3}	1.18×10^{-3}
3	1.02 (±0.04)	1.56 (±0.001)	1.08×10^{-3}	1.08×10^{-3}
4	1.18 (±0.04)	1.67 (±0.005)	1.04×10^{-3}	1.02×10^{-3}
5	1.57 (±0.02)	1.78 (±0.09)	9.46×10^{-4}	7.77×10^{-4}
6	1.74 (±0.01)	1.67 (±0.05)	8.50×10^{-4}	6.09×10^{-4}
7	1.66 (±0.03)	1.45 (±0.034)	7.28×10^{-4}	5.98×10^{-4}

This data represents the mean (SEM) of triplicate

After 4 min the activity in the supernatant decreased, probably due to denaturation of the active protein (Figure 4.6). Throughout the sonication period, there was a reduction in the specifc activity of the pellet, due to the protein being solubilised and released from the pellet (Figure 4.6).

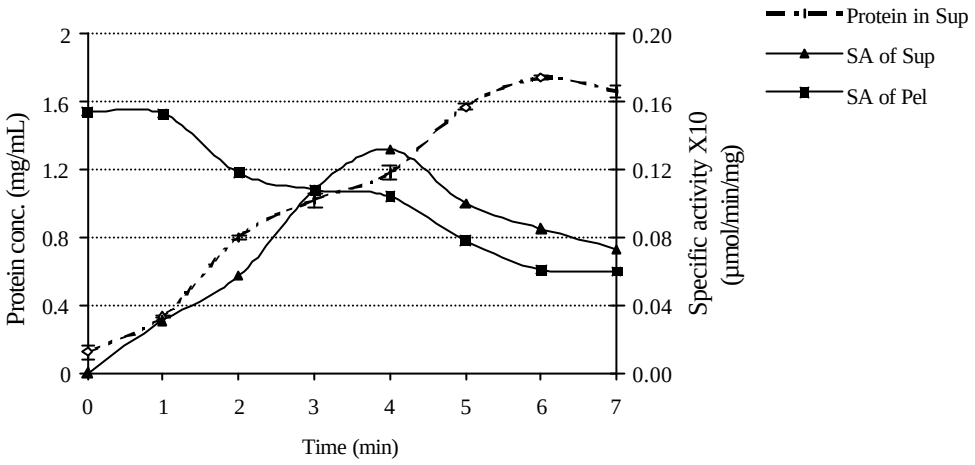


Figure 4.6: Changes in the protein content of the supernatant and specific activities of the supernatant and pellet with an increase in sonication time of cells of RU-KM1

4.3.2.3 Non-enzymatic disruption of RU-KM1 cells

The use of chemicals to permeabilise cell walls and membranes is well documented Neugebauer (1990), and is usually conducted in conjunction with either mechanical or enzymatic disruption processes. In experiments to enhance the release of the enzymes from the membranes of lysozyme-treated RU-KM1 cells, solubilisation of the cell membranes by addition of various surfactants: polyethylene glycol (PEG), polyoxyethylene ether (W1), Cetyltrimethyl-ammonium bromide (CTAB), and chloroaminopropyldimethyl-ammonio-1-propane sulfonate (CHAPS) was investigated (Figure 4.7). Toluene, an organic solvent, was also tested. It is evident (Figure 4.7) that the only surfactants to have a significant effect were W1 and CHAPS, which resulted in increased hydantoin-hydrolysing activity of 12.9 and 17.4 $\mu mol.h^{-1}.mL^{-1}$ respectively. The

other surfactants reduced hydantoinase activity. The surfactant, PEG, had little effect on hydantoin-hydrolysing activity, possibly due to the ratio of the detergent to the membrane protein. This factor has been shown to be important when using surfactants to solubilise membrane bound proteins (Hjelmeland, 1990). Further experimentation would need to be done, increasing the ratio of surfactant. Toluene resulted in an insignificant change in hydantoin-hydrolysing activity of the RU-KM1 extract.

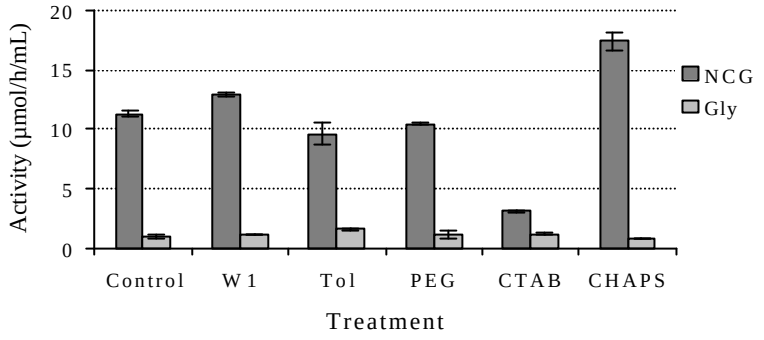


Figure 4.7: The changes in hydantoinase and *N*-carbamoylase activities in RU-KM1 cells that have been disrupted with 1 mg.mL^{-1} of lysozyme (control) and incubated with 0.1 % W1, 2.5 % Tol, 2.5 % PEG, 2.5 % CTAB and 10 mM CHAPS

4.3.3 Purification of RU-KM1 hydantoinase

4.3.3.1 Ammonium sulphate precipitation

Ammonium sulphate precipitation was conducted to further prove the location of the hydantoinase enzyme within the cell, as well as removing extraneous contaminating proteins from the sonicated crude extract. Figure 4.8 shows the hydantoinase activity of the different fractions using ammonium sulphate. Most of the activity was obtained in the 40 % pellet. However, to improve yields the 20 - 50 % fractions were pooled, freeze-dried and dialysed against buffer, for further use in hydrophobic interaction chromatography (HIC).

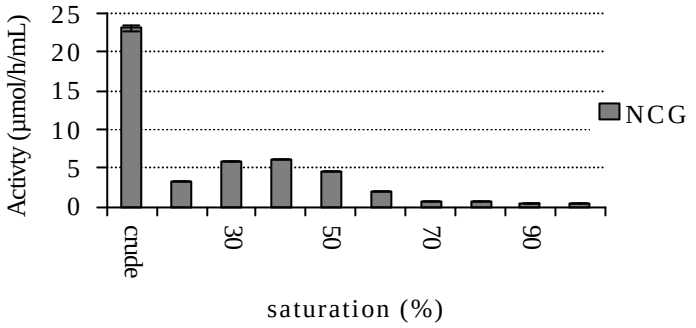


Figure 4.8: Hydantoinase activity detected in the different fractions after salting out with ammonium sulphate

4.3.3.2 Hydrophobic Interaction Chromatography (HIC)

The dialysed samples obtained from the ammonium sulphate precipication were applied to a Phenyl-Sepharose column and the proteins separated according to their hydrophobicity using a stepwise change in ionic strength of the equilibration buffer. Figure 4.9 shows the elution profile of the sample produced from the 20-50 % ammonium sulphate fractionation. After gradient elution, the fractions containing protein were assayed for hydantoin-hydrolysing activity. The elution profile (Figure 4.9) showed 7 protein peaks, and of these, hydantoin-hydrolysing activity was detected in four. Peak 5, which corresponded to fractions 31-34, displayed the greatest specific activity of 6.8 x 10^{-4} µmol.min^{-1}.mg^{-1}. Peak 4, also displayed high specific activity of 4.2 x 10^{-4} µmol.min^{-1}.mg^{-1}, whilst peak 1 and 2 both showed low specific activities of 1.2 x 10^{-6} µmol.min^{-1}mg^{-1}. Peak 5 was removed, dialysed against 0.05 M phosphate buffer pH 8.0 for 12 hours and applied to an equilibrated Butyl-Toyopearl 650M column. Fractions showing protein content were assayed for hydantoin hydrolysing activity. No activity was detected in any of the samples eluted from the column.

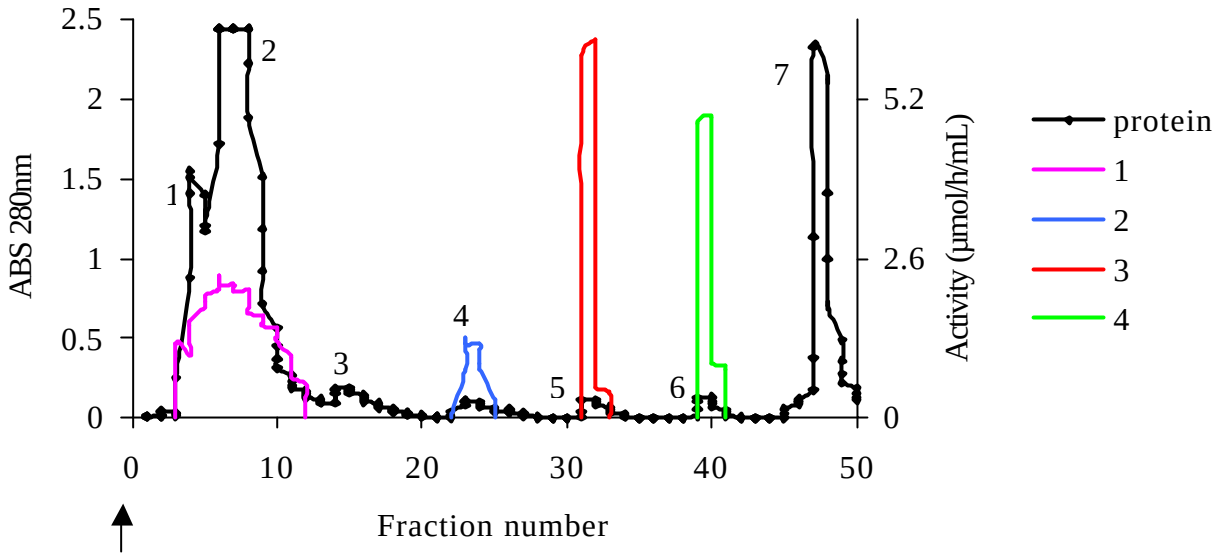


Figure 4.9: Elution profile of the proteins collected from the 20-50 % ammonium sulphate fractionation

Samples from the ammonium sulphate fractionation and the first chromatographic steps that showed the greatest hydantoin-hydrolysing activity were separated using a 10 % denaturing PAGE gel (Plate 2). However, there was very little difference in the banding pattern, indicating that there was no substantial purification of the protein.

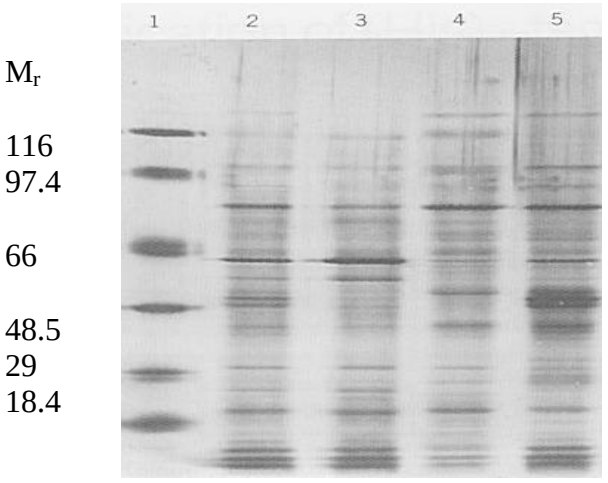


Plate 2: SDS-PAGE of RU-KM1 crude extract. Standard proteins: â-lactoglobulin (M_r 18400), carbonic anhydrase (M_r 29000), ovalbumin (M_r 48500), serum albumin (M_r 6600), phosphorylase B (M_r 974000), Galactosidase (M_r 116000) (lane 1), crude extract (lane 5), 20 - 50 % amonium sulphate cut (lane 4), protein peak #5 (lane 3) and protein peak #5 second elution (lane 2)

4.3.3.3 Acetone precipitation of RU-KM1 hydantoinase from sonicated protoplasts

Organic solvents are occasionally used to precipitate soluble proteins. With the two-step HIC protocol described in section 4.3.3.2, there was no detectable hydantoinase activity in the second stage of the chromatography. The lack of detectable activity was probably as a result of the active protein being too dilute in the eluted samples to be detected by the Ehrlichs reagent. The protoplasting procedure coupled to the precipitation procedure was developed as an alternative method for partial purification of hydantoinase from RU-KM1 cells. Table 4.4 shows the hydantoinase activities measured in the samples during the protoplasting and precipitation procedure. The majority of the solubilised protein remained in the supernatant after the sonication (Table 4.3). The addition of an equal volume of cold acetone precipitated most of the extraneous cell debris and polysaccharides from the mixture. The specific activity of the supernatant increased from 2.2 x 10^{-3} $\mu mol.min^{-1}.\mu g^{-1}$ to 2.30 x 10^{-2} $\mu mol.min^{-1}.\mu g^{-1}$ after freeze drying. The proteins in this sample were separated using a 10 % SDS-PAGE (Plate 3).

Table 4.3: Measured biocatalytic activities of different fractions prepared during the acetone precipitation of sonicated protoplasts of RU-KM1 cells

Sample ID	Protein ($\mu g.mL^{-1}$)	Activity ($\mu mol.h^{-1}.mL^{-1}$)	Specific Activity ($\mu mol.min^{-1}.\mu g^{-1}$)
Crude Extract	246.9	5.36	3.62 x 10^{-4}
Protoplasts	147.82	17.69	2.0 x 10^{-3}
Pellet	147.82	7.63	8.6 x 10^{-4}
Freeze-dried pellet	359.27	4.10	1.0 x 10^{-4}
Supernatant	60.16	8.03	2.22 x 10^{-3}
Acetone ppt^{n} supernatant	494.56	1.53	5.2 x 10^{-5}
Freeze-dried supernatant	4.85	6.57	2.3 x 10^{-2}

Plate 3 shows a SDS-PAGE gel. Lanes 2 and 6 are duplicate samples of the resultant preparation after the purification process. Lanes 3 and 7 are duplicates of the RU-KM1 crude extract. Substantial purification of the sample is evident when comparing the crude extract and the semi-purified sample. There are numerous bands between 48.5 and 66 kDa in size. Of these there are two distict bands below 66 kDa, which would correspond to all the reported molecular weights of the *Pseudomonas* hydantoinases that have been purified to date (Morin *et al*., 1986a, 1990; LaPointe *et al*., 1994; Chein *et al*., 1998; Chen and Tsai, 1997). Further purification using non denaturing conditions would be needed to allow for complete biochemical and kinetic studies.

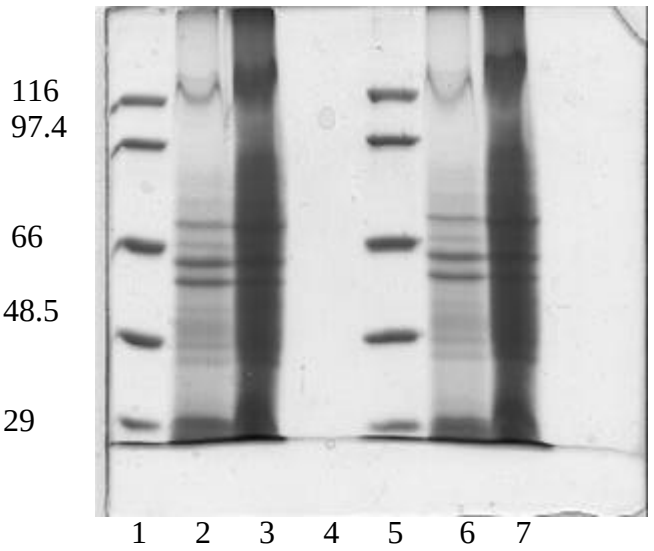


Plate 3: SDS-PAGE of freeze-dried supernatant; Lane 1 and 5 molecular markers: carbonic anhydrase (M_r 29000), ovalbumin (M_r 48500), serum albumin (M_r 6600), phosphorylase B (M_r 974000), Galactosidase (M_r 116000), lane 2 and 6 freeze-dried supernatant and lane 3 and 7 RU-KM1 crude extract

Table 4.4 sumarises the purification procedures used and a comparison of the specific activities measured during the different purification processes.

Table 4.4: Summary of different purification procedures to produce a semi-purified hydantoinase from RU-KM1 cells

Purification Technique	Protein ($\mu g.mL^{-1}$)	Activity ($\mu mol.h^{-1}.mL^{-1}$)	Specific activity ($\mu mol.min^{-1}.\mu g^{-1}$)	Yield (%)	Purification factor
Crude Extract (100 $mg.mL^{-1}$)	1350	11	1.4×10^{-4}	100	1
$(NH_4)_2SO_4$: 20%	378	1.9	8.3×10^{-5}	28	0.6
30 %	417	3.7	1.4×10^{-4}	30.88	1
40 %	400	4.4	1.8×10^{-4}	29.62	1.3
50 %	353	2.6	1.2×10^{-4}	21.15	0.9
60 %	357	1.1	5.1×10^{-5}	26.44	0.4
Phenyl Sepharose	22	0	4.5×10^{-4}	6.1	3.3
Butyl-Toyopearl	nd	nd	Nd	nd	nd
Acetone precipitation	4.85	6.57	2.2×10^{-2}	0.4	58

4.4 Conclusions

For complete biochemical characterisation and kinetic studies, purified protein is needed for meaningful results. However, from a process perspective pure protein is is not a necessity, and is in all likelihood not a real option. However, the purification was conducted with the view of removing extraneous proteins and polysaccharides to facilitate immobilisation and enhancing the specific activities.

Strain RU-KM1 produced large amounts of extracellular polysaccharide (EPS) when cultured on a glucose carbon source. Not only is EPS a "waste" of carbon that may be utilised for cellular metabolism and reproduction, it also has serious implications in the utilisation of this organism or its enzymes for the biocatalytic production of amino acids, because the EPS is difficult to remove using mild conditions that would not denature proteins or kill the cells. Further, it may limit the transfer of substrates into and products out of the cell. The use of a poor carbon source, (mannitol) resulted in a reduction of EPS produced by the cell under these conditions. There was a small loss in biomass yield and the fermentation time was substantially longer (72 hrs), but no reduction in the activities of the hydantoin-hydrolysing enzymes was noted. In fact the specific activities of the samples increased when the mannitol was used a carbon source.

For downstream processing (DSP), if bacterial cells are mechanically or chemically disrupted, the polysaccharide is not normally removed, and this has major cost implications in a process design for separating the substrates, products, reactants, and biocatalysts. Thus EPS can present a major problem for the efficient use of this microorganism. The use of a manitol as an alternative carbon source for RU-KM1 was

useful for the reduction of EPS. However, there were disadvantages incurred in the duration of the fermentation time. Thus, the medium would need to be optimised for biomass production and reduction in EPS. (This will be addressed in later chapters). However, for purification studies, the use of the manitol-based medium did overcome the production of EPS without incurring any loss of activity.

The use of lysing enzymes to digest cell wall components has been widely reported and documented, and their use has potential for aiding further purification procedures. In this study, lysozyme showed the best results in the release of active hydantoin-hydrolysing enzymes within the cells. The use of sonication as a mechanical means of disrupting RU-KM1 cells was successful in disrupting the cells and releasing the proteins and hydantoinase into the supernatant. However from a process perspective sonication is not a scalable option so the use of this technique was limited to a lab-scale experiment.

In preliminary investigations, no *N*-carbamoylase activity was detected in the pellet or the supernatant of the RU-KM1 cells, and this organism shows little *N*-carbamoylase activity when assayed as whole cells. However, during sonication there was an increase in the *N*-carbamoylase activity with time, indicating not only the presence of the enzyme but also possible a mass transfer problem into and out of the cell. This method of releasing protein was useful for two reasons; it was successful in disrupting the cells and releasing the membrane bound hydantoinase from the RU-KM1 cells.

The use of surfactants and solubilising agents for the enhanced release of the hydantoinase and *N*-carbamoylase from the crude extract showed limited success. The only surfactants that had significant effects were the W1 and the CHAPS, which were shown to disrupt the

membranes of the RU-KM1 cells and increase the activity. The use of these two surfactants on an industrial scale is not feasible due to their cost, so again, this experiment is confined to a laboratory-scale experiment that would facilitate in yielding only small scale results that may have implications at a larger scale, for example kinetics studies.

A protocol was developed for the partial purification of the RU-KM1 hydantoinase. This involved ammonium sulphate precipitation, hydrophobic chromatography using two different colomns and gel electrophoresis. This process proved successful in removing significant amounts of contaminating protein from the sample, but low yields were obtained from this protocol.

A further purification protocol involving the precipitation of solubilised proteins was developed. Protoplasts were prepared from RU-KM1 cells, and intracellular proteins liberated by protoplast lysis. The removal of the cellular debris resulted in the removal of a large proportion of contaminating proteins. Subsequent precipitation of the proteins from the supernatant yielded in a semi-pure hydantoinase, with a specific activity of 2.3×10^{-2} $\mu mol.min^{-1}.mg^{-1}$, and a molecular weight between 48.5 and 66 kDa. This molecular weight is similar to that reported by other for *Psedomonas* hydantoinases (Morin *et al*., 1986a, 1990; LaPointe *et al*., 1994; Chein *et al*., 1998; Chen and Tsai, 1997).

From a process point of view, further purification would probably not be needed, as pure protein has been shown not to be necessary for hydantoinase activity. Further purification would only be needed to elucidate the kinetics of the enzyme which may influence substrates used and could imput on the bioreactor design.

Chapter 5

5. Introduction

5.1 General Introduction

Once a bacterial strain producing industrially useful hydantoinase and *N*-carbamoylase enzymes has been isolated and characterised, the optimisation of enzyme production is the next logical step in the development of the biocatalyst (Schmid *et al*., 2001). Biocatalytic processes differ from conventional chemical processes in that biocatalyst stability is an important component of an operational process.

Several reports have indicated that the production of the enzymes involved in hydantoin-hydrolysis is regulated by growth conditions, and maximum enzyme activity in a complex growth medium is only detected during late logarithmic or early stationary phase. This activity is also dependent upon the presence of inducers in the growth medium (Möller *et al*., 1988; Meyer and Runser, 1993; Syldatk *et al*., 1990a). This suggests the two main factors regulating the production of hydantoin-hydrolysing enzyme activity, are nitrogen control and induction. The general consensus of reported literature is that the production of hydantoin-hydrolysing activity requires induction by hydantoin analogues in the growth medium (Runser *et al*., 1990; Meyer and Runser, 1993; Louwrier and Knowles, 1996, Durham and Weber, 1996).

Hydantoin can serve as both a carbon and nitrogen source, however D-stereoselective hydantoin hydrolysis has mostly been linked to the nitrogen metabolism in bacteria (Vogels and van der Drift, 1976; Yokozeki *et al*., 1987a; Ogawa and Shimizu, 1997; Runser *et al*., 1990). Repression of hydantoin-hydrolysing enzyme production by carbon catabolites has not been reported to date, and hence hydantoin-hydrolysing activity seems

to be regulated principally by nitrogen availability in the cell. (Syldatk *et al*., 1990a; Deepa *et al*., 1993),

Research into the best synthetic growth medium for production of hydantoin-hydrolysing activity showed that ammonium was not the optimal nitrogen source for enzyme production, and that non-preferential nitrogen sources provided better hydantoin-hydrolysing enzyme activity (Deepa *et al.*, 1993; George and Sadhukhan., 1996; Achary *et al*., 1997). This also suggested the regulation of hydantoin-hydrolysing activity by nitrogen catabolite repression.

Understanding the mechanisms that regulate hydantoin-hydrolysing enzyme production in RU-KM1 was necessary for both optimisation of growth conditions and for the physiological manipulation of the regulatory systems for enhanced enzyme biocatalyst production and activity. Section 2.2.4 illustrated that the production of hydantoinase and NCAAH activity in RU-KM1 only reached high levels after early stationary phase in complete medium. This led to the hypothesis that the hydantoin-hydrolysing enzyme systems of RU–KM1 might be regulated by nitrogen control pathways and an induction system.

Having established the commercial potential of the D-stereoselective hydantoin-hydrolysing enzyme system of RU-KM1 (Burton *et al.*, 1998), the development of a production process was required. This chapter focuses on the biological, physiological and chemical factors that affect the design of the bioprocess and aims to define an operational window within which a hydantoinase biocatalyst could operate effectively.

5.2 Materials and Methods

5.2.1 Chemicals

N-Succinyl-ala-ala-pro-phe-*p*-nitroanilide and hydantoin were purchased from Sigma-Aldrich. D- and L- Methylhydantoin was purchased from Toronto Reasearch Chemicals, Canada. The commercial protease, Solvay proteolytic L-1000 was obtained from Solvay Enzymes. Comercial D-hydantoinase was obtained from Roche Pharmaceuticals.

5.2.2 Steroselectivity and *ee* of RU-KM1 Hydantoinase

Strain RU-KM1 was grown in a 1 L Erlenmeyer flask containing 200 mL HMM for 2-3 days at 29 °C with shaking. The cells were harvested and washed according to the standard procedure (Section 2.1.4), and resuspended in 0.1 M phosphate buffer at pH 8.0 to a give a final concentration of 0.1 $g.ml^{-1}$. The cells were reacted with a racemic mixture of 1 % 5-n-butylhydantoin for 22 h at 40 °C with shaking. The reaction was terminated by heating at 100 °C for 10 min, and the solution was clarified by centrifugation on a benchtop microfuge for 10 min. The resulting supenatant was diazotisation with $NaNO_2$ by cooling to room temperature and while stirring rapidly, adjust to pH 1.0 by the rapid addition of 25 % HCl. While stirring at 4°C, slowy add 1.2 mol $NaNO_2$ for every 1 mol hydantoin. After 1.5 h the diazotisation reaches 98 % conversion. The pH was adjusted to the isoelectric point at pH 4.3 with 30 % NaOH. The resulting solution is clarified by centrifugation and analysed by chiral HPLC. The commercial D-hydantoinase was subject to the same procedure as a positive control. The enantiomeric selectivity and enantiomeric excess of the RU-KM1 hydantoinase was determined by the chiral analysis of the amino acids produced (Appendix A4).

5.2.3 Determination of chemical racemisation rates

10 mM D-methylhydantoin solutions were prepared in 0.1 M phosphate buffers at pH 7.0, 8.0 and 9.0. The initial specific rotation was determined using a sodium lamp (589 nm) polarimeter with a path length of 1dm at a constant temperature (40 °C). The solutions were incubated at 40 °C for 15 h, and the optical rotation was measured over time using the same conditions. The specific rotation was calculated according to the following formula (Hendrickson *et al*., 1990):

Specific rotation: $[\alpha]^{40}_{589} = \alpha / (l \times c)$ ……………………………………(1)

where: α = measured optical rotation
l = path length (dm)
c = concentration of **PURE** substrate (%)

5.2.4 Temperature stability of the biocatalyst

5.2.4.1 Effect of freeze-thaw in hydantoin-hydrolysing activity

Strain RU-KM1 was grown in a 1 L Erlenmeyer flask containing 200 mL HMM for 2-3 days at 29 °C with shaking. The cells were harvested and washed according to the standard procedure (Section 2.1.4), and resuspended in 0.1 M phosphate buffer at pH 8.0 to a give a final concentration of 0.1 g.mL^{-1}. A crude extract was produced using lysozyme (Section 2.1.4). Aliquots (10 mL) were removed and frozen in liquid nitrogen. On a daily basis all the samples were removed and thawed by immersing them in water at room temperature. A single sample was retained each day for hydantoinase and *N*-carbamoylase activity analysis using 100 mM hydantoin as a substrate while the remaining samples were refrozen in liquid nitrogen (Section 2.1.5).

5.2.4.2 Effects of temperature and storage duration on hydantoinase stability

Starter cultures were grown to stationary phase (18 h) in NB supplemented with 0.1 % hydantoin as inducer. The starter culture was inoculated into a 2 L fermenter operated at 29 °C. The pH was maintained at 7.0 by the addition of NaOH. Dissolved oxygen was regulated at 40 % saturation by cascade control of the agitator. The fermentation was harvested after 17 h and the cells washed twice with 0.1 M potassium phosphate buffer pH 8.0. The cells were resuspended in the same buffer to a final concentration of 0.1 $g.mL^{-1}$. Aliquots (100 mL) of the suspension were removed and lysed according to the procedure described in section 3.2.11.

Aliquots (5 mL) of the crude extract and the resting cells were sealed and placed at –20, and 4 °C, for 24 and 168 h. Further aliquots of the same crude extract and resting cells were placed at 25 °C and 40 °C for 4, 8 and 24 h each. After the specified incubation period, the samples were removed and reacted at 40 °C for 1 h using 100 mM hydantoin and 50 mM NCG as substrates.

5.2.5 Determination of protease activity

An 18 h starter culture (200 mL) of RU-KM1 cells grown in nutrient broth supplemented with 0.1 % hydantoin as an inducer were inoculated into a 2 L fermentor containing 1.4 L of PP2 medium (Appendix A2). The culture was grown and harvested as described above (Section 5.2.3.2). The cells were lysed using lysozyme according to the protocol described in section 3.2.11. Samples were prepared from the fermentation broth, the supernatant after the second wash, the supernatant after cell lysis, and the supernatant after 1 h substrate incubation.

All samples were clarified by centrifugation at 13 000 rpm for 10 minutes in a benchtop microcentrifuge. All solutions and equipment were pre-warmed to 25 °C. 0.935 mL 0.1 M Tris-HCl buffer was added to 0.05 mL of each of the samples. The mixture was gently mixed and allowed to equilibrate for 10 minutes at 25 °C whilst monitoring the baseline at 410 nm. 15 µL of a stock solution (15.4 mM N-succinyl-ala-ala-pro-phe-*p*-nitroanilide) was added to the reaction mix and gently mixed using a pipette. The activity was measured over a 10 min interval. A known protease (Solvay proteolytic L-1000) was included in the experiment as a positive control. The activity was determined as a change in absorbance over time. The protease activity was defined as: 1 unit = the amount of *p*-nitroaniline produced from N-succinyl-ala-ala-pro-phe-*p*-nitroanilide per minute per mL reaction.

5.2.6 Ammonia shock, recovery and inhibition of RU-KM1 hydantoinase and *N*-carbamoylase

5.2.6.1 Ammonia shock and recovery

Induced cells were grown in HMM to mid log phase, harvested by centrifugation, washed twice in 0.1 M potassium phosphate buffer pH 8.0, and resuspended in "ammonium shock medium" (ASM) (Appendix A2) at an OD_{600} = 0.5 - 0.8, (ASM: MM containing 0.1 % $(NH_4)_2SO_4$). Half the culture was disrupted by passing it through the French press according to the protocol described in section 3.2.11. The remaining culture was incubated with shaking at 200 rpm, at 29 °C for 30 min, before being assayed for enzyme activity under standard (resting cell) biocatalytic reaction conditions (Section 2.1.4). Optical density (600 nm) was measured after the 30 min incubation to determine if the cells were still in stationary phase (the cells were considered to be still in stationary phase if the OD

increase was 0.1). The ammonium shocked cells were harvested by centrifugation, washed with a 0.1 M phosphate buffer pH 8.0 and resuspended at an OD_{600} = 0.5 - 0.8 in induction medium (HMM). After one hour of incubation with shaking at 29 °C, enzyme activity was determined. A small increase in optical density was detected during this period (OD 600 nm increase 0.2) in the whole cells.

5.2.6.2 Ammonia inhibition

RU-KM1 cells were grown and disrupted as above (Section 52.5.1). Ammonium sulphate was added to give concentrations of 1, 5 and 10 mM. The standard assay was conducted to quantify the results (Section 2.1.5).

5.3 Results and Discussion

5.3.1 Stereoselectivity of RU-KM1 hydantoinase

Reaction of 5-n-butylhyantoin and the Roche enzymes yields an NC-norleucine. Diazotisation of this product yields nor-leucine. The Roche enzyme is D specific, hence only D-norleucine will be produced from this reaction. Figure 5.1 shows a typical chromatogram of diazotised products (NC-norleucine) to form D-norleucine.

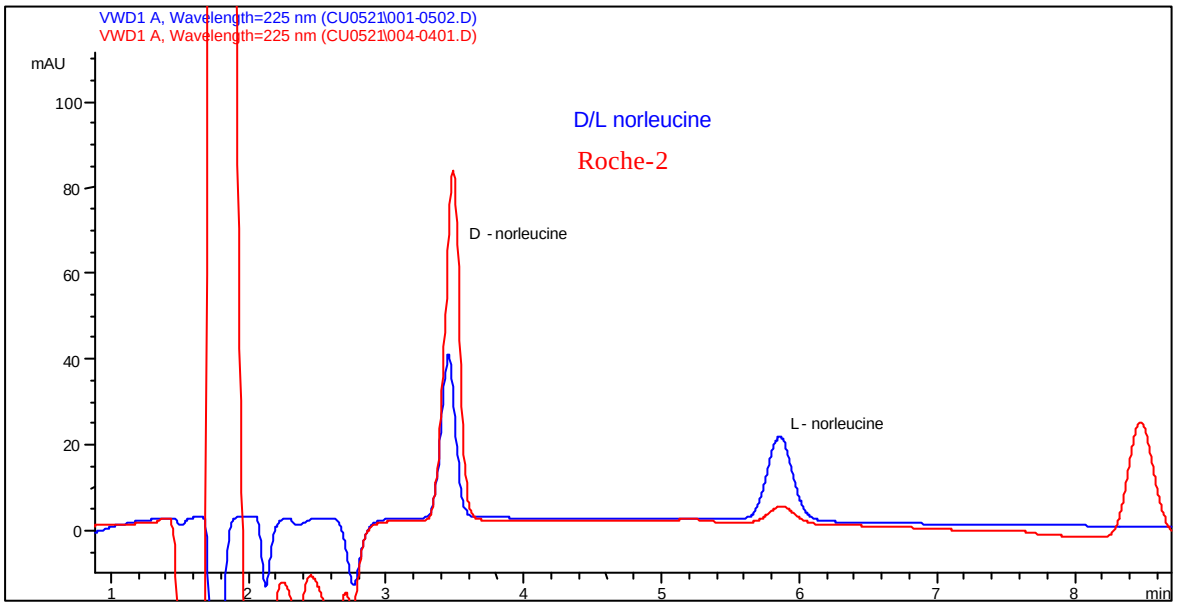


Figure 5.1: Typical chromatogram showing the production of the D-norleucine after diazotisation

To further validate the chiral methodology, the Roche enzymes were reacted with 1 % 5-n-butylhydantoin under identical conditions to those reported by Roche data sheets (Hyd-2: 21.5 hours with 127 $U.mg^{-1}$). Chiral analysis of the diazotised reaction products showed a D selectivity of both enzymes with an *ee* greater than 83 % (Roche Hyd-2) and 99.9 % (Roche Hyd-1)

Table 5.1: Comparison of the *ee* data reported by Roche and the results of a chemical diazotisation of the reaction products obtained by reaction of the Roche enzymes and 5-n-butylhydantoin

Sample ID	Measured *ee* (%)	Expected *ee* (%)
Roche 2	84.6	> 83
Roche 1	> 99.9	> 99

The results indicate that the use of this method to show chirality of the enzymes involved in the hydrolysis of the substituted hydantoins is accurate under the conditions used and with in the detection limits of the HPLC.

RU-KM1 and the two commercial D-hydantoinases were reacted with 5-n-butylhydantoin for 22 h. The supernatant was diazotised and the products were quantified by chiral HPLC (Table 5.2). The results obtained from this experiment indicate that the RU-KM1 hydantoinase is D-specific and has an *ee* of 97.3. This result is consistant with the literature on the selectivity of other *Pseudomonas putida* strains that have been characterised (Morin *et al*., 1986a, 1990; LaPointe *et al*., 1994; Chein *et al*., 1998; Chen and Tsai, 1997; Takahashi *et al*., 1978).

Table 5.2: Comparison of the *ee* data reported for the Roche D-hydantoinases and RU-KM1 hydantoinase from the chemical diazotisation of the reaction products obtained by reaction with 5-n-butylhydantoin for 22h

Sample ID	D-norleucine ($\mu mol.h^{-1}.mL^{-1}$)	L-norleucine ($\mu mol.h^{-1}.mL^{-1}$)	*ee*
Roche 1	13.2	1.1	84.6
Roche 2	13.8	0.002	>99.9
RU-KM1	8.83	0.12	97.3

5.3.2 Chemical racemisation

It has been reported that racemisation of chiral substituted hydantoins is possible at elevated pH's (> 8.0) (Bommarius *et al*., 1998), and the chemical racemisation of 5-D-methylhydantoin at elevated temperature and under alkaline conditions was also reported by Tsuji *et al*. (1987) and Takahashi *et al*. (1986). This occurs through keto-enol-tautomerism, where the rate constant of racemisation depends strongly on the 5-substitution on the hydantoin ring, and larger substitutents show lower racemisation rates (Bommarius *et al*., 1998; Pietzsch *et al*., 2000). Racemisation of the substrate could be an exploitable attribute, alleviating the need for optically pure substrates for enantioselective enzymes. The equilibrium between the D- and L- forms would thus favour the racemisation to the correct isomer, by the continual enzymatic hydrolysis of the substituted hydantoin. This investigation was conducted to determine the rate of racemisation of the substrate 5-D-methylhydantoin at the pH that the reaction has been conducted at.

Chemical racemisation rates of 5-D-methylhydantoin were compared at different pH values over a 15 h duration (Table 5.3). At pH 7.0 and pH 8.0 there was no change in the optical rotation of the 5-D-methylhydantoin after 900 min of incubation at 40 °C. However, after 900 min incubation at pH 9.0, the sample displayed nearly 50 % reduction in optical rotation. Thus under these conditions, chiral products would racemise slowly at pH 9.0, but would not racemise at pH 7.0 or 8.0. Thus in the proposed system to develop a chiral product, where the proposed pH would be 8.0, yields would not be reduced by the racemisation of the products.

Table 5.3: Chemical racemisation of 5-methylhydantoin at 40 °C, at pH 7, 8 and 9

pH	Optical Rotation (°)		Change in Optical Rotation ($°.min^{-1}$)
	0 min	900 min	
7	(+) 35.09	(+) 35.09	0
8	(+) 35.09	(+) 35.09	0
9	(+) 35.09	(+) 17.47	0.020

5.3.3 Temperature stability

5.3.3.1 The effect of freeze-thaw on hydantoin-hydrolysing activity of RU-KM1

The effects of freeze-storage on enzyme activity have not previously been investigated. The aim of this work was to investigate freeze-thaw as a means of disrupting membranes and liberating membrane-associated proteins, and thereby exposing or liberating the hydantoinase enzymes, and allowing improved enzyme/substrate interaction. Table 5.4 illustrates the changes in activity of RU-KM1 crude extract over a 7 d period with a daily freeze thaw cycle. The total activity (NCG + Gly) increased with sequential freeze-thaw cycles, as did the hydantoinase activity. This might be attributed to the fracturing of the phospholipid bilayer of the membrane, and hence the release of the hydantoinase from the membrane. However, the *N*-carbamoylase activity started to decline after day 2 and after day 7 there was only residual *N*-carbamoylase activity detectable. It has been previously demonstrated that *N*-carbamoylase is a relatively unstable enzyme (Ragnitz *et al*., 2001a; Siemann *et al*., 1993a,b). Thus the value of physical freeze-thaw to liberate the hydantoin-hydrolysing enzymes is limited to the hydantoinase, as this enzyme showed an increase in activity over the 7 day freeze-thaw cycle.

Table 5.4: Changes in hydantoinase and *N*-carbamoylase activity of RU-KM1 crude extract after 7 freeze-thaw cycles

Day	Hydantoinase ($\mu mol.h^{-1}.mL^{-1}$)	NCAAH ($\mu mol.h^{-1}.mL^{-1}$)	Total Hydantoinase ($\mu mol.h^{-1}.mL^{-1}$)	Percentage change (%)
0	12.3 (±0.23)	4.6 (±0.99)	16.9	0
1	12.1 (±0.12)	5.0 (±0.12)	17.1	1.2
2	13.4 (±0.09)	4.5 (±0.33)	17.9	6.2
3	14.5 (±0.10)	3.2 (±0.91)	17.7	4.5
4	15.0 (±0.90)	2.2 (±0.09)	17.2	2.0
5	15.5 (±0.02)	1.9 (±0.16)	17.4	3.0
6	16.1 (±0.35)	1.2 (±0.34)	17.3	2.4
7	16.2 (±0.20)	1.0 (±0.97)	17.2	1.8

This data represents the mean (SEM) of triplicate assays after a 1 h reaction.

5.3.3.2 The effect of storage temperature and storage duration on hydantoin-hydrolysing activity of RU-KM1

The effects of storage temperatures and times are important factors in understanding the durability of the biocatalyst. This may become particularly important in down-stream processing, when reactants, products and biocatalyst need to be separated by processes involving changes in temperature for precipitation or evaporation. Figures 5.2 and 5.3 show the changes of total hydantoinase activity of whole cells (Figure 5.2) and crude extract (Figure 5.3) after different incubation periods at various temperatures. After incubation, the samples were reacted at 40 °C for 1 h with hydantoin as a substrate. An internal positive control, assayed immediately, was included for comparison with the stored samples. Figure 5.2 shows that the biocatalyst is stable for up to 24 h at temperatures between –20 and 40 °C. There was a small loss in activity in the sample stored at 40 ° for 24 hours. This loss represented a 13.4 % reduction in total activity, while

samples stored at 25 °C showed a significant increase (38.1- 49.1 %) in activity with incubation period between 4 and 24 h. After 24 h of incubation at 4 °C, the activity of the whole cells was similar to that of the control. This is consistent with the known instability of the *N*-carbamoylase at low temperatures for extended periods (Section 5.3.2). A similar result was observed in the sample that was held at –20 °C for 168 h.

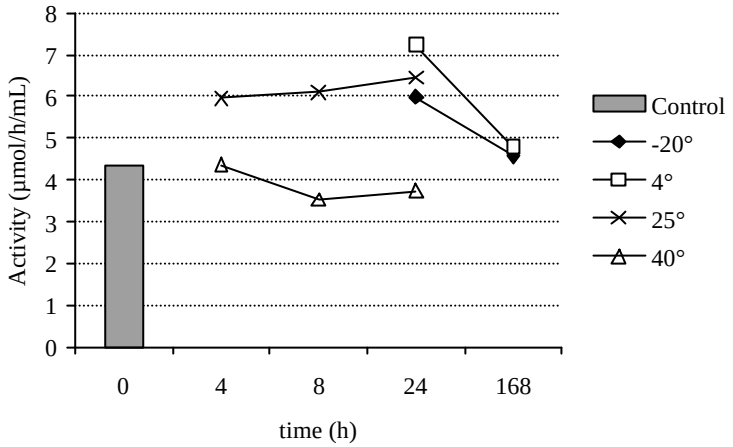


Figure 5.2: Changes in whole cell hydantoinase activity after incubation at –20° and 4° C for 24 and 168 h, and 25° and 40° C for 4, 8 and 24 h

The crude extract (Figure 5.3) showed similar results to those obtained in the storage experiments using whole cells, but the activities obtained in the crude extracts were all higher than those obtained from the whole cells, presumably because the disruption of the cell membranes makes the substrate more accessible to the enzymes. There were no deleterious effects on enzyme activity for storage periods of up to 24 h at temperatures between –20 and 40 °C (Figure 5.3). The sample stored at 40 °C showed an insignificant increase in activity (5.9 %) in comparison to the crude extract control. However, the sample stored at 25 °C showed a significant increase (6.9 – 49.4 %) in activity with the increasing periods (4 -24 h) of storage, which could be attributed to further release from the membranes. After 24 h of storage, the samples stored at 4 and –20 °C again showed a

decrease in activity consistent with the known instability of *N*-carbamoylase. This has implications for the use of the biocatalyst, as the storage over extended periods at low temperature leads to a decrease in hydantoinase activity, but shorter storage periods would be feasible.

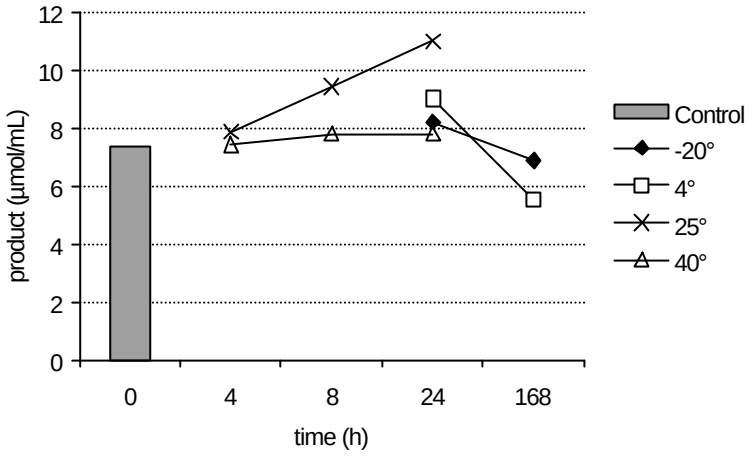


Figure 5.3: Changes in crude extract hydantoinase activity after incubation at -20 °C and 4 °C for 24 and 168 h, and 25 °C and 40 °C for 4, 8 and 24 h

5.3.4 Determination of protease activity in fermentation broth and crude extract of RU-KM1

The presence of protease activity in the biocatalytic reaction could seriously reduce the hydantoin-hydrolysing capacity and longevity of the system, affecting process economics and feasability. The presence of extracellular proteases was previously demonstrated (data not reported), using agar plates containing casein as a substrate for proteases, where "zones of clearing" around colonies were indicative of protease activity. Protease activity data from different extracts of RU-KM1 and the fermentation broth are shown in Table 5.5. These data show that under the fermentation conditions, only a small amount of protease was produced. The sample with the highest activity was the fermentation broth

(FermB), which showed an activity of 36 units.mL^{-1}. Washing of the sample removed most of the extracellular proteases. Lysis of the cells did not liberate any significant intracellular proteases into the reaction mixture. Thus the potential problem of proteases was reduced through two washes in buffer.

Table 5.5: Protease activity detected in fermentation broth (FermB), post-wash supernatant (Supt1), post lysis supernatant (Supt2), post substrate reaction supernatant (Supt3). A positive control (Solvay proteolytic L-1000) was included

Sample ID	Protease Activity (Units/min)*
Solvay proteolytic L-1000	378.5
FermB	37.2
Supt1	8.3
Supt2	5.3
Supt3	3.4

1 unit = the µmol of *p*-nitroaniline produced per minute per mL protease

5.3.5 Ammonia inhibition of RU-KM1 crude extract hydantoinase and *N*-carbamoylase

The final by-products of cellular hydantoin hydrolysis are CO_2 and NH_4^+. It has been shown that the *N*-carbamoylase is subject to product inhibition (Runser *et al*., 1990; Hartley *et al*., 1998). To investigate the effect of ammonia (NH_4^+), a by-product of hydantoin hydrolysis, on enzyme activity, cells were harvested in late log phase and assayed for hydantoinase and *N*-carbamoylase activity. High levels of activity were

detected for both enzymes when cells were grown in media containing 1 % hydantoin as a sole nitrogen source, as this is a growth-rate limiting nitrogen source ($\mu < 0.05$ OD Uh^{-1}) for RU-KM1 (Table 5.6). The activity of both enzymes was repressed or uninduced during growth with ammonium sulphate, as ammonium sulphate is a non growth-rate limiting nitrogen source for RU-KM1 ($\mu > 0.1$ OD Uh^{-1}).

To further investigate the effect of NH_4^+ on the hydantoin-hydrolysing activity of RU-KM1, the cells were subjected to "ammonium shock" to determine whether enzymes were reversibly inhibited by ammonia. The cells were grown in HMM to late log phase and harvested, and then resuspended in buffer containing 0.1 % NH_4^+ for 30 min. After 30 min incubation in this ammonia shock medium (ASM) the hydantoinase activity was significantly reduced, as compared with activity in the HMM (Figure 5.4). No significant reduction in activity was observed with the *N*-carbamoylase. These data indicated that not only did non-growth limiting nitrogen sources (NH_4^+) have repressive effects on the hydantoin-hydrolysing enzymes of RU-KM1, but this repression was reversible. The effect of the NH_4^+ on the cells was termed "ammonia shock" (Hartley, 2001). When ammonium-shocked RU-KM1 cells were resuspended in HMM, the hydantoinase activity returned to approximately original levels (Figure 5.4).

Table 5.6: Effect of ammonia on hydantoin-hydrolysing enzyme activity of whole cells and crude extract of RU-KM1 grown in HMM or ammonim sulphate containing medium

Nitrogen Source for RU-KM1	Hydantoinase ($\mu mol.h^{-1}.mL^{-1}$)		*N*-carbamoylase ($\mu mol.h^{-1}.mL^{-1}$)	
	Whole cells	Crude Extract	Whole cells	Crude Extract
1.0 % HMM	7.2 (±0.16)	12.7 (±0.14)	1.5 (±0.21)	6.1 (±0.01)
0.1% NH_4^+	2.3 (±0.15)	3.4 (±0.02)	0.9 (±0.72)	1.9 (±0.18)

This data represents the mean (SEM) of 3x triplicate

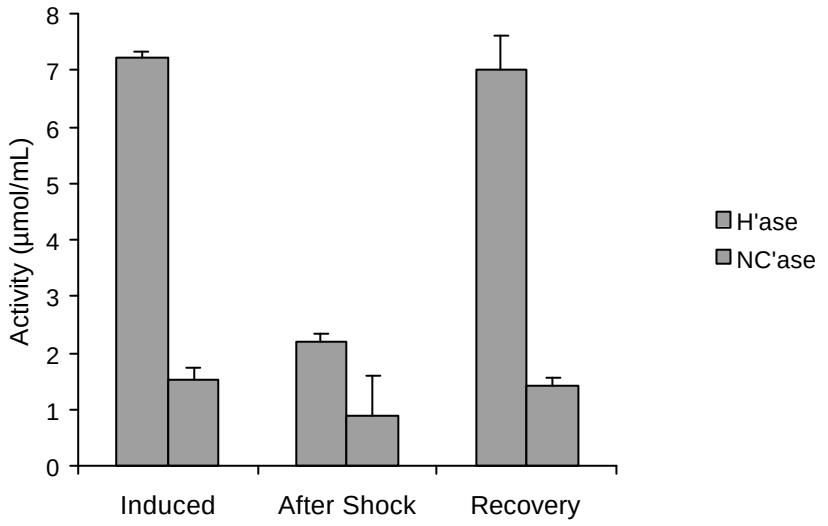


Figure 5.4: Ammonia shock effect on hydantoinase and *N*-carbamoylase activities after 30 minutes in ammonia shock medium, and the recovery of hydantoin-hydrolysing activity after resuspension in a growth-limiting nitrogen medium (HMM). Error bars represent SEM of 9 separate measurements

5.3.6 Ammonia inhibition of RU-KM1 crude extract hydantoinase and *N*-carbamoylase activity

In the conversion of hydantoins to their corresponding amino acids, CO_2 and NH_4^+ are produced as by-products of the hydrolysis (Runser *et al.*, 1990; Kim *et al*., 1994; Ogawa and Shimizu, 1997; Hartley *et al*., 1998). To determine whether the ammonia caused product inhibition, which might be concentration-dependent, the crude extract was incubated in the presence of a range of concentrations of ammonium sulphate and then assayed for enzyme activity. Concentrations of ammonium sulphate below 1 mM in the reaction mixture had a no significant inhibitory effect on either the hydantoinase and *N*-carbamoylase activity of RU-KM1 crude extract (Table 5.7).

Table 5.7: Hydantoinase and *N*-carbamoylase activity in crude enzyme extracts of RU-KM1 in the presence of ammonium sulphate

$[NH_4^+]$ (mM)	Hydantoinase Activity ($\mu mol.h^{-1}.mL^{-1}$)	*N*-carbamoylase Activity ($\mu mol.h^{-1}.mL^{-1}$)
0	13.5 (±0.13)	5.2 (±0.05)
1	12.0 (±0.03)	6.0 (±0.59)
5	2.5 (±0.01)	4.0 (±0.42)
10	2.6 (±0.12)	3.2 (±0.21)

This data represents the mean (SEM) of triplicate

5.4 Conclusions

In the development of a bioprocess where the substrates and products are chiral molecules, special attention needs to be given to the possibility of the reactants or products racemising. This could reduce the yields of the product of interest, and hence result in requirement for further steps, for instance to chemically racemise the substrate of the incorrect chirality, to that of the correct chirality. The results of this work indicated that there was potential for racemisation of the hydantoin substrates at elevated temperature and pH (40°C and pH 9.0). From a process perspective, the racemisation of the substrate could be a useful attribute to exploit as this might alleviate the need for optically pure substrates. The equilibrium between the D- and L- forms would favour the racemisation to the correct isomer, by the continual hydrolysis of that substituted hydantoin. Thus, there is a theoretical possibility of a 100 % conversion of the substrate. In an optimised process, it is likely that a racemic substrate would be used. Operating the reaction at a pH greater than 9 would be advantageous both in terms of substrate racemisation and the alkaline pH optima for the enzymes. However, this is only valid if the product does not racemise or can be removed from the process. This study showed that racemisation of the substrates could be exploited at elevated temperatures and alkaline conditions, and this could result in an increase in the yield of the product of the correct chirality.

The effects of multiple freeze-thaw cycles were investigated in order to determine the durability of the enzyme. The results obtained indicated that the hydantoinase enzyme was able to withstand multiple freeze-thaw cycles. This durability may be due to an association of the enzyme with the membrane, and the freeze-thaw process may have fractured the membrane, thereby facilitating the increased exposure of hydantoinase within the membrane, or allowing better interaction of the substrate and the enzyme. Further to this

the continual freeze-thaw may also have have helped in liberating the hydantoinase from the membrane into the solution, thereby increasing the hydantoinase activity.

The *N*-carbamoylase did not show an increase in activity with subsequent freeze-thaw cycles. There was an initial increase in *N*-carbamoylase activity with a single freeze-thaw cycle, possibly an apparent effect due to the disruption of the membrane and release of the hydantoinase to hydrolyse more hydantoin. The increased production of *N*-carbamyl glycine from the hydantoin, which is the substrate for the *N*-carbamoylase, would allow an increase in the *N*-carbamoylase activity. Subsequent to this, the *N*-carbamoylase activity decreased with the freezing cycles.

The stability of the enzymes was also investigated in terms of storage times at different temperatures. These results would indicate the storage durability of the biocatalyst at different temperatures, which is important from a process point of view because a more robust biocatalyst would be an advantage in a process in terms of its ability to withstand possible fluctuations in temperature. Whole cells and crude extracts showed similar stability trends. The hydantoinase and *N*-carbamoylase enzymes were more active in the crude extract than the whole cells, possibly due to the greater availability of the substrate to the enzyme in the disrupted cells, and no loss of activity was observed over a 24 h period. After this, there was a significant decrease in total hydantoinase activity (NCG +Gly) in the whole cells and the extract, presumably due to the molecular instability of the *N*-carbamoylase.

Storage stability is an important consideration when producing a biocatalyst, as it allows for less stringent measures of temperature regulation and control when using the

biocatalyst in a process situation or when transporting it. The data showed that whole cells and crude extract samples showed no change in activity for the samples stored at –20, 4 and 25 °C for up to 24 h. There was a small decrease in activity in the sample stored at 40 °C for 24 h. After 24 h there was a significant loss in activity in the samples. Thus from this data it was concluded that the biocatalyst, in whole cell or crude extract form showed significant storage durability over a wide temperature range, for a maximum of 24 h. This was an important result from a process point of view, as it this durability may be needed in the down-stream processing, where, the reactants, products and biocatalysts need to be separated by a process that may involve temperature changes for precipitation or evaporation.

Low levels of extracellular protease activity were detected in the fermentation broth at the end of a 17-hour fermentation. There was no significant protease activity detected in a suspension of lysed cells. Thus, it was possible to remove most of the protease activity present in the system during the harvesting and washing of the cells, and thereafter the activity in the supernatants was minimal. From a process point of view, this result was advantageous as the presence of proteases in the system would require the addition of protease inhibitors, addition of large amounts of other pure protein as a preferential substrate or even the partial purification of the hydantoin-hydrolysing enzymes.

Literature has not reported extensively on the regulation of hydantoin-hydrolysing enzymes by nitrogen catabolite repression mechanisms. However, the delayed onset of hydantoinase activity in a non growth-rate limiting culture media implicates a nitrogen catabolite repression-type regulation of transcription (LaPointe *et al*., 1994; Watabe *et al*., 1992b). This was confirmed to be the case in the regulation of hydantoin-hydrolysing

activity in RU-KM1 by the low levels of hydantoin-hydrolysing activity during log growth in a non growth-rate limiting nitrogen source, but optimal activity measured when grown in a growth rate limiting nitrogen source.

Further investigation into the effect of a repressive nitrogen source indicated that repression, of the hydantoin hydrolysing activity could involve the direct repression of the hydantoin-hydrolysing enzymes, through catabolite repression, or the effect could be an ammonium sulphate specific response, which causes the "ammonium shock", resulting in the rapid but reversal of the previously induced hydantoin-hydrolysing activity. This regulatory effect protects the cell from rapid depletion of energy when the efficient ammonium metabolism enzyme systems begin to operate at a maximal level, by rapidly shutting down other pathways involved in nitrogen catabolism (Merrick and Edwards, 1995).

In addition to these effects, product inhibition is a cellular regulatory mechanism. Ammonia ions have been previously reported to inhibit hydantoin-hydrolysing activity, particularly affecting the *N*-carbamoylase enzyme (Runser *et al.*, 1990; Kim *et al.*, 1994; Ogawa and Shimizu, 1997, Hartley, 2001). Similar results were observed with RU-KM1 cells, where the presence of ammonia inhibited the hydantoin-hydrolysing activity of the cells. This inhibition was reversible by further incubation in growth-limiting medium. At a concentration of greater than 1.0 mM of ammonium sulphate, the crude extracts of RU-KM1 cells showed significant inhibition of the hydantoin-hydrolysing activities. This suggested that the ammonium "shock" effect may be separate from the product inhibition effect seen with the resting-cell biocatalytic reactions, but more evidence, such as measurement of mRNA, protein and activity under ammonia shock conditions would be

needed to prove this conclusively. Thus, it is evident from the results that for the production of the amino acids from hydantoins, the removal of the ammonia would be an important part of the process. This could be achieved by the removal of the products in a continuous system by immobilisation of the biocatalyst and then removal of the products by chromatography. Any unused substrate could be recycled into the process.

Chapter 6

6. Introduction

6.1 General Introduction

The method developed for the cultivation of RU-KM1 was to inoculate into HMM and incubate for between 3 and 5 days depending on the carbon source. This seed culture was then transferred to a fermentation vessel containing HMM, where it was cultured until it had reached stationary phase. This process would yield approximately 3.0 $g.L^{-1}$ dry cell weight (DCW) biomass. Once harvested, biocatalytic assays showed hydantoinase activities ranging between 8 and 12 $\mu mol.h^{-1}.mL^{-1}$ and 2 and 3 $\mu mol.h^{-1}.mL^{-1}$ for the *N*-carbamoylase activity. These cells could then be subjected to a variety of procedures to disrupt or destroy the cell wall to liberate the intracellular enzymes. From production purposes, this protocol was not ideal, as the fermentation time could be more than 7 days and the yields of biomass too low to be viable. A cheap alternative to the costly HMM medium was therefore required. This growth medium would need to be scalable in terms of cost of ingredients, comparable yield and/or higher enzyme activities. In addition, the fermentation time would ideally be reduced to hours. This chapter describes the optimisation of the fermentation medium to maximise the yields of biomass and enzyme activity.

6.2 Materials and Methods

6.2.1 Chemicals

Hydantoin were purchased from Sigma-Aldrich. All other chemicals were of industrial grade and were purchased from local suppliers.

6.2.2 Seed culture media evaluation

The standard inoculum train for the cultivation of RU-KM1 consisted of transferring a loopful of culture from an HMM plate into a 50 mL volume of hydantoin minimal media. This culture was transferred at an OD of 0.5 – 0.8 into 200 mL HNB media. The inoculum train was shortened by almost 3 days by omitting the HMM step. Different media (chemically rich medium and a production medium) were investigated in order to optimise the biomass yield and enzyme production from a shortened fermentation cycle.

6.2.2.1 Rich media

Benchmarking, in terms of growth and enzyme profiles, was performed on RU-KM1 using the standard culturing procedure and media (Section 2.1.3). RU-KM1 was cultured from HMM agar plates containing 1 % hydantoin into 50 mL HMM. The 50 mL HMM was incubated at 30 °C with shaking (200 rpm) to an $OD_{(600nm)}$ of between 0.5 - 0.8. The seed culture was sub-cultured into 3 different media. Medium (1) contained per litre: sucrose, 10 g; peptone, 5 g; yeast extract, 5 g; KH_2PO_4, 1 g; NaCl 0, .5 g; $MgSO_4$, 0.1 g; $MnSO_4$, 0.1 g; and either uracil, 0.5 g or hydantoin 10 g. Medium (2) contained per litre: yeast extract 5 g, glycerol 5 g, and K_2HPO_4 2 g. Medium (3) contained nutrient broth and 1 % hydantoin as an inducer (HNB1). 4 HNB fermentations were run simultaneously. The organisms were grown to early stationary phase, and the cultures were sampled at 5 h intervals from the time of inoculation to determine $OD_{(600nm)}$. Colorimetric assays were

performed to determine the hydantoinase and carbamoylase levels during growth (Section 2.1.4).

6.2.2.2 Production media

The *Pseudomonas putida* fermentation medium reported by Lee *et al.* (1999) was modified to achieve a biomass loading of 30 $g.L^{-1}$ DCW in batch fermentation. The maximum biomass obtained from RU-KM1 on a rich medium had been 10.4 $g.L^{-1}$ DCW. To determine whether the low biomass and enzyme activity were due to the media composition, RU-KM1 was cultivated on this *P.putida* medium. A loopful of RU-KM1 was inoculated into 1 L Erlenmeyer flasks containing nutrient broth medium with 1 % hydantoin (200 mL), and incubated on a rotary shaker at 200 rpm at 30 °C. The inoculum was transferred after 18 h into a 2 L batch fermenter containing 1.4 L *Pseudomonas* medium ver. 1 (PP1) (Lee *et al*., 1999) consisting of (per L): Citric acid, 1.6 g; $(NH_4)_2HPO_4$, 6.0 g; $MgSO_4.7H_2O$, 2.8 g; KH_2PO_4, 8.0 g; Yeast extract, 10 g; $(NH_4)_2SO_4$, 10 g; antifoam, 1mL; trace element solution, 20 mL. The trace element solution consisted of (per L): $FeSO_4.7H_2O$, 10 g; $CaCl_2.2H_2O$, 2.6 g; $ZnSO_4.7H_2O$, 2.2 g; $MnSO_4.4H_2O$, 0.5 g; $CuSO_4.5H_2O$, 1 g; $(NH_4)Mo_7O_{24}.4H_2O$, 0.1 g; $Na_2B_4O_7.10H_2O$, 0.02 g; conc. HCl, 160 mL and dH_2O, 840 mL. Glucose (70 $g.L^{-1}$) and hydantoin (14 $g.L^{-1}$) feeds were added continuously to the fermenter from the onset of the fermentation. The pH was maintained at 7 with 32 % NH_4OH solution and the DO_2 at 40 % saturation with cascade control of the agitation. The temperature was maintained at 28 °C and the aeration was 1vvm. Samples were taken every 3 h and duplicate DCW and cell density determined (OD 600nm) values determined. Enzyme activities were determined at the beginning of stationary phase (Section 2.1.4).

Nitrogen Source Optimisation

A loopful of RU-KM1 was inoculated into 4 1 L Erlenmeyer flasks containing 200 mL HNB with 1 % hydantoin (Appendix 2) and incubated on a rotary shaker at 200 rpm at 30 °C. The inocula were transferred after 12 h into 2 L batch fermenters containing PP1 medium (Appendix 2), with the following changes; glucose (20 $g.L^{-1}$), varied yeast extract (YE) and ammonium sulphate (AS) concentrations, ie. YE (20 $g.L^{-1}$), YE + AS (10 + 10 $g.L^{-1}$), AS (10 $g.L^{-1}$) and AS (20 $g.L^{-1}$). The pH was maintained at 7 with 32 % NH_4OH solution and the DO_2 at 40 % saturation with cascade control of the agitation. The temperature was maintained at 28 °C and aeration at 1 vvm (volume/volume/minute). Samples were taken every 3 h and duplicate DCW and cell density determined (OD 600nm) values determined. Enzyme assays for hydantoinase activity were performed at appropriate time intervals (Section 2.1.4)

Carbon Source Optimisation

The PP1 fermentation media was further modified to determine the effect of different carbon sources on the growth and enzyme expression of RU-KM1. Organic acids and other carbon sources were tested as energy sources. PP1 media was prepared as above replacing the glucose with 0.035 M oleic acid, succinic acid or sucrose. The growth of the cultures was followed by monitoring the $OD_{(600nm)}$. DCW yields of each fermentation was determined. The production medium PP1, showed excessive foaming, and it was decided to further modify the media to reduce the foaming of the medium. The new medium PP2 contained (per litre): Citric acid, 0.8 g; $MgSO_4.7H_2O$, 1.4 g; KH_2PO_4, 4.0 g; Yeast extract, 10 g; hydantoin, 1.0 g; oleic acid, 10 g; antifoam, 1 mL; trace element solution, 10 mL. The trace element solution consisted of (per L): $FeSO_4.7H_2O$, 10 g; $CaCl_2.2H_2O$, 2.6 g; $ZnSO_4.7H_2O$, 2.2 g; $MnSO_4.4H_2O$, 0.5 g; $CuSO_4.5H_2O$, 1 g; $(NH_4)Mo_7O_{24}.4H_2O$, 0.1 g;

$Na_2B_4O_7.10H_2O$, 0.02 g; conc HCl, 160 mL and dH_2O, 840 mL. A loopful of RU-KM1 was inoculated into 1 L Erlenmeyer flasks containing nutrient broth media with 1 % hydantoin (200 mL), and incubated on a rotary shaker at 200 rpm at 30 °C. The inoculum was transferred into 2 L batch fermenter2 containing 1.4 L PP1, PP2 and nutrient broth with 1 % hydantoin as an inducer after 18 h growth. Growth was monitored by measuring optical density at 600nm and the specific growth rates were determined using the following formula:

$\mu = (\log_{10}Z - \log_{10}Z_0)\ 2.303/\ (t-t_0)$,

where Z is the amount of any cellular component: *t* is time (Stanier *et al*., 1976).

6.2.3 Hydantoin-hydrolysing profile during stationary phase

Results from previous investigation (Section 6.2.2.2) indicated that enzyme activity was detected only in mid-stationary growth phase. The experiments were repeated to evaluate nitrogen starvation as a means of enhancing enzyme activity. Residual hydantoin concentrations in the fermentation broth were measured by reverse phase HPLC (Appendix A4) from the onset of the fermentation.

6.2.4 Scale-up of production medium

Scale-up fermentations using PP1 and PP2 media with increased carbon and nitrogen levels were evaluated. Seed cultures of RU-KM1 were prepared as above in a 1 L Erlenmeyer flasks containing 200 ml HNB medium (Appendix A2) and incubated on a rotary shaker at 200 rpm and 30 °C. The HNB inocula (200 ml) of RU-KM1 were transferred after 12 h into 15 L batch fermenters containing 9.8 L PP2 with oleic acid

(Appendix A2) or PP1 medium with oleic acid (Appendix 2). The pH was maintained at 7, using 32 % NaOH or NH_4OH for PP2 and PP1 medium respectively. The temperature was maintained at 30 °C and the DO_2 at 40 % with cascade control of the agitator. Growth was followed by optical density at 600nm and samples were taken for enzyme activities, 10 h into stationary growth phase. The DCW was determined gravimetrically for each sample.

6.3 Results and Discussion

The commercial production of biomass has become possible through the development of fermentation technology, where the conditions within the fermentation vessel can be controlled precisely. This technology has allowed for the production of high-density biomass that would not normally be possible when using flask cultures. The potential of this technology was employed for the production of high-density biomass for hydantoin hydroloysis. The approach involved the modification of the original minimal medium to various rich media by alteration of the carbon and nitrogen sources, and the use of a published *Pseudomonas putida* high-density production medium (Lee *et al*., 1999). The specific aims of these changes were to increase biomass yields and to maintain or improve the hydantoinase and *N*-carbamoylase activity of the biomass.

6.3.1 Media Evaluation

6.3.1.1 Rich media

Chapter 2 describes investigation of the culture conditions for RU-KM1 in HMM, where low enzyme yields were obtained after 4-5 day fermentation period. Subsequently, different medium compositions were investigated as an alternative to HMM as seed cultures for the inoculum train. The typical growth period for RU-KM1 in HMM (mannitol) to stationary phase was between 30 an 40 h with a yield of approximately 3 $g.L^{-1}$ (dry cell mass) (Figure 6.1). Enzyme activities ranged from 8-12 $\mu mol.h^{-1}.mg^{-1}$ for hydantoinase activity and 2-3 $\mu mol.h^{-1}.mg^{-1}$ for *N*-carbamoylase activity. The duration of this fermentation is suitable in terms of the biomass yields and the activity obtained, for small-scale laboratory experiments. However, optimisation of the media was required for

an industrial scale fermentation process to address the problems of fermentation time and poor yields associated with the media.

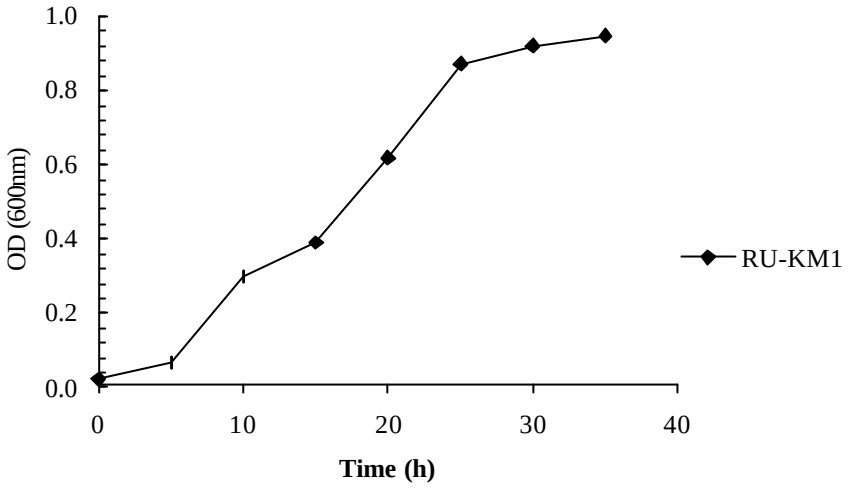


Figure 6.1: Typical growth curve for RU-KM1 grown in hydantoin minimal medium

The use of nutrient broth containing 1 % hydantoin as an inducer was considered a starting point for medium optimisation, as this medium was previously used as a seed culture, and showed the cells obtained had hydantoin-hydrolysing activity. Figure 6.2 illustrates the identical growth of 4 fermenters inoculated with a seed culture, and containing nutrient broth and 1 % hydantoin as an inducer. The fermentations entered stationary phase after 15 h. This was a significant reduction in the total fermentation time when compared to the time in HMM.

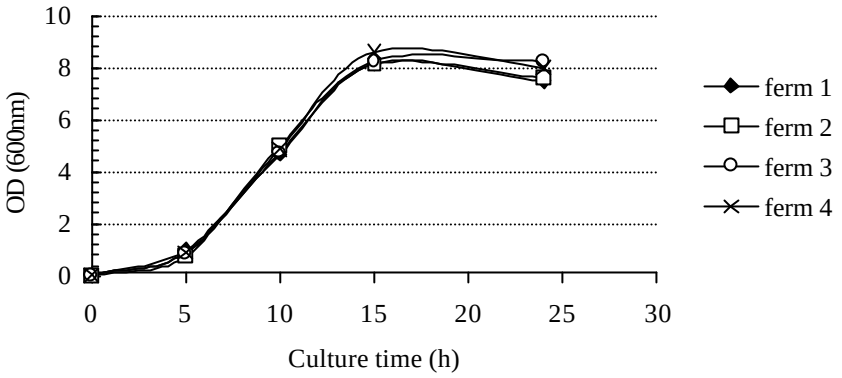


Figure 6.2: Growth curves of RU-KM1 in nutrient broth supplemented with 1 % hydantoin as an inducer, in 4 fermenters with pH and DO_2 control

The biomass yield from hydantoin nutrient broth media (Figure 6.3) showed good reproducibility, with all four fermenters yielding between 8.9 and 9.4 $g.L^{-1}$ dry cell weight (DCW) after 25 h of fermentation period. This represented a 6 $g.L^{-1}$ increase in biomass compared to the HMM, which yielded approximately 3 $g.L^{-1}$ after 40 h fermentation.

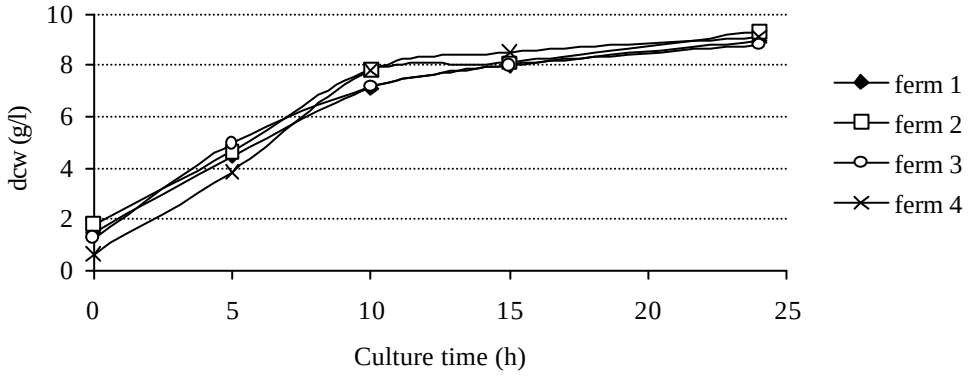


Figure 6.3: Biomass yields of RU-KM1 in hydantoin broth supplemented with 1 % hydantoin as an inducer, from the 4 fermenters with pH and DO_2 control

Enzyme activities from the fermentation samples of RU-KM1 revealed that the hydantoin-hydrolysing activity was present in all samples taken at 25 h (Figure 6.4). Samples from fermenters 1 and 3 were reacted with substrate (100 mM hydantoin) for 3 h and showed a

specific activity of 1.2 µmol.min^{-1}.mg^{-1} and 1.1 µmol.min^{-1}.mg^{-1} respectively. The samples from fermenters 2 and 4 were reacted with hydantoin for 1 h and yielded a specific activity of 2.22 µmol.min^{-1}.mg^{-1} and 2.1 µmol.min^{-1}.mg^{-1} respectively.

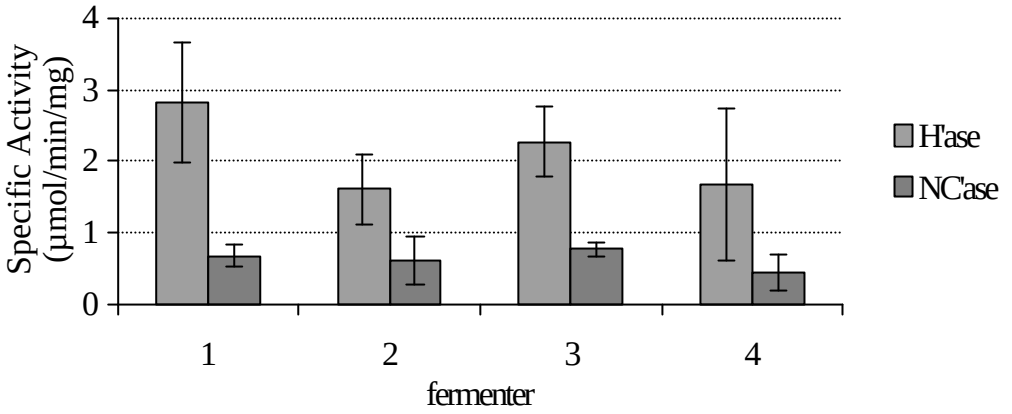


Figure 6.4: Specific activity of whole cells of RU-KM1 grown in nutrient broth with 1 % hydantoin as an inducer from the 4 fermenters and reacted with hydantoin for 3 h (fermenters 1 and 3) and for 1 h (fermenters 2 and 4)

From results shown in Figures 6.2 - 6.4 it is evident that a significant reduction in fermentation time, a 3-fold biomass increase and good enzyme activities were achieved using HNB in small-scale fermentors (compared to that of the the HMM grown cells).

The results obtained in the nutrient broth experiments (Section 6.3.1.1), showed that it was possible to eliminate the use of the HMM step for active biomass production in fermentations of RU-KM1. However, nutrient broth is too expensive to be used as a commercial-scale production medium. Further optimisation of the growth medium was aimed at the use of alternative cheaper carbon and nitrogen sources. Three different growth media investigated for further optimisation of RU-KM1 culture conditions (Figure 6.5). The growth of the three cultures was followed by OD measurement. Each culture reached stationary phase within 8 hours. This was great reduction in fermentation time

compared to that observed with the organism HMM (Figure 6.1), and the HNB (Figure 6.2). Biomass yields of approximately 6.8 $g.L^{-1}$ were obtained, compared to approximately 9 $g.L^{-1}$ achieved on HNB. However, enzyme assays performed after 10 hrs of fermentation, using whole cells in a 6 hr substrate reaction and using 100 mM hydantoin as a substrate, yielded no detectable activity. Medium 1H contained per litre: sucrose 10 g, peptone 5 g, yeast extract 5 g, KH_2PO_4 1 g, NaCl 0.5 g, $MgSO_4$ 0.1 g $MnSO_4$ 0. 1 g and hydantoin 10 g. Medium U was identical to Medium1H except the hydantoin was replaced by uracil 0.5 g. Medium (2) contained per litre: yeast extract 5 g, glycerol 5 g, and K_2HPO_4 2 g

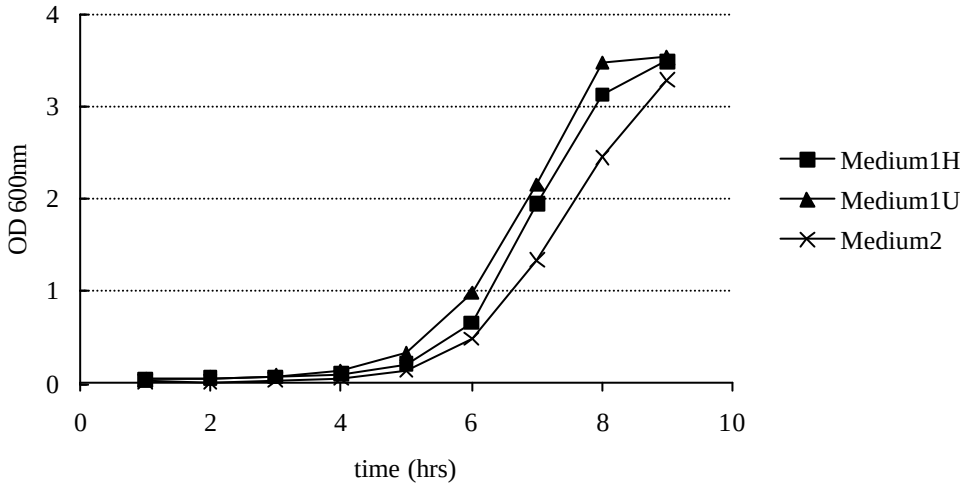


Figure 6.5: Growth of RU-KM1 in different growth media

Table 6.1 summarises the growth media, with the different hydantoin-hydrolysing activities. The biomass yields and time to reach stationary phase are compared.

Table 6.1: Summary of different rich medium conditions for the use as a seed culture in the initial stage of the fermentation cycle or as a growth medium. HMM fermentation results are included as a reference

Medium	DCW ($g.L^{-1}$)	Hydantoinase ($\mu mol.h^{-1}.mg^{-1}$)	NCAAH ($\mu mol.h^{-1}.mg^{-1}$)	Total	Time to stationary phase
HMM	3	8 -12	2 – 3	10 - 15	3-5 days
M1 + uracil	6.8	0	0	0	8 hrs
M1 + 1% hydantoin	6.8	0	0	0	8hrs
M2	6.8	0	0	0	8hrs
1% HNB	8.9 - 9.4	4.8 - 5.01	1.3 – 1.8	6.31 – 6.6	15 hrs

Key:

- M1: sucrose 10g, peptone 5g, yeast extract 5g, KH_2PO_4 1 g, NaCl 0.5g, $MgSO_4$ 0.1g $MnSO_4$ 0.1g and either uracil 0.5g or hydantoin 10g.
- M2: yeast extract 5g, glycerol 5g, and K_2HPO_4 2g.
- HNB: nutrient broth supplemrnted with 1% hydantoin
- HMM hydantoin minimal medium.

6.3.1.2 Production media

A more defined medium (PP1) based on the *P. putida* medium of Lee *at al*. (1999), was prepared and the fermenters inoculated with an 18 h seed culture grown in 1 % hydantoin nutrient broth. The resulting growth was monitored (Figure 6.6) and showed that RU-KM1 entered stationary phase after 10 h from inoculation. Cell samples from the fermenters taken after 15 h were washed and assayed for hydantoinase activity as the fermentation entered stationary phase. The results (Figure 6.7) showed comparable activity to those obtained in the HNB (Figure 6.4).

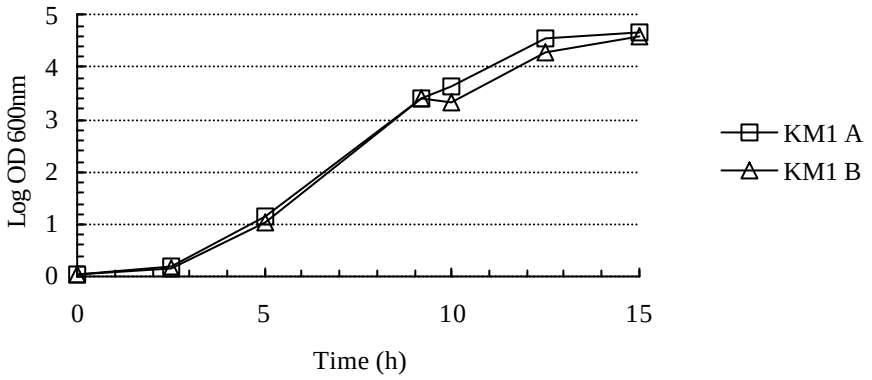


Figure 6.6: Growth of RU-KM1 in two 2 L batch fermenters with production medium PP1 (Lee *et al*., 1999)

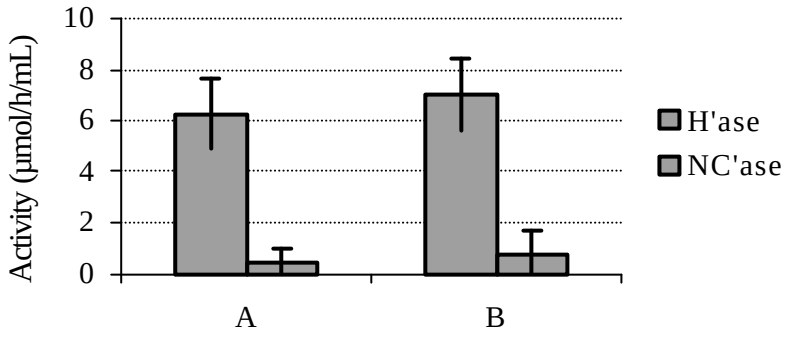


Figure 6.7: Hydantoinase and *N*-carbamoylase activities measured in duplicate (A and B) 2 L batch fermentations using PP1 medium

Nitrogen Source Manipulation:

Further optimisation of the *P. putida* medium, using different concentrations of yeast extract and ammonium sulphate was evaluated. A biomass yield of 9.2 $g.L^{-1}$ was obtained when using 20 $g.L^{-1}$ yeast extract (YE) (Figure 6.8). This was a 2 $g.L^{-1}$ improvement in comparison to the biomass produced when using a mixture of 10 $g.L^{-1}$ each of yeast extract and ammonium sulphate (AS) as in the PP1 medium. No hydantoinase activity was detected after 15 h of fermentation. When the AS concentration increased from 10 gL^{-1} to 20 $g.L^{-1}$, no significant change in biomass (5.5 $g.L^{-1}$ compared to 4.3 $g.L^{-1}$ respectively) occurred, and no enzyme activity was detected in samples taken after 15 h of fermentation.

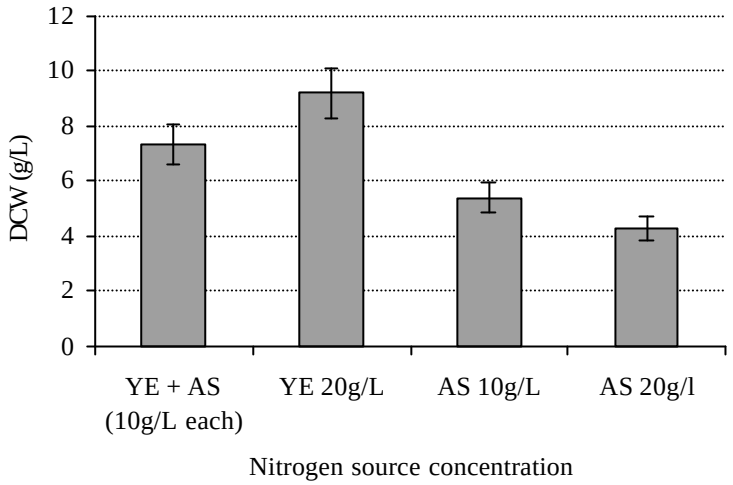


Figure 6.8: The effects of different combinations and concentrations of nitrogen sources; yeast extact (YE) and ammonium sulphate (AS) on the biomass yield of RU-KM1

Carbon Source Manipulation:

Over the past two decades many studies have been conducted on the energetics of growth. The central issue has revolved about the theoretical maximum yield of biomass from a single carbon source (Gommers *et al*., 1986). It is well known that during assimilation of carbon to biomass, it is inevitable that some substrate is converted to CO_2. In addition, different carbon sources are more or less readily utilised by different organisms as an energy source. Thus different carbon sources were evaluated for use in the production medium. The data obtained (Figure 6.9) showed that oleic and succinic acids supported the highest biomass production, yielding approximately 4.0 $g.L^{-1}$ DCW.

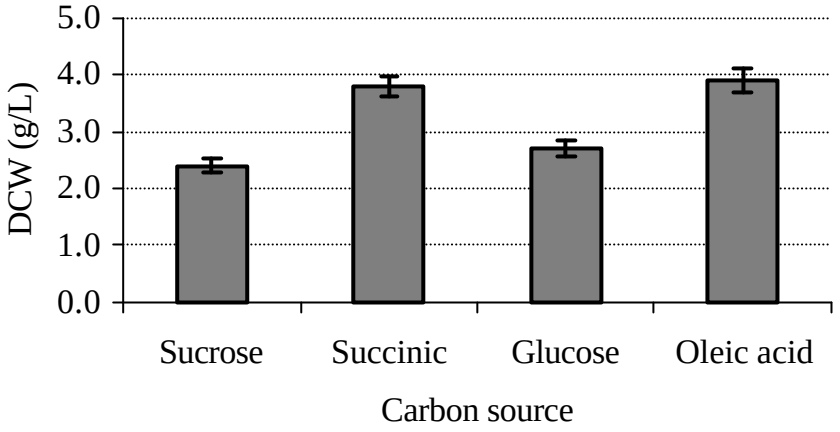


Figure 6.9: Biomass yields obtained during carbon source optimisation using different sugars and organic acids as an alternative carbon source, for the use in the production medium for the cultivation of RU-KM1 cells

The growth of the RU-KM1 was followed over the duration of the carbon source optimisation experiment and the optical density was measured to determine the stage of the culture (Figure 6.10). All fermentations entered stationary phase between 4 and 8 h. Similar growth rates were observed over the first 4 h, after which the sucrose based fermentation entered stationary phase. The fermentations containing succinic acid, glucose and oleic acid, entered stationary phase after 8 h.

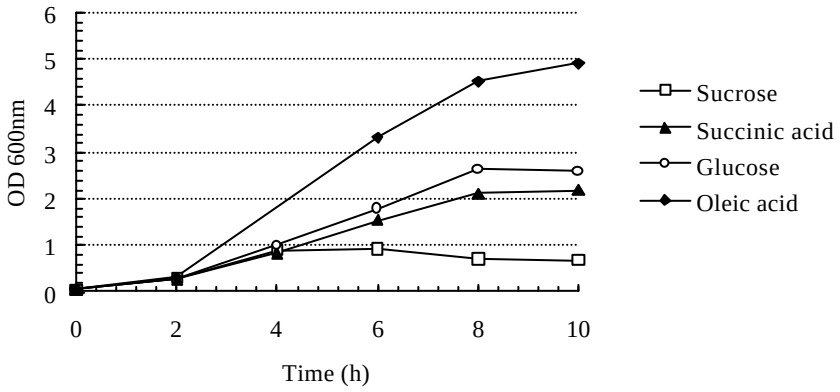


Figure 6.10: The growth of RU-KM1 using sucrose, glucose, succinic acid and oleic acid as different carbon sources

Further optimisation of the PP1 medium led to the development of the PP2 medium, where the composition was modified to remove ammonia as a nitrogen source. The rationale for this was that the lower nitrogen concentration in the production media would reduce the fermentation time by having the nitrogen becoming growth limiting, hence forcing the induction of hydantoin-hydrolysing enzymes for the utilisation of the hydantoin as a nitrogen source. Figure 6.11 shows the growth curves of RU-KM1 grown in nutrient broth with 1 % hydantoin (HNB), PP1 and PP2 media. The growth cycles of the cultures grown in the production media were similar, with all cultures entering stationary phase after 5 to 6 h of growth. The HNB culture started to enter stationary phase after about 2 h from inoculation. The two production media (PP1 and PP2) gave a greater cell density based on measurement of the optical density of the culture, and an increase in DCW yields from the different culture media was observed (8.6 to 10.4 and 17.2 $g.L^{-1}$ for the HNB, PP1 and PP2 media respectively).

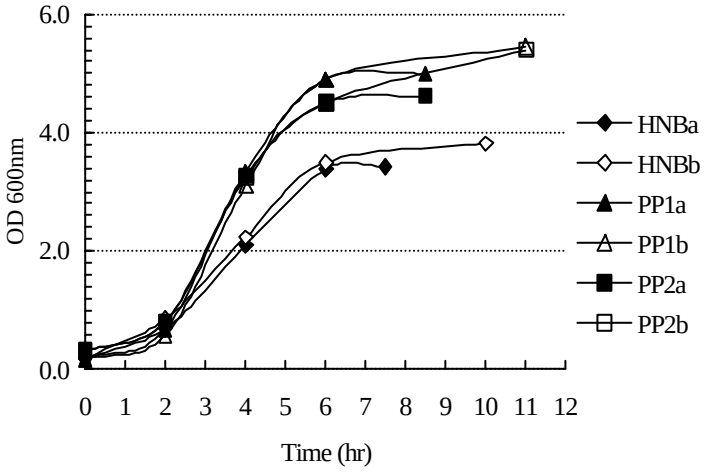


Figure 6.11: Growth of RU-KM1 grown in a rich medium, HNB, and 2 production media, PP1 and PP2. Culture flasks indicated with an "a" were sampled 3.5 h into stationary phase, and culture flasks indicated with a "b" were sacrificed 6 h into stationary phase

Specific growth rates in the production media were greater than in the rich media (Figure 6.12). The two production media showed similar growth rates and reached stationary phase after 4 h of growth with a μ_{max} of 0.8 h^{-1} for PP1 and 0.71 for PP2. The HNB culture had a μ_{max} of 0.7 h^{-1} after 2 h of growth.

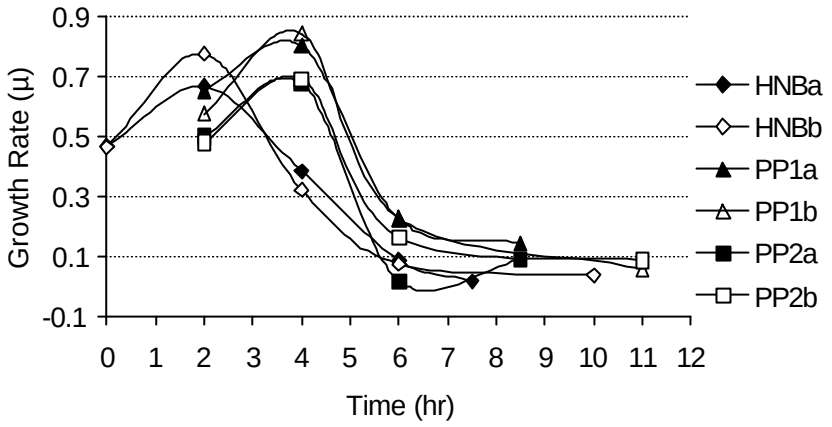


Figure 6.12: Specific growth rates of RU-KM1 in rich medium (HNB) and the two production media PP1, and PP2

Analysis of enzyme activities after 3.5 h and 6 h of growth showed different enzyme activities for cells cultured in the different media (Figure 6.13). It also indicated that the duration for which the culture had been in stationary phase was important in determining the levels of hydantoin-hydrolysing activity, since an increase in activity was observed in all media with an increase of period into stationary phase. This may be attributed to the levels of nitrogen remaining in the growth media before the organism was required to utilise the hydantoin as a nitrogen source. Thus, it was observed that the longer the organism was in stationary phase, the greater the hydantoin-hydrolysing activity. As discussed in Section 5.3.4, the levels of nitrogen in the fermentation broth may inhibit hydantoin-hydrolysing activity, and thus, extended periods in stationary phase would lead

to utilisation of the remaining nitrogen and remove the repression of the hydantoin-hydrolysing enzymes.

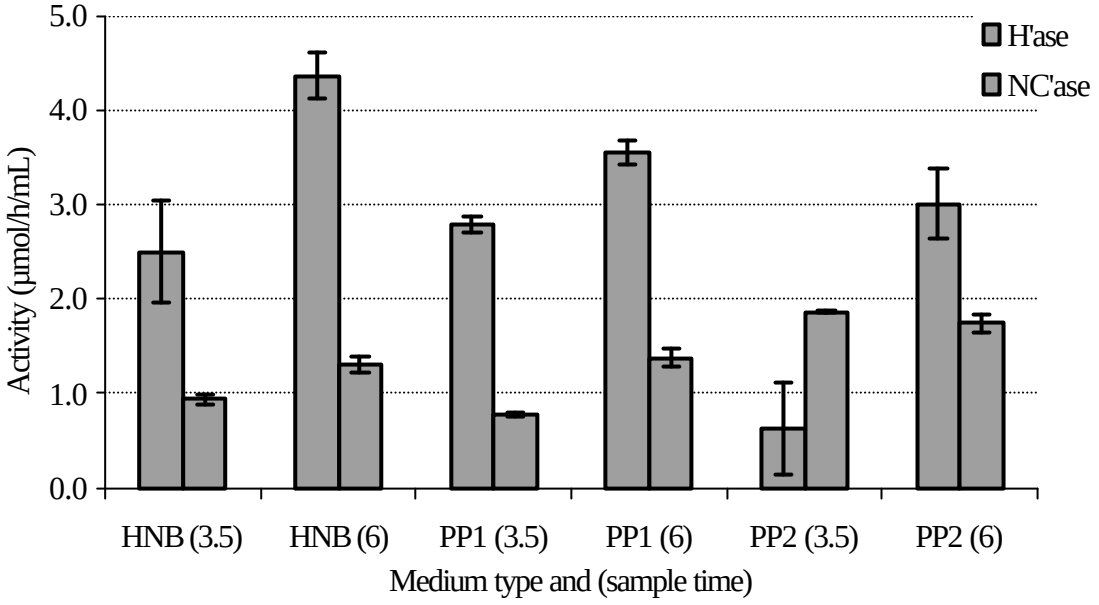


Figure 6.13: Hydantoin-hydrolysing activity determined at different times (3.5 and 6 hours) in the growth cycle of RU-KM1 using HNB, PP1 and PP2 media

6.3.2 Hydantoin-hydrolysing activity in stationary phase

The results from the experiments discussed in the previous section (6.3.1.2) indicated that greatest enzyme activity was detected well into stationary phase. In view of the change in total hydantoin-hydrolysing activity (3.4 µmol.h^{-1}.mL^{-1} to 4.7 µmol.h^{-1}.mL^{-1}) from late log phase until to 2 h into stationary phase, a profile of the growth of the organism and the activity measured was investigated. RU-KM1 was cultured in batch fermentation in HNB and PP2 media to determine the onset of stationary phase and optimal time for the production of the hydantoin-hydrolysing enzymes in order to evaluate the feasibility of nitrogen starvation as a means to increase enzyme activity. Results (Figure 6.14) indicated that there was an increase in hydantoin-hydrolysing activity in the cells up to 14 h into

stationary phase. Figure 6.15 shows the decrease in the residual hydantoin measured in the fermentation broth. There was a small decrease in concentration of residual hydantoin over the first 3 h of fermentation, and thereafter a rapid reduction was measured in the fermentation broth. This is probably due to the organism initially utilising the ammonium sulphate as a nitrogen source, and once this started to become limiting, it switched to the hydantoin as a nitrogen source. The highest activity under these fermentation conditions was measured 14 h into stationary phase, and from a process point of view, this could mean that the fermentation would need to be transferred to a holding tank for this period, this may affect the ultimate design of a the process as this would represent additional costs for the process.

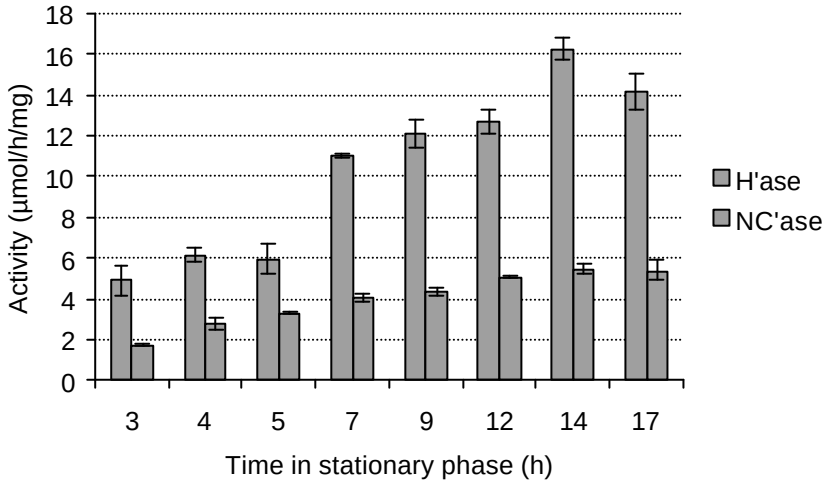


Figure 6.14: Increase in hydantoin-hydrolysing activity during stationary phase culture in PP2 medium

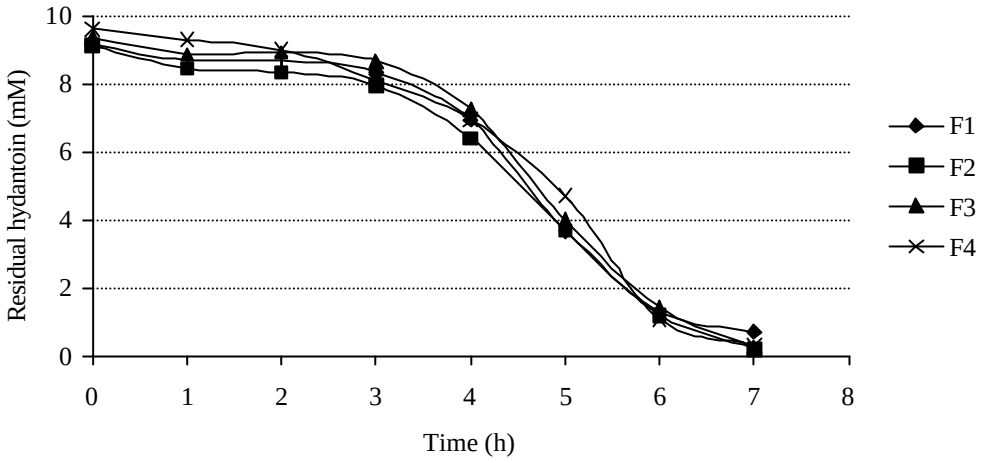


Figure 6.15: Decrease in residual hydantoin in the fermentation broth of RU-KM1 cells cultured in PP2

6.3.3 Scale-up to 15 L batch fermentations

Previous fermentations had been carried out on a 2 and 10 L scale. This was scaled up to 15 L batch fermentation, in order to produce sufficient active biomass from a single fermentation, to be used for many biocatalytic reactions without batch-to-batch variability. At this scale biomass production in PP1 medium was slightly higher than that observed in PP2 medium with a maximum biomass of 18.7 $g.L^{-1}$ and 15.6 $g.L^{-1}$ respectively. Significantly, maximum *N*-carbamoylase activity in PP2 medium was 8.1 $\mu mol.h^{-1}.mL^{-1}$, nearly 5-fold higher than observed on PP1 medium, which yielded 1.9 $\mu mol.h^{-1}.mL^{-1}$ (Figure 6.16). Hydantoinase activity was also sensitive to the medium composition with activities of 20.6 $\mu mol.h^{-1}.mL^{-1}$ and 12.1 $\mu mol.h^{-1}.mL^{-1}$ for PP2 and PP1 media, respectively.

Carbon dioxide and oxygen concentration was determined in the off-gas using a Hartman and Braun Uras 10E gas analyser (Figure 6.17). This figure shows a rapid increase in CO_2 production in the first 4 hours of the fermentation. This is coupled to a decrease in O_2 in

the off gass. The production of CO_2 and use of the O_2 is as a result of cellular metabolism. After 4 h of fermentation the CO_2 release and O_2 utilisation reach a maximum, thereafter, as the cells enter stationary phase there is a reduction in the CO_2 liberated, and an increase in the O_2 concentration. The culture remains in stationary phase for 15 h, after this time there is a rapid decrease in the CO_2 production. This is probably due to cell lysis, as the fermentation becomes very viscous. The optimum time for the production of the hydantoinase enzymes was determined to be 14 h into the fermentation (Section 6.3.2). Thus from this data a strong correlation between the time in stationary phase, cellular metabolism and production of hydantoin-hydrolysing enzymes can be seen. This further supports the hypothesis that the production of the hydantoin-hydrolysing enzymes is as a result of the culture having exhausted the nitrogen within the medium.

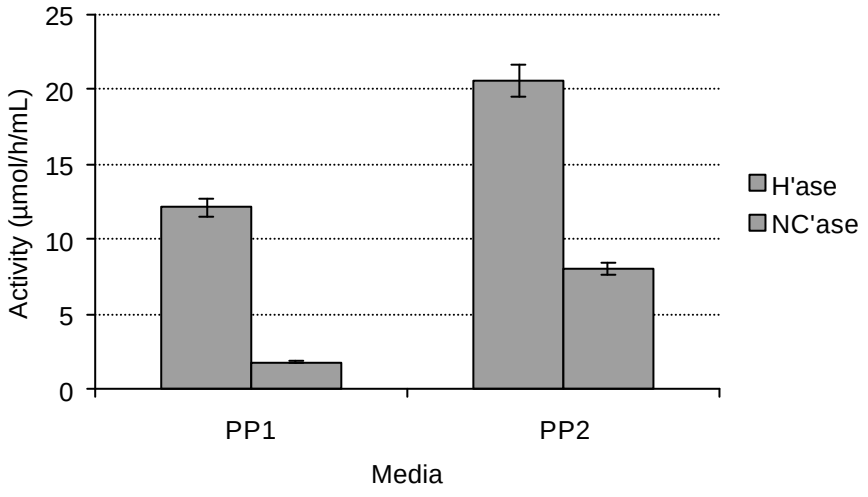


Figure 6.16: Hydantoinase and *N*-carbamoylase activities measured from samples removed from 15 L fermentation after 14 h into stationary phase

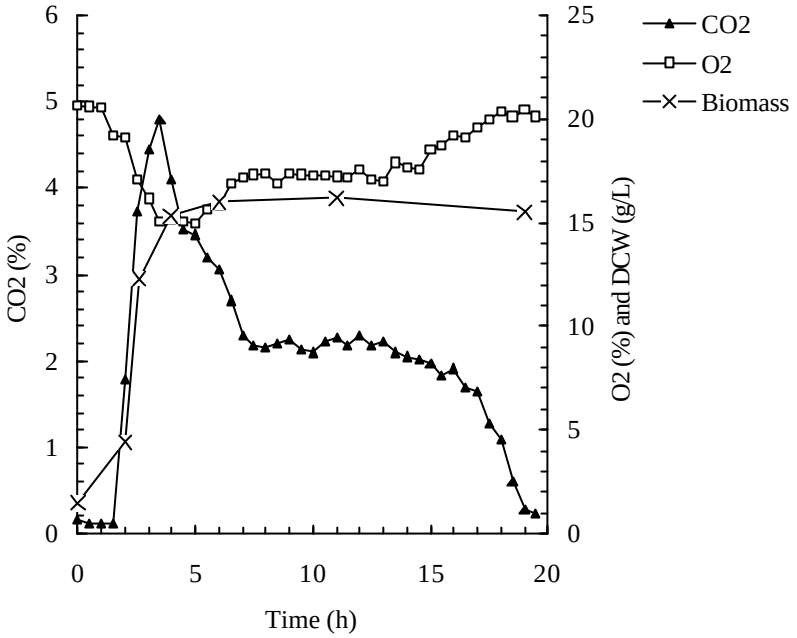


Figure 6.17: RU-KM1 metabolic relationship between CO_2 production, O_2 utilisation and biomass production over a 20 h fermentation in PP2 medium

Table 6.2 summarises the results of the production medium optimisation and the scale-up using the PP2 medium, giving a comparison of the different carbon and nitrogen sources, biomass yields and hydantoin-hydrolysing activities.

Table 6.2: Hydantoin-hydrolysing activities of RU-KM1 measured in the optimisation of two production media

Media	DCW ($g.L^{-1}$)	Hydantoinase ($\mu mol.h^{-1}.mL^{-1}$)	NCAAH ($\mu mol.h^{-1}.ml^{-1}$)	Time to stationary phase (h)
PP1 ($20g.L^{-1}$ YE)	±9.2	6.1 – 6.9	0.4 – 1.0	10
PP1 $10g.L^{-1}$ (YE+AS)	±7.2	nd	Nd	4
PP1 ($10g.L^{-1}$ AS)	±5.4	nd	Nd	4
PP1 ($20g.L^{-1}$ AS)	±4.3	nd	Nd	4
PP1 (glucose)	±2.7	-	-	4

Table 6.2 (Cont): Hydantoin-hydrolysing activities of RU-KM1 measured in the optimisation of two production media

Media	DCW ($g.L^{-1}$)	Hydantoinase ($\mu mol.h^{-1}.mL^{-1}$)	NCAAH ($\mu mol.h^{-1}.ml^{-1}$)	Time to stationary phase (h)
PP1 (oleic acid)	±3.9	-	-	4
PP1 (succinic acid)	±3.8	-	-	4
PP1 (sucrose)	±2.4	-	-	4
PP2 ($10g.L^{-1}$ YE + oleic acid)	17.4	12.6	5.1	6
* PP1 ($20g.L^{-1}$ YE + oleic acid)	±18.7	12.1	1.88	4
* PP2 ($10g.L^{-1}$ YE + oleic acid)	±15.6	20.6	8.1	6

Key : nd = not detected
- = not measured
* denotes 15 L ferementation

Enzyme activities were sampled 12 h into stationary phase, and standardised to a 1 h reaction

6.4 Conclusions

Biomass yield is an important factor in industrial processes (Verduyn, 1991), especially when the price of a single raw material makes up a large fraction of the cost of the final product (Schröder and Weide, 1974). The possibility of using a rich medium as a seed culture for the fermentations of RU-KM1 was assessed. Three different rich media were compared, with respect to the biomass yields, hydantoin-hydrolysing activity, and to results obtained from culturing in HMM and nutrient broth supplemented with 1 % hydantoin as an inducer. All three media resulted in rapid growth of RU-KM1, with the cultures entering stationary phase after 8 h of growth. However, enzyme assays showed no detectable activity in any of the cultures, as a result of the organism being cultured in a rich medium.

A production medium was needed to overcome the long fermentation time that was observed initially when using HMM. It would need to be a cheaper alternative to the more costly HMM, and scalable in terms of ingredients. Enzyme activities and biomass yields would need to be improved using this new medium. Lee *et al*. (1999), reported a growth medium designed specifically for the fermentation of *Pseudomonas putida*, and this medium was compared in terms of DCW yields and hydantoin-hydrolysing activity. It yielded a biomass of 9.2 $g.L^{-1}$ DCW, and hydantoin-hydrolysing activity was measured at 6.5 – 7.9 $\mu mol.h^{-1}.mL^{-1}$. However, the culture took 10 h to reach stationary phase, so the nitrogen and carbon sources were changed in order to reduce the fermentation time. The use of 10 $g.L^{-1}$ each of yeast extract and ammonium sulphate, reduced the fermentation time to 4 h, but there was a reduction in biomass yield. Further manipulation of the

nitrogen source to 10 or 20 $g.L^{-1}$ ammonium sulphate resulted in a further reduction in the biomass yields.

The process was scaled-up in 15 L fermenters containing PP2 and PP1 media. The biomass yields reached 15.6 $g.L^{-1}$ and 18.7 $g.L^{-1}$ in the PP2 and PP1 media respectively, and enzyme activities showed significant increases with the hydantoinase activity reaching 12.1 and 20.6 $\mu mol.h^{-1}.mL^{-1}$ in PP1 and PP2 respectively. The *N*-carbamoylase activity measured in RU-KM1 cells from PP2 medium was the highest of any fermentation trial (8.1 $\mu mol.h^{-1}.mL^{-1}$).

The hydantoin-hydrolysing activity was shown to be directly linked to the period for which the culture was in stationary phase. Using PP2 medium, the optimal time to harvest the culture was 14 h into the stationary phase. This represents a total 18 h fermentation time, excluding the 10 h seed culture. In terms of the development of a suitable fermentation procedure to produce biomass, this protocol has been successful in producing large quantities of active biomass in a significantly reduced time period.

Chapter 7

7. Introduction

7.1 General Introduction

The utilisation of immobilised biocatalysts to perform biotransformations is an important field of biotechnology (Ramakrishna and Prakasham, 1999; Venkatsubramian, 1980). Biocatalysts can be used either in a suspension, or as an aggregated or immobilised system, where the biocatalyst is adsorbed, entrapped, or encapsulated in a solid or a suspended substrate, as a means of retaining the catalyst in the reactor. The immobilisation of the biocatalyst orientates the biocatalyst in a specific phase, enabling an interaction with the bulk phase containing the substrate for the reaction, whilst it remains separate from the bulk phase. The phase containing the biocatalyst, termed the support, is usually a water insoluble, high molecular weight polymer, which restricts the movement of the biocatalyst, is chemically inert, and normally does not take part in the chemical reaction (Tramper, 1985). A variety of supports, including gels, membranes, powders and foams, have been reported (Laskin, 1985; Scott, 1995; Ryu *et al.*, 1997; Gekas, 1986; Tramper, 1985).

The advantages of using immobilised biocatalysts, as opposed to soluble systems, include; more convenient handling, ease of separation of the biocatalyst and product, and reuse of the biocatalyst (Nanba *et al.*, 1998b). All of these advantages increase the economic viability of a potential process, especially if it is to be conducted on a large scale (Tischer and Kasche, 1999). Immobilisation may also enhance the thermostability, durability and activity of enzymes in particular, and is often the most viable option when using expensive enzymes or continuous processes (Ramakrishna and Prakasham, 1999; Gekas, 1986).

Before immobilisation, certain factors must be considered (Marshall and Woodley, 1995). One of the most important is the form in which the biocatalyst is immobilised – as whole cells or as isolated enzymes. Single enzymes will catalyse a specific reaction, converting substrate into a closely related chemical product. On the other hand, whole cells represent a very complex system of enzymes and may carry out a whole sequence of reactions, the products of which may be distinctly different from the substrate. Furthermore, a single substrate may be converted to a range of products, or the desired product may be further converted into yet another product by a different enzyme. Whole cells are generally used in cases where the biotransformation is a multi-step process or the enzyme is unstable (Marshall and Woodley, 1995).

Immobilisation of whole cells represents a convenient localisation of a group of enzymes, co-enzymes, and cofactors. These systems are advantageous in that there are no extra costs in purification of the enzyme, but they are not suitable for polymer or insoluble substrates due to mass transfer and permeability constraints.

Isolated enzymes are incapable of self-replication, whereas whole cells can continuously replicate and replenish themselves. This has important consequences in terms of using a substrate for maintenance and growth, or alternatively in terms of the need to reload the reactor with fresh biocatalyst. In whole cell systems, there may be increased operating costs due to the necessity of supplying nutrients as maintenance feed, in addition to the substrate of the reaction. Separating the product from the nutrient matrix may also add costs to the process and downstream processing.

In an immobilised biocatalyst system, catalyst separation can be based on physical exclusion at the point of product removal, because the solid support particles are usually macroscopic. Rigid and well-structured particles are advantageous because they prevent blocking of filters. A process involving such removal of the catalyst has the advantage of stopping the reaction instantaneously, which may be useful for reaction control. The recovered catalyst can then be reused by contact with fresh substrate. In addition, the product stream is free of protein and cell debris, simplifying separation downstream. Purer products may be obtained and substrate utilisation may be more efficient if the mass-transfer problems associated with intact cells can be reduced, and good access to the substrate can be achieved. There are still potential problems associated with loss of activity in an immobilised biocatalyst, due to shear forces, and the need for cofactor recycling, if an enzyme is removed from its natural environment (Tischer and Kasche, 1999). Proteolytic attack and microbial contamination may also produce problems when operating such a system (Tramper, 1985).

There are four basic modes in which biocatalysts can be immobilised on a support:

1. ***Adsorption***, where the biocatalyst is held on the surface of the carrier by non-covalent interactions (van der Waals forces, hydrophobic interactions, hydrogen bridges, or ionic bonds);
2. ***Covalent bindin****g*, where the biocatalyst and support are linked by means of covalent bonds;
3. ***Cross-linking***, where the individual biocatalytic units (enzymes, organelles, whole cells) are joined to one another with the aid of bi- or multi-functional reagents;

4. ***Entrapment***, where the biocatalyst is embedded in natural or synthetic polymers, generally giving a gel structure.

The matrix of the support used and the procedure of immobilisation are an important consideration (Laskin, 1985; Scott, 1995; Ryu *et al*., 1997). Table 7.1, summarises the advantages and disadvantages of each immobilisation technique (Atkinson and Mavituna, 1991; Hartmeier, 1986).

Table 7.1: Comparison of immobilised enzyme techniques (Atkinson and Mavituna, 1991; Hartmeier, 1986).

Method	Advantages	Disadvantages
Adsorption	Simple, no modification of enzyme. Regeneration of carrier possible. Cheap technique.	Changes in substrate, ionic strength or temperature may cause desorption. Enzyme subject to microbial or proteolytic enzyme attack.
Ionic binding	Simple, with mild coupling procedure. Ion exchanger can be regenerated.	Binding weaker than covalent. Susceptible to interference from other ions. Binding highly dependent on ionic strength and pH.
Covalent attachment	Not affected by pH, ionic strength of the medium, or substrate concentration.	Active site may be modified. Costly process.
Cross-linking	Enzyme strongly bound, thus unlikely to be lost.	Loss of enzyme activity during preparation. Not effective for macromolecular substrates. Regeneration of carrier not possible
Entrapment	No chemical modification of enzyme. Enzyme not subject to microbial or proteolytic action.	Diffusion effects affect transport of substrate/product to and from active site. Preparation difficult and often results in enzyme inactivation. Continuous loss of enzyme due to distribution of pore size. Not effective for macromolecular substrates.

Some additional novel immobilisation techniques have been developed, such as the use of enzymes enclosed in reverse micelles, allowing for application in organic systems (Laskin, 1985; Scott, 1995). The enzyme is held in the inner, polar region of the reverse micelle, while the outer layer is in contact with an organic bulk solvent of the reaction containing the substrate. The enzyme-catalysed reaction occurs at the interface of the polar and organic regions.

Whatever the mode employed, it is essential that the three dimensional structure of the catalyst should not be altered, as this would effect the catalytic activity. The choice of immobilisation procedure is not limited only by the biocatalyst characteristics, but also by factors such as the reactivity of the matrix, the reagents used in the reaction and the cost of the matrix (Tischer and Kasche, 1999). The eventual form of the biocatalyst will be determined then, according to the more economically feasible approach.

The aims of the work reported in this chapter were to investigate different immobilisation techniques to allow the development of a small-scale bioreactor using RU-KM1 as the biocatalyst. The conditions used for developing the reactors were based on all the previous results obtained in investigating optimal conditions for growth and application of RU-KM1 as a biocatalyst. This bioreactor would represent a proof of concept, demonstrating the feasibility of the bioreactor for use in a biocatalytic process to produce amino acids.

7.2 Materials and Methods

7.2.1 Chemicals

Flat sheet membranes were purchased from Separation Scientific, SA or synthesised at the Polymer Science Institute, Stellenbosch, SA; Medium-viscosity alginate was purchased from Sigma-Aldrich. All other chemicals were purchased from local suppliers and were of analytical grade.

7.2.2 Immobilisation of RU-KM1 using flat-sheet membranes

Unless otherwise stated, for all immobilisation experiments using membranes, the cells were grown in 1 L Erlenmeyer flasks containing 200 mL of HMM with mannitol as the carbon source. The cells were inoculated from an HMM plate into the HMM and grown for 4 d at 29 °C with shaking at 200 rpm. The cells were harvested and washed according to the standard procedure described for whole cells (Sections 2.1.4.1) and for crude extracts (Section 2.1.4.2).

Four flatsheet membrane types, namely polysulphone, polypropylene, polycarbonate and nylon (33 mm diameter) were washed in distilled water and equilibrated in 0.1 M potassium phosphate buffer pH 8.0 for 24 h. After equilibration, the wetted membranes were placed in a stirred cell reactor (34 x 130 mm). The cell was closed and washed with 30 mL water by applying a slight pressure with nitrogen gas. The membranes were retained in a moist state. A 10 mL cell suspension (0.01 $g.mL^{-1}$, dry weight) was added to the stirred cell reactor. The reactor was sealed and pressurised using nitrogen gas. The mixture was stirred gently and the solution forced through the membrane by applying

constant pressure inside the cell. This procedure was repeated using all the membranes. Each membrane was examined under scanning electron microscope for the presence of RU-KM1 cells.

The effect of adding a crosslinking agent (2 % glutaraldehyde) was investigated using the same procedure, but glutaraldehyde was added to the cell suspensions before the system was pressurised. The cells were washed in 0.1 M potassium phosphate buffer pH 8.0 containing 2 % glutaraldehyde and allowed to incubate with membrane for 2 h. Following this, each membrane was assayed for hydantoinase activity by incubation in a petri dish containing 3 mL of 50 mM hydantoin as a substrate and then the supernatant was assayed for NCG and glycine.

7.2.3 Immobilisation of RU-KM1 whole cells using hollow fibre membranes

A 10 cm, single hollow-fibre, externally skinless, polysulphone membrane (Plate 7.1), produced at the Polymer Science Institute in Stellenbosch, was sealed in rubber tubing between two glass T-junctions with epoxy resin (Figure 7.1). A 10 mL cell suspension of RU-KM1 cells was pumped into the area surrounding the membrane and the system kept under pressure to force the cell suspension into the membrane, trapping the cells in the macrovoids of the hollow fibre. The liquid moving through the membrane was removed from the system via the lumen. After immobilisation, the membrane was removed from the system and cut into 10 mm sections and substrate incubated containing 3 mL of 50 mM hydantoin. A single 10 mm section was dried and examined under a scanning electron microscope.

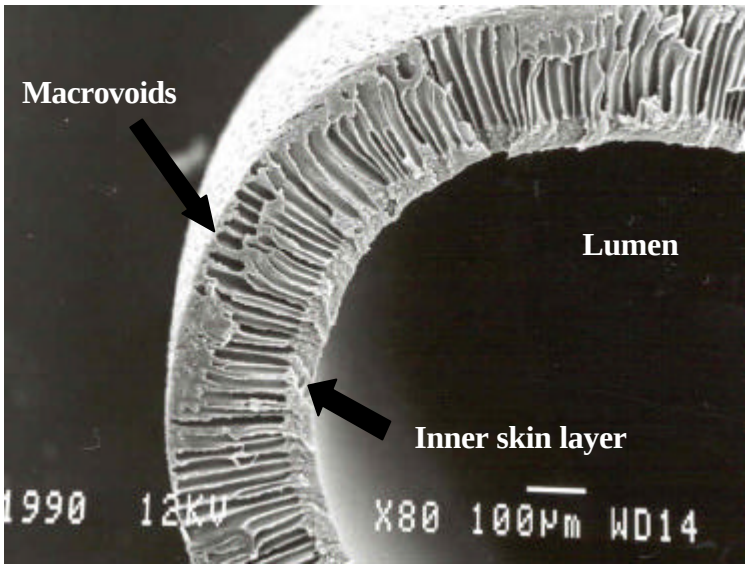


Plate 4: Scanning electron micrograph of ultrastructure of externally skinless hollow fibre polysulphone membrane

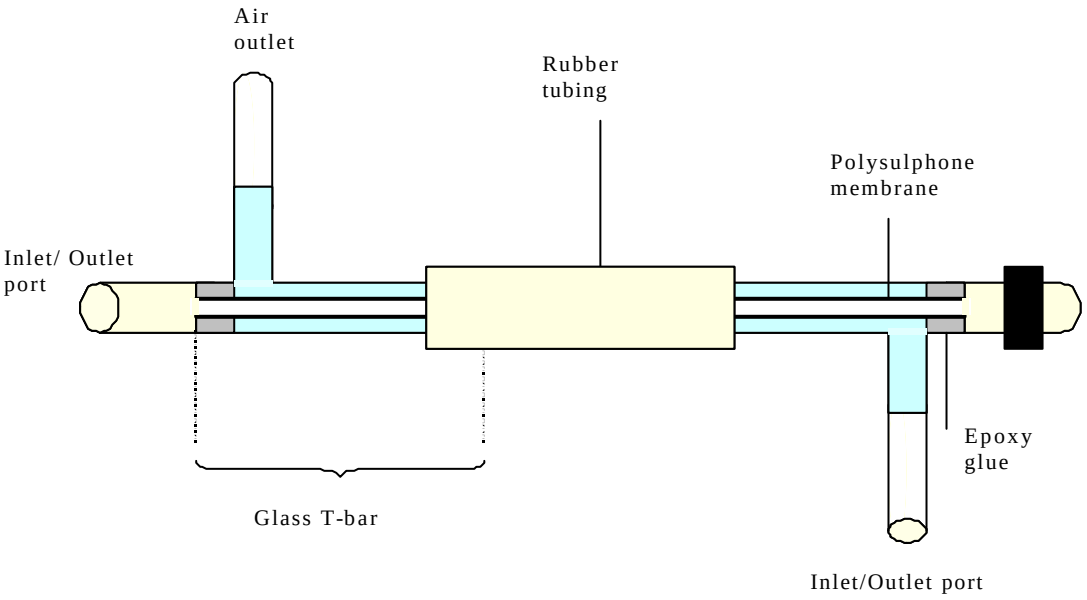


Figure 7.1: Schematic diagram of the immobilised bioreactor using a single hollow-fibre membrane

7.2.4 Bioreactor design for biotransformation using RU-KM1

7.2.4.1 Transverse Flow Hollow Fibre Membrane Bioreactor (TFHFMB)

A bioreactor for growing strain RU-KM1 was developed, using the Transverse Flow Hollow Fibre Membrane Bioreactor (Jacobs and Leukes, 1996). This bioreactor consisted of wafers containing sixteen, 10 cm, single-spaced skinless polysulphone membranes (see above) embedded in a resin (Figure 7.2). Ten of these wafers comprised the core of the membrane bioreactor. Wafers were positioned in such a manner that the membranes of adjacent wafers were perpendicular to each other, creating a transverse flow system. In addition to the membranes, each wafer had four channels, at right angles to each other. Two of the channels (marked A and B in Figure 7.2) feed into the lumen of the polysulphone membranes, while the other channels (C and D) drain from the extra-membrane/ shell space. The ten wafers were sandwiched together by two manifolds offering rigid support to the membrane wafers and allowing fluid flow in and out of the reactor. The manifolds were approximately 2 cm greater in diameter than the wafers and made from polypropylene (Figure 7.3).

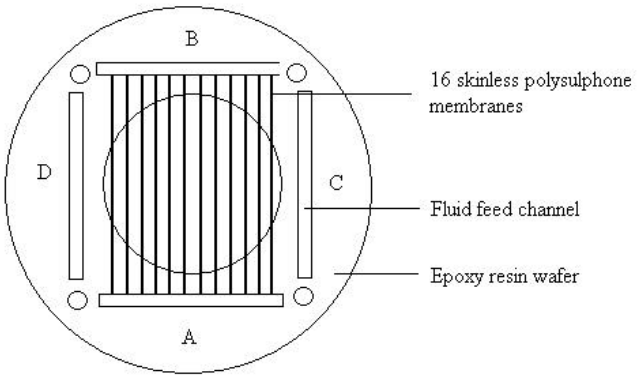


Figure 7.2: Schematic representation of a single membrane wafer

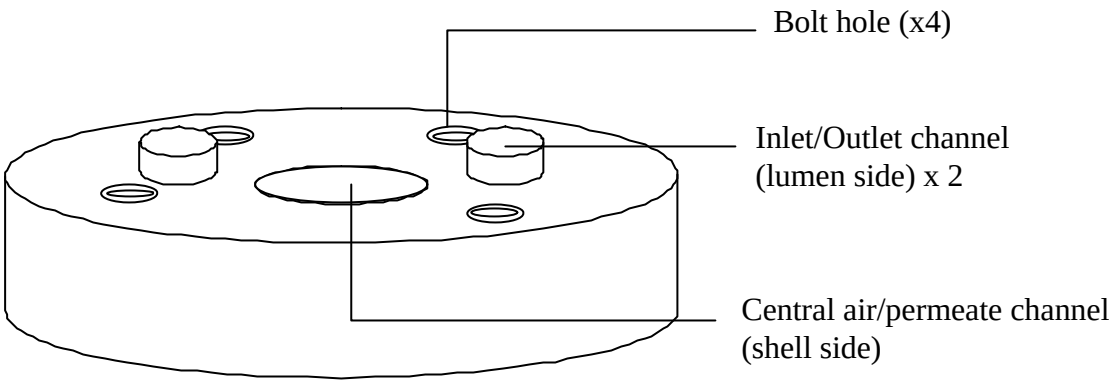
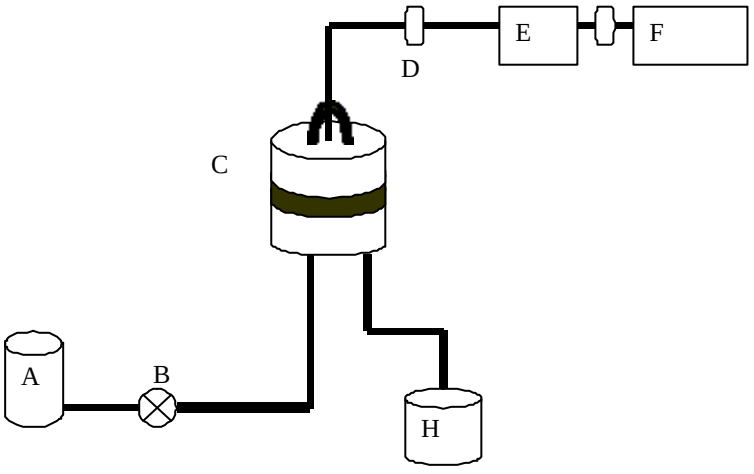


Figure 7.3: Diagrammatic representation of manifold used in Bioreactor assembly

The bioreactor was assembled (Figure 7.4) and pressure-tested with water to identify leaks. The bioreactor and piping were sterilised by pumping 200 mL 4 % formaldehyde through the system and allowing it to stand for 24 h. After sterilisation, the system was drained and flushed with 10 volumes of sterile distilled water to remove residual formaldeyhde.



A: Medium/Substrate supply
B: Pump connected to Inlet tubes
C: Bioreactor module
D: Air filters
E: Humidifier
F: Air pump
G: Outlet pipes
H: Permeate collection vessel

Figure 7.4: Operational configuration for the TFHFMB used for the immobilisation of RU-KM1

Cultures were harvested and resuspended in sterile 0.1 M phosphate buffer pH 8.0, to give a final concentration of less than 1 $mg.mL^{-1}$ wet cell mass to reduce the fouling of the membrane. The inoculation of the module was conducted from the shell side towards the lumen. The inlet pipe was closed off and the cell suspension was pumped at a flow rate of 0.13 $mL.min^{-1}$ overnight, up through the permeate channel and into the extra-membrane space, to expel all air from the chamber. Once the chamber was filled the pump was stopped, and the air inlet sealed and the inlet pipe opened. Pumping continued and, due to dead end flow, the cell suspension was forced into the macrovoids, towards the inner skin and lumen. Inoculation was carried out at the lowest flow rates possible until all the inoculum had been pumped through the system.

Once inoculation was complete, the humidifier was connected, the air inlet pipe opened and the air pump started. HMM was pumped into the system and, once the reactor was filled, it was allowed to stand overnight. The next day the pump was restarted and fresh medium fed into the reactor. This fresh medium was also allowed to stand overnight. This 'pump and stand' procedure continued for 3 d, to allow the culture time to acclimatise to reactor conditions and to begin forming a biofilm. The optimal parameters for maximum hydantoinase activity were shown (Edkins, 2000) to be a temperature of 40 °C, a flow rate of 0.45 $mL.min^{-1}$ and a reactor feed of 50 mM hydantoin. Samples were collected from the bioreactor thrice daily and assayed for the production of NCG and glycine.

7.2.4.2 Immobilisation of RU-KM1 crude extract using sodium alginate beads

A culture of RU-KM1 was harvested and protoplasts produced by the addition of lysozyme according to the procedure described in section 3.2.11 to produce crude extract. Crude extract (1 g) of RU-KM1 was resuspended in 15 mL of cold Tris-HCl pH 7.0 buffer. This mixture was added to a 50 mL solution of medium viscosity sodium alginate (2.5 %). The solution was placed in a separating funnel suspended over a beaker of $CaCl_2$ (200 mL). Immobilised biocatalyst (beads) was prepared by the slow, drop-wise addition of the alginate mixture to a gently stirring cold, 2 % $CaCl_2$ solution. After preparation the beads were removed from the $CaCl_2$ solution and washed in 3 volumes of the same buffer and stored at 4 °C until required.

Free and immobilised crude extracts from approximately 6 g of immobilised cells (equivalent to 20 mg cells per reaction) were assayed for activity using 50 mM hydantoin and 25 mM NCG as substrates. The reaction was incubated at 40 °C for 24 h with shaking. Following incubation, the supernatants were assayed colorimetrically for the production of NCG and glycine.

The effect of immobilisation on the pH optima was investigated. The assays were performed as above except that the following buffers were used: 0.1 M phosphate buffer, pH 5-8; 0.1 M Tris-HCl buffer, pH 8-9; and 0.1 M carbonate buffer, pH 9-10.

The effect of immobilisation on the temperature optima was investigated as above except that the beads were incubated with the substrates over a 24 hour period in a reaction vessel with a temperature control unit to maintain the temperatures ranging from 30-70 °C.

Stability of the immobilised enzymes and crude extract was determined by storage at 4 °C for a 4 week period in sealed reaction bottles. The residual activity was monitored on a weekly basis in order to evaluate the stability of the free and immobilised crude extract.

7.2.5 Up-flow fluidised-bed bead bioreactor (UFBB)

Crude extracts of RU-KM1 and RU-OR were immobilised separately in alginate beads according to the methods described in section 7.2.4.2 above.

Initially, a single bioreactor consisting of a single glass columns (2.6 x 22cm) attached at the base to a peristaltic pump using tubing (ID 0.5mm) was assembled. The peristaltic pump fed 50 mM hydantoin through the bioreactor at a flow rate on 0.045 $mL.min^{-1}$. Samples were collected in batches from the effluent stream and assayed for hydantoin-hydrolysing activity on a daily basis. The entire bioreactor was maintained in a 40 °C constant environment room.

A combination bioreactor was developed, consisting of a glass chromatography column packed with immobilised RU-KM1 and RU-OR crude extract. Samples were collected in batches over 24 h periods from the effluent stream of the column, and analysed for hydantoin-hydrolysing activity.

The use of a substituted hydantoin was investigated using D,L-5-HPH. The conversion of D,L5-HPH to D-HPG in the combination bioreactor containing the immobilised RU-OR and RU-KM1 biocatalysts combined was evaluated using 20mM D,L-5-HPH in Tris-HCl buffer (0.1M, pH 8) fed at a flow rate of 0.045 $mL.min^{-1}$, except 100mL substrate solution was used. NC-HPG and D-HPG concentrations were determined colorimetrically.

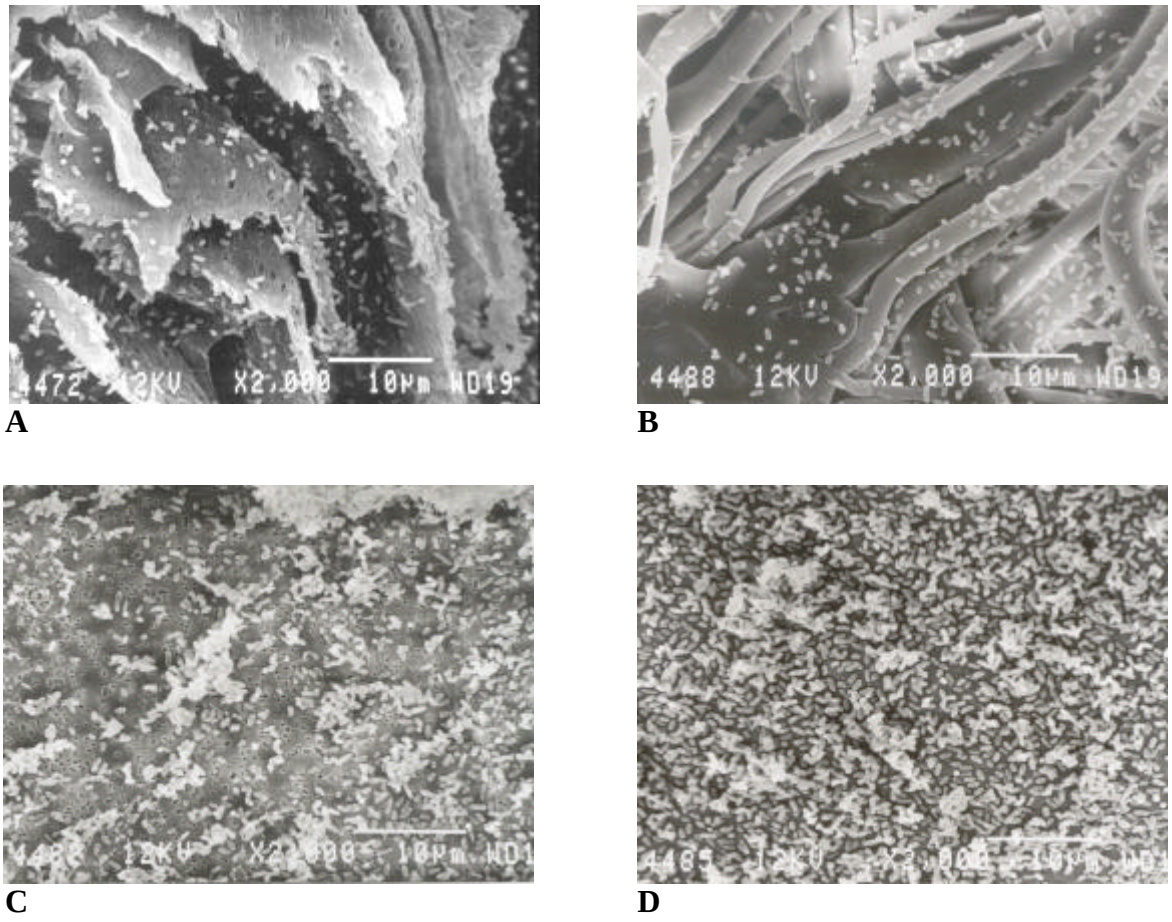
7.3 Results and Discussion

Different methods of immobilisation of the biocatalyst RU-KM1, and different reactor designs for the production of amino acids were investigated. The selection of the best bioreactor configuration would be essential for maximum productivity of the biotransformation. The use of membrane bioreactors as a replacement for the conventual batch systems was based on the separation of the biocatalyst and the substrate by a semi-permeable selective barrier. The configuration of the membrane would ultimately determine the design of the reactor (Marshall and Woodley, 1995), and the productivity of the bioreactor is influenced by the type of fibre used (Lloyd *et al*., 1997). One of the major advantages of a continuous membrane bioreactor is the ability to run the reaction continuously to increase economic viability. Continual removal of products and inhibitors from the system may overcome many downstream processing (DSP) problems associated with batch reactions (Gekas, 1986; Prazeres and Cabral, 1994). Membrane reactors are also advantageous as they possess a high surface area to volume ratio, allowing for smaller production modules (Adikane and Nene, 1995).

7.3.1 Immobilisation of RU-KM1 on flat-sheet membranes

In a pressurised reaction cell, equivalent amounts of crude extract or whole cells (to those used in the non-immobilised reactions (Section 2.1.4)) were adsorbed on the membranes by utilising the pressure cell. This facilitated the direct comparison of the activities, and thus showed the effect of the immobilisation on the activity of the hydantoin-hydrolysing activity of the whole cells or the crude extract. Table 7.2 shows the product yields detected using 50 mM hydantoin as a substrate. All the membranes showed similar hydantoin-

hydrolysing activities when whole cells or crude extract was immobilised. It was concluded that the immobilisation had no effect on the hydantoinase activity of RU-KM1 whole cells or crude extracts. Similar samples of RU-KM1 cells immobilised on the membranes were prepared for microscopy to confirm the presence of cells (Plate 7.2).



A **B**

C **D**

Plate 5: Cells of RU-KM1 immobilised by adsorption on flat sheet membranes of polysulphone (A), polypropylene (B), nylon (C) and polycarbonate (D)

Table 7.2: Immobilisation of RU-KM1 whole cells and crude extract by adsorption on different flat sheet membranes

Biocatalyst Support	Whole Cells		Crude Extract	
	Hydantoinase ($\mu mol.h^{-1}.mL^{-1}$)	Specific activity* ($\mu mol.min^{-1}.mg^{-1}$) $x10^{-4}$	Hydantoinase ($\mu mol.h^{-1}.mL^{-1}$)	Specific activity ($\mu mol.min^{-1}.mg^{-1}$) $x10^{-4}$
None	9.9	4.5	14.8	6.9
polysulphone	9.3	4.2	14.8	6.8
polypropylene	10.0	4.6	14.8	6.9
nylon	10.8	5.0	15.1	7.0
polycarbonate	9.8	4.5	13.9	6.5

*per mg cells
Assays performed using 50 mM hydantoin for 1 h

The addition of the cross-linking agent, glutaraldehyde, resulted in significant decreases in hydantoin-hydrolysing activity in all the membranes and the free cells (Table 7.3). The activity in the immobilised cells showed the greatest reduction in activity; the immobilisation reduced the activity in the polysulphone, polypropylene, nylon and polycarbonate membranes by 89.7, 98.1, 89.7 and 81.1 % respectively. Whole cells showed approximately 60 % loss in activity. This was attributed to some form of toxicity or inhibition caused by the glutaraldehyde, as the effect was seen in either the free biocatalyst and the crude extract, and similar results have been reported in the literature (Ragnitz *et al*., 2001; Bryjak *et al*., 1993).

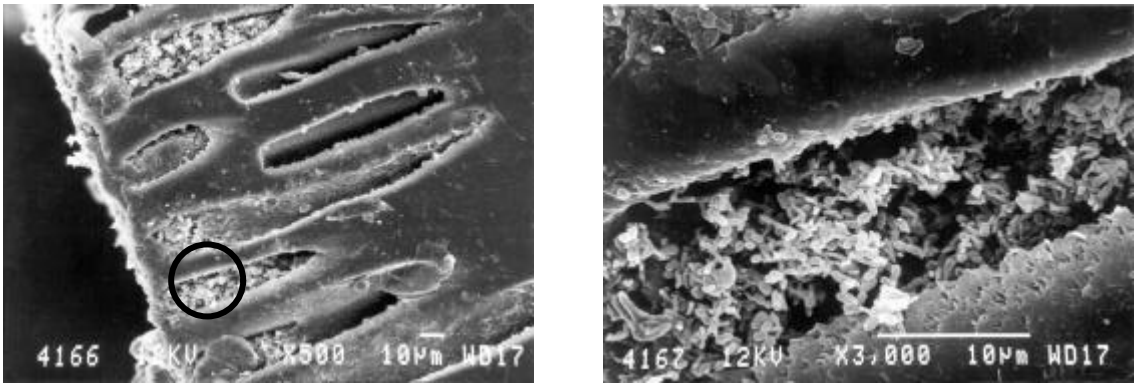
Table 7.3: Immobilisation of RU-KM1 whole cells and crude extract by cross-linking with 2 % glutaraldehye for 2 h on different flat sheet membranes

Biocatalyst Support	Whole Cells		Crude Extract	
	Hydantoinase ($\mu mol.h^{-1}.mL^{-1}$)	Specific activity* ($\mu mol.min^{-1}.mg^{-1}$) $x10^{-5}$	Hydantoinase ($\mu mol.h^{-1}.mL^{-1}$)	Specific activity* ($\mu mol.min^{-1}.mg^{-1}$) $x10^{-5}$
None	3.60	16.7	1.23	5.70
Polysulphone	0.85	3.94	1.03	4.75
Polypropylene	0.20	0.9	1.65	7.66
Nylon	1.11	5.12	1.32	6.11
Polycarbonate	1.84	8.52	1.05	4.87

*per mg cells
Assays performed using 50 mM hydantoin for 1 h

7.3.2 Immobilisation of RU-KM1 whole cells using hollow fibre membranes

The hollow-fibre polysulphone membrane bioreactor used, was originally designed for water treatment using filamentous fungi (Leukes *et al*., 1995). The application of this system as a bacterial bioreactor had not previously been demonstrated; and the development of this technique for the production of amino acids was thus a novel investigation. After immobilisation, the membranes were removed and a small section examined for the presence of cells in the macrovoids. Plate 7.3 shows that hollow-fibre membranes could be successfully used for the immobilisation of RU- KM1 cells in the macrovoids.



A B

Plate 6: Low powered SEM (A) of RU-KM1 cell immobilised in the macrovoids of the hollow-fibre polysulphone membrane. SEM of the indicated area showing RU-KM1 cells forming a biofilm in the macrovoid

Single hollow-fibre reactors were used to investigate the feasibility of immobilisation of bacteria in the hollow fibre membranes for the production of amino acids. After immobilisation, the reactor was dismantled and the membrane was cut into smaller sections and added to 50 mM hydantoin solution. Both NCG and glycine were detected in the supernatant, and the total activity measured (7.8 $\mu mol.h^{-1}.mL^{-1}$) was comparable to that obtained (8.3 $\mu mol.h^{-1}.mL^{-1}$) on the flat sheet polysulphone membrane above. This result showed that the use of a hollow fibre for the immobilisation of a bacterial culture was possible, and that development of a bioreactor with a larger surface area to volume ratio was feasible.

7.3.3 Immobilisation of RU-KM1 crude extract in sodium alginate

The utilisation of sodium alginate beads as an entrapment matrix offered an alternative immobilisation technique for RU-KM1 as a biocatalyst. This method of immobilisation would allow the production of small beads containing the biocatalyst, resulting in a large surface-area to volume ratio. This would allow for simple mass transfer of substrates and

products, and the removal of the biocatalyst from the reaction mixture millieu may be achieved by a simple filtration step.

Immobilisation of the crude extract in sodium alginate beads resulted in a 456 % increase in *N*-carbamoylase activity, from 1.6 to 7.3 $\mu mol.h^{-1}.mL^{-1}$ (Figure 7.5). No significant change in hydantoinase activity was observed. The effect was further investigated by incubation of the crude extract in the presence of the separate components used for the immobilisation; *viz* $CaCl_2$, Na-alginate and Tris-HCl pH 7.0 buffer. The activity of the crude extract in the presence of the different components was measured using 100 mM hydantoin and 50 mM NCG over a 1 h period (Table 7.4). No change in any of the activities was observed, suggesting that the immobilisation procedure (rather than the components) in some way enhanced the activity of the *N*-carbamoylase enzyme. This might result from stabilisation of the enzyme, further illustrating the instability of the *N*-carbamoylase when removed from the intracellular environment.

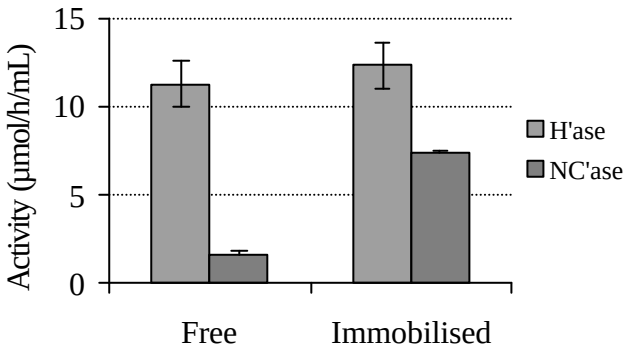


Figure 7.5: The effect of immobilisation in sodium alginate, on the hydantoinase and *N*-carbamoylase activities of RU-KM1 crude extracts

Table 7.4: The effect of components used in the sodium alginate immobilisation of RU-KM1 on the hydantoin-hydrolysing activity of RU-KM1

Component	Total Hydantoinase ($\mu mol.min^{-1}.mg^{-1}$)	NCAAH ($\mu mol.min^{-1}.mg^{-1}$)
Free crude extract	14.3 (± 0.01)	4.3 (± 0.2)
$CaCl_2$	14.1 (± 0.004)	4.1 (± 0.06)
Na-alginate	14.5 (± 0.02)	3.9 (± 0.05)
Tris-HCl pH 7	14.4 (± 0.01)	4.4 (± 0.07)

This data represents the mean (SEM) of triplicate assays

7.3.3.1 The pH stability of the sodium-alginate immobilised RU-KM1 crude extract

Immobilisation procedures have been reported to shift enzyme pH optima (Laskin, 1985; Scott, 1995). The pH optimum of the RU-KM1 crude extract had been determined (Section 3.3.6), and this section of work was to determine whether immobilisation affected the pH optima. Different buffers were utilised to determine the pH profile of the immobilised RU-KM1 crude extract. Hydantoinase and the *N*-carbamoylase activities were determined using 50 mM hydantoin and 25 mM NCG as substrates. The pH optima for the hydantoinase and *N*-carbamoylase of the immobilised RU-KM crude extract (Figure 7.6) were essentially identical to those of the soluble crude extract (Section 3.3.6; Figure 3.11). However, the physical nature of the beads was affected by the buffering system used. In phosphate buffer, the beads disintegrated at pH 7 whilst the beads remained intact when a Tris-HCl buffer was used.

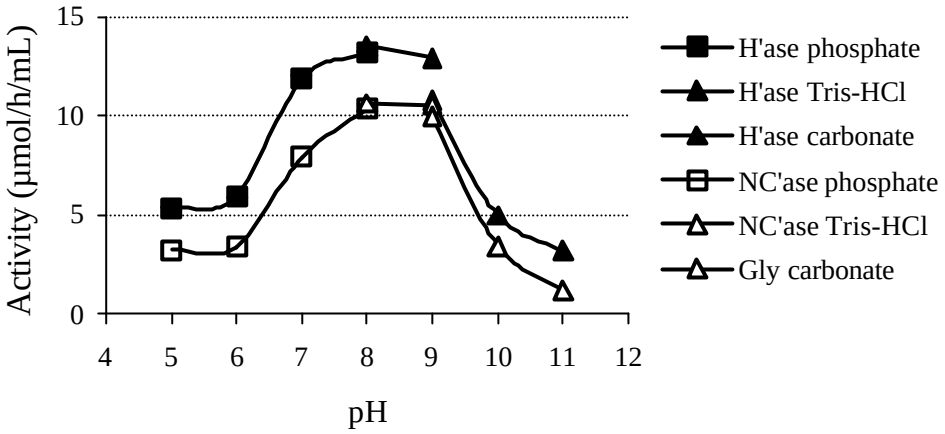


Figure 7.6: The effect of pH on the hydantoinase and *N*-carbamoylase activities of RU-KM1crude extracts of immobilised in sodium alginate beads

7.3.3.2 The temperature stability of the sodium-alginate immobilised RU-KM1 crude extract

The temperature optima and range were investigated in order to assess whether immobilisation of the crude extract of RU-KM1 had altered these parameters. The temperature optimum for the sodium-alginate immobilised RU-KM1 crude extract (Figure 7.7) was unchanged compared to the free crude extract (Figure 3.10). Hydantoinase activity was maximal at around 70 °C. At greater temperatures no activity was detected. The immobilised *N*-carbamoylase activity was maximal at around 60 °C.

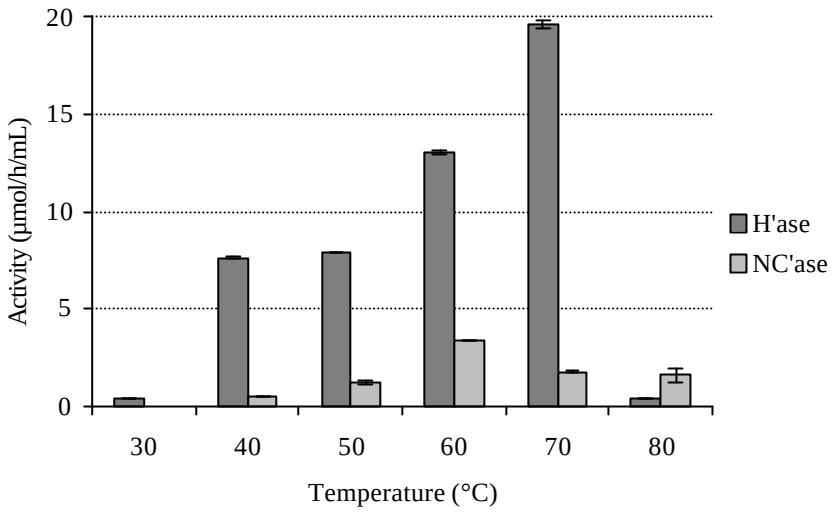


Figure 7.7: The effect of temperature on the hydantoinase and *N*-carbamoylase activities of crude extract of RU-KM1 immobilised in sodium alginate beads

7.3.3.3 Storage stability of the sodium-alginate immobilised RU-KM1 crude extract

For a process to be viable, it is important for the immobilised biocatalyst to be robust, and to be retained on/in the support matrix for as long a period as possible, whilst retaining the biocatalytic activity. The aim of this investigation was to determine the duration that the beads could be stored at 4 °C and retain their hydantoin-hydrolysing activity.

Periodic assay data (Figure 7.8) showed that the hydantoinase activity of the unimmobilised crude extract was retained over the first two weeks (3.8 to 4.6 µmol.h^{-1}.mL^{-1}), after which no hydantoinase activity was detectable. A similar trend was noted for the free *N*-carbamoylase. The hydantoinase activity of immobilised crude extract was higher (5.4 µmol.h^{-1}.mL^{-1}) than that observed for the free extract (3.8 µmol.h^{-1}.mL^{-1}), and remained constant over the 4 week period. A similar trend was observed for *N*-carbamoylase activity. This result is consistent with results obtained previously (Section

7.3.3), where a significant increase in activity was observed when the crude extract was immobilised.

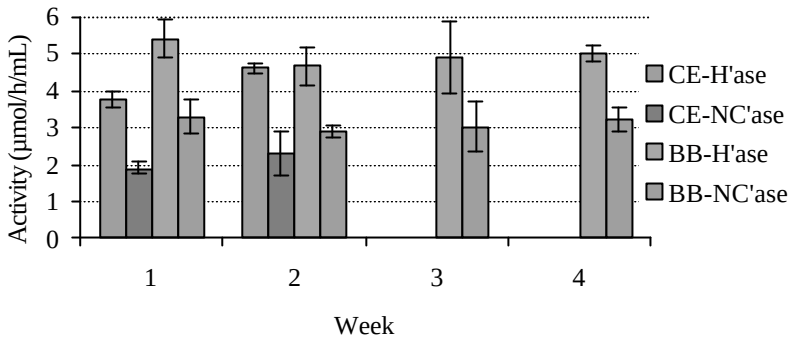


Figure 7.8: The effect of storage at 4 °C on hydantoinase and *N*-carbamoylase activities of free crude extract (CE) and alginate immobilised crude extract (BB) over a 4 week period

7.3.4 Bioreactor development

Two methods of immobilisation (polysulphone membranes adsorption and calcium-alginate bead encapsulation) showed potential for use in a bioreactor. These two immobilisation techniques were further developed and scaled-up to a bench-scale size reactor. The membrane system was developed for the use with whole cells, and the alginate beads were scaled-up for development of a bioreactor containing immobilised crude extract, as this preparation was too viscous for use in the membrane reactor.

7.3.4.1 Transverse flow hollow fibre membrane bioreactor (TFHFMB)

The transverse flow hollow fibre membrane bioreactor (TFHFMB) consisted of ten wafers (Figure 7.2) at right angles to each other, sandwiched between two manifolds (Figure 7.3). After sterilisation and inoculation of the bioreactor (Section 7.2.5.1), the substrate solution (50 mM) was pumped throught the reactor from the lumen side of the membrane and the

resulting permeate was collected in fractions over a 100-hour period and analysed for the production of NCG and glycine (Figure 7.9). The initial lag (40 h) in hydantoin-hydrolysing activity is due to the residence time of the substrate in the bioreactor. The influent substrate was diluted by the buffer remaining within the bioreactor. Only once the substrate solution had replaced the buffer from the bioreactor was activity detected, demonstrated by the rapid rise in product concentration detected in the permeate. The bioreactor reached a "steady state" after 73 h where the concentration of product in the permeate reached a maximum of 1.96 $\mu mol.h^{-1}.mL^{-1}$. A combined NCG and glycine concentration of above 1.5 $\mu mol.h^{-1}.mL^{-1}$ was maintained for 24 h after this steady state was reached. This value was significantly lower than the assays of unimmobilised cells assayed in the same manner, possibly as a result of poor bicatalyst/substrate interaction within the bioreactor due to uneven immobilisation of the biocatalyst on the membranes. The low concentrations of products produced in the bioreactor containing cells of RU-KM1, suggesting that the bioreactor system may not be as efficient as previously hoped and would need to be optimised further. This could be achieved by increasing the biomass loading or increasing the residence time of the substrate in the bioreactor. Nevertheless, the bioreactor-based biotransformation of hydantoin resulted in continuous product formation. The advantage of a continuous system is that the production of the desired product does not necessarily have to be maximal, so long as the reaction can be maintained, to result in efficient yields. This membrane-based bioreactor system for the continuous production of amino acids had not been achieved to date, and this finding has shown significant potential in the utilisation of this technology for a small-scale production unit.

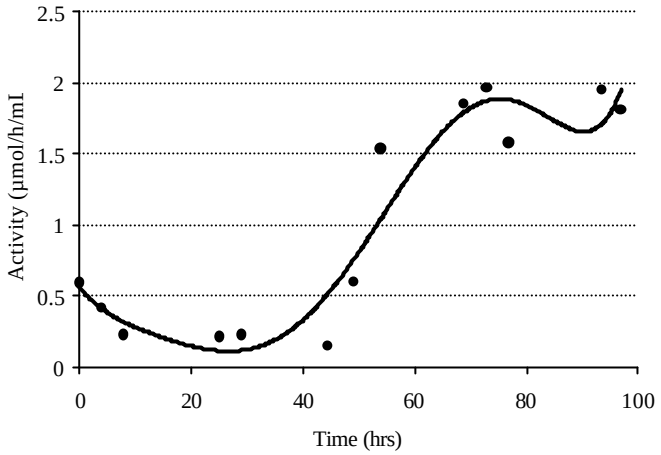


Figure 7.9: Hydantoin hydrolysis detected in permeate of the bioreactor, supplied with 50 mM Hydantoin at 40 °C with a flow rate of 0.045 mL.min^{-1}

7.3.4.2 Fluidised-bed bead bioreactor (FBB)

The aim of this work was to develop a "proof-of-concept" bioreactor for hydrolysis of hydantoin to glycine. Such a bioreactor would ideally be more efficient than a free enzyme process and robust enough to run continuously for an extended period. In view of the comparatively low results obtained in the transverse flow membrane bioreactor (Section 7.3.4.1), the issue of poor conversion, possibly due to poor interaction of the biocatalyst/substrate, was addressed by concentrating the biocatalyst in a relatively small volume compared to that of the membranes. Using the data obtained from experiments on the crude extract immobilised in alginate beads (Section 7.3.3), a single-biocatalyst bioreactor was designed for the production of amino acids from hydantoin, containing immobilised RU-KM1 crude extract.

The single-biocatalyst bioreactor, containing alginate-immobilised crude extract from RU-KM1, was supplied with 50 mM hydantoin at 0.04 ml.min^{-1} with recycle, and the effluent was sampled daily in batches for 15 days for the production of NCG and glycine. Figure 7.10 shows a gradual increase in NCG and glycine production up to day 10 (for glycine

production) and day 12 (for NCG production). The *N*-carbamoylase production remained constant from day 10 and showed only a small decrease thereafter, as indicated by a slight decrease in product formation. The hydantoinase activity remained constant from day 12 until day 15 with only a slight decrease in activity observed. At day 10 when there was an equal production of NCG and glycine, the reactor was apparently operating at its maximal productivity, as the rates of both the NCG and glycine produced were equal (Table 7.5). At this time the reactor had reached a steady state, where it remained until terminated at day 15. When this stage was reached, the NCG (22.9 µmol.mL^{-1}) and glycine (24.6 µmol.mL^{-1}) detected, represented a 95 % yield of the 50 mM hydantoin to NCG and glycine.

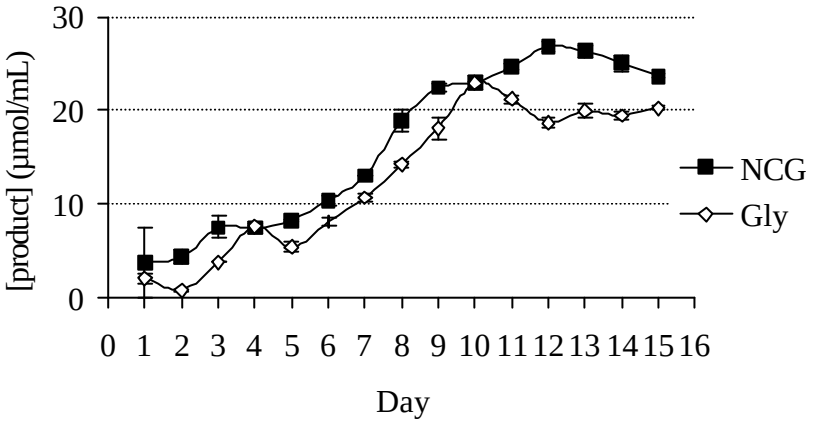


Figure 7.10: NCG and glycine detected in the single bioreactor of sodium alginate immobilised crude extract of RU-KM1

Table 7.5: The rate of product formation with sodium alginate-immobilised RU-KM1 crude extract using 50 mM hydantoin

Day	Rate ($\mu mol.min^{-1}.mg^{-1}$) x 10^{-3}	
	NCG	Glycine
1	2.6	1.45
2	1.5	0.2
3	1.7	0.8
4	1.3	1.3
5	1.0	0.7
6	1.2	0.9
7	1.3	1.06
8	1.6	1.2
9	1.7	1.4
10	1.6	1.6
11	1.6	1.3
12	1.6	1.1
13	1.4	1.1
14	1.2	1.1
15	1.2	1.0

RU-KM1/RU-OR combination bioreactor:

It was proposed that the combination of two biocatalysts, the hydantoinase enzyme from RU-KM1 and the *N*-carbamoylase enzyme from RU-OR, immobilised separately in alginate beads and placed in series in a bioreactor, would be an effective bench-scale system for the production of amino acids. The rationale for this was the known high activity and good stability of the hydantoinase from RU-KM1 and the unusually high activity of the *N*-carbamoylase of RU-OR. A bioreactor was assembled using a singlre chromatography columns of sodium alginate-immobilised RU-KM1 and RU-OR crude extract.

The combination bioreactor of RU-KM1 and RU-OR (Figure 7.11) yielded increasing hydantoinase products from day 3 to day 15. The *N*-carbamoylase activity of both biocatalysts stabilised at day 7 as shown by glycine concentrations ranging from 9.2 to 13.2 $\mu mol.mL^{-1}$, for the following 7 days of the experiment, and by the constant rates of product formation (Table 7.6). The hydantoinase activity of the biocatalysts showed constant increase in production of NCG over the 15 days, as seen in figure 7.11 and table 7.6.

The combined yields of the bioreactor system showed a 94 % conversion of the hydantoin after day 14. From Figure 7.11, it is evident that the production of NCG by the RU-KM1 hydantoinase was faster than the utilisation of the NCG to produce glycine by the RU-OR *N*-carbamoylase. The results demonstrated that the combination bioreactor was capable of hydrolysing hydantoin over an extended period of time (15 days).

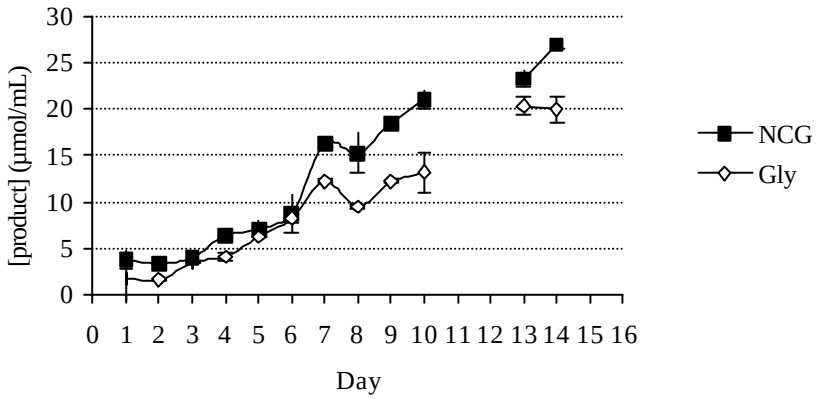


Figure 7.11: NCG and glycine measured in the combination bioreactor with RU-KM1 and RU-OR crude extract immobilised separately

Table 7.6: The rate of product formation with sodium alginate immobilised RU-KM1/RU-OR crude extract using 50 mM hydantoin

Day	Rate ($\mu mol.min^{-1}.mg^{-1}$) x 10^{-3}	
	NCG	Glycine
1	2.6	1.
2	1.2	0.6
3	0.9	0.8
4	1.1	0.7
5	1.0	0.9
6	1.0	1.0
7	1.6	1.2
8	1.3	0.8
9	1.4	0.9
10	1.5	0.9
11	nd	Nd
12	nd	Nd
13	1.2	0.6
14	1.5	0.6
15	2.6	0.6

nd – not determined

The data generated from this bioreactor is evidence of a novel method to produce amino acids from hydantoins using immobilised biocatalysts. This work has shown that is is possible to produce amino acids using a continuous bioreactor system, that out-performs similar reactions using unimmobilised biocatalysts. This system has been developed to a bench-scale prototype, which was run continuously for 15 days.

The use of substituted hydantoins was investigated using the same system. The application of this bioreactor would only be commercially viable if the hydantoins that were hydrolysed yielded economically valuable amino acids. The conversion of 5-hydroxyphenylhydantoin (5-HPH) yields hydroxyphenylglycine (HPG), which is a valuabe fine chemical. The bioreactor was washed and 5-HPH was pumped through the system and the production of NC-HPG and D-HPG was measured (Figure 7.12).

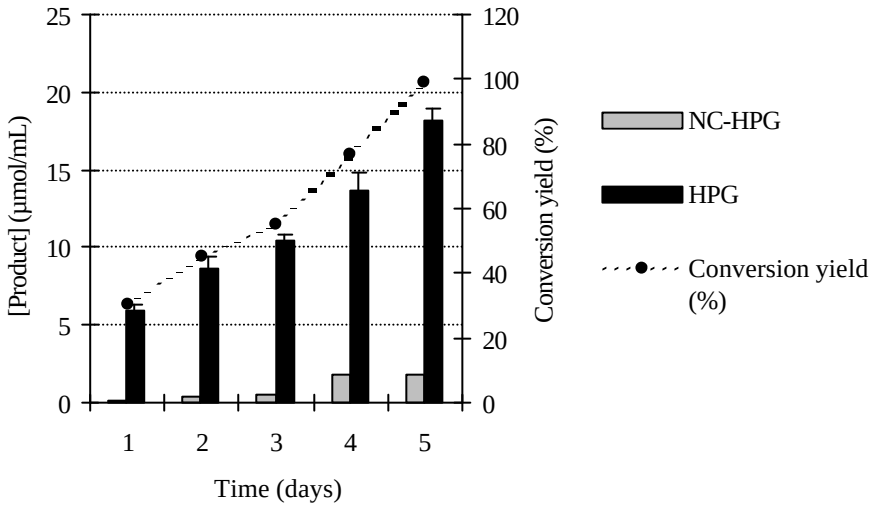


Figure 7.12: Production of NC-HPG and HPG using immobilised RU-KM1 and RU-OR crude extract

Figure 7.12 shows the conversions of HPH to HPG over a five-day period. From the graph, it is evident that this bioreactor was successful in converting 100 % of the substituted hydantoin to HPG.

Table 7.8 summarises the different activities measured for the different immobilisation techniques. All the alginate immobilised biocatalysts showed greater than 90 % conversion of the substrate. The combination reactor with HPH showed the greatest

conversion with a conversion yield of greater than 99 % for the immobilised crude extracts.

Table 7.8: Comparative activities of Biocatalyst immobilisation techniques

Immobilisation teqhnique	Substrate used	Hydamtoinase (µmol.mL^{-1})	*N*-carbamoylase (µmol.mL^{-1})	Total (µmol.mL^{-1})	Substrate converted (%)
Free (RU-KM1)	Hydantoin	11.3	0	11.3	23
Flat sheet (RU-KM1)	Hydantoin	6.9	1.3	8.2	16
Hollow fibre (RU-KM1)	Hydantoin	6.8	1.0	7.8	16
TFHFMB (RU-KM1)	Hydantoin	1.2	0.8	2.0	4
Alginate (RU-KM1)	Hydantoin	26.8	18.7	45.5	96
Alginate RU-KM1/RU-OR)	Hydantoin	26.9	11.2	38.1	94
Alginate RU-KM1/RU-OR)	HPH	1.7	18.14	19.8	99.2

7.4 Conclusions

The work discussed in this chapter focussed on the development of a bench-top bioreactor that was capable of converting hydantoin to glycine. This was not intended to be a fully optimised bioprocess, but rather, a proof of concept, showing the continuous production of amino acids from hydantoin using immobilised RU-KM1 as the biocatalyst. The work involved the investigation of some immobilisation procedures and their evaluation as potential methods for the further development of a bioprocess. Two successful immobilisation techniques, polysulphone membranes and alginate beads, were used as examples for possible application in a bioreactor for the conversion of hydantoins to amino acids.

Initial immobilisation studies were conducted using four flat sheet membranes, made of polysulphone, polypropylene, nylon and polycarbonate. The use of a pressure cell to force the biocatalysts into the macrovoids proved successful, and biomass was localised on the membrane surfaces as demonstrated by the SEM pictures (Plate 1). More important and fundamental to this work was the detection of activity from the immobilised biomass. The whole cells that were immobilised by this adsorbtion process showed similar and in some case slightly greater activity than free whole cells assayed under the same conditions (4.5×10^{-4} and 5.0×10^{-1} $\mu mol.min^{-1}mg^{-1}$ respectively). The crude extract showed higher activity in comparison to the whole cells (7×10^{-4} and 4.5×10^{-1} $\mu mol.min^{-1}mg^{-1}$ respectively), and this was attributed to different mass transfer effects. The crude extract immobilised on flat sheet membranes showed only a slight improvement in activity over the non-immobilised form. Ragnitz *et al*. (2001a,b) has shown immobilisation of a

hydantoinase and a *N*-carbamoylase via the crosslinking of the carboxylic acid groups of the enzymes and the amino group of the carrier.

The use of a cross-linking agent to enhance the immobilisation process was considered as a possible method to increase the immobilisation efficiency. The addition of glutaraldehyde resulted in significant decreases in activity in immobilised and free biocatalysts. In the case of the whole cells, the reduction in activity was about 60 % and in the crude extract, the loss of activity was about 90 %, This loss of activity was attributed to a possible inhibitory or toxicity effect of the glutaraldehyde. Thus, it was evident that an immobilisation procedure requiring glutaraldehyde was unlikely to be suitable for bioprocess for the conversion of hydantoins to amino acids using hydantoin-hydrolysing enzymes of RU-KM1. Similar results were reported for other hydantoin-hydrolysing enzymes (Bryjak *et al*., 1993; Ragnitz *et al*., 2001a,b).

A different configuration for the adsorption on membranes was considered as a further possibility. An externally skinless polysulphone hollow-fibre membrane bioreactor configuration was used, in a small-scale experiment. This immobilisation procedure proved successful and biofilm formation was demonstrated using scanning electron microscopy. When equivalent amounts of biomass were used, the activity detected from samples in the mini-reactors was comparable to that detected with the flat sheet membranes and the free whole cells.

A larger bioreactor with a greater membrane surface area, incorporating transverse-flow hollow-fibre membranes, was developed. This technology was already available in our laboratory, having been developed as a support matrix for fungal biomass. However

demonstration of a viable and enzymatically active bacterial biofilm in these membrane reactors, represents a novel result and is being submitted as a publication. The biofilm was shown to be capable of hydantoin hydrolysis, but the biocatalytic yields obtained were very low in comparison to the mini-reactors. The scale-up and optimisation of this bioreactor proved more difficult than anticipated as equal distribution of the biocatalyst, dilution of the substrate and an extended lag phase had caused lower than anticipated yields in comparison to other results, and further work would be necessary. Nevertheless, this novel approach has much potential. Optimisation of the membrane bioreactor system would require, principally, increasing the active biomass in the reactor

The need for a simple bioreactor then led to the investigation using alginate beads to entrap the biocatalyst. These biocatalytic beads proved to be physically and catalytically stable. The enzymes in the beads showed no adverse effects at temperatures up to 70 °C. Activity was detected after 4 weeks of storage at 4 °C, while under similar conditions free extract samples showed no activity after 2 weeks. Immobilised *N*-carbamoylase showed significant increase in activity and stability, with the immobilisation. This may possibly be linked to some form of conformational stabilisation. The pH optima of the encapsulated enzymes did not alter and the hydantoinase showed greatest activity at pH 8.0, whilst the *N*-carbamoylase showed optimal activity at pH 9.0, this was the same as the optima determined for the crude extract and whole cells.

These findings led to the development of a fluidised bed bioreactor. This bioreactor was initially assembled using only RU-KM1 crude extract as the immobilised biocatalyst. This bioreactor was operated continuously for 15 day and showed a 96 % conversion was achieved after 10 days. A further modification led to the development of the combined

bioreactor, consisting of alternating columns of immobilised RU-KM1 and RU-OR crude extracts. The rationale for this was the use of the RU-KM1 hydantoinase to produce NCG and the use of the RU-OR *N*-carbamoylase to convert the NCG to glycine. This bioreactor was operated for 14 days and showed a 94 % conversion at day 14. The use of substituted hydantoins as substrates in this bioreactor was also successful. When HPH was pumped throughthe system, a greater that 99 % product yield was obtained.

The hypothesis of using the two immobilised biocatalysts in a single bioreactor was successfully demonstrated in this investigation, and the development of a novel bench-scale bioreactor for the production of amino acids was successfully proven. The bioreactor showed an increase in productivity of the enzymes over an extended period of time due to the immobilisation process. This bioreactor also showed a greater than 95 % product yield in 10 days of operation, which is the best reported to date. Ragnitz *et al*. (2001a,b) have shown yields of 90 %.

With further development, these findings could lead to a more industrially applicable bioreactor with a better immobilisation matrix for the continuous production of amino acids. This could represent the first such membrane biorteactor using either immobilised bacterial cells of enzymes from two different organisms for the simultaneous production of amino acids.

Chapter 8

8.1 Final Conclusions Future Work

A number of organisms were isolated locally, which differed in their ability to utilise hydantoins with different 5-substituent groups, as substrates. The isolation procedure successfully provided a strain collection of 145 different strains capable of growth with hydantoin as a nitrogen source. Two of these showed the ability to produce *N*-carbamylamino acids and amino acids from hydantoin in crude extracts. *Pseudomonas* sp., RU-KM1, was selected as a suitable strain for further study due to its very active hydantoinase activity. *Agrobacterium tumefaciens*, RU-OR, was selected for its high *N*-carbamoylase activity. Literature reports a large selection of hydantoin-hydrolysing bacteria with widely varying characteristics (Syldatk *et al*., 1992a; Morin *et al*., 1987). The Pseudomonad and Agrobacterium species investigated in this study differed in temperature and pH optima, but show similarities in substrate selectivities and stereoselectivity (Runser *et al.*, 1990; Nanba *et al.*, 1999; Olivieri *et al.*, 1979). Other factors such as the inducer requirements and inhibitors of enzyme activity vary considerably between the different organisms (Hartley *et al.*, 2001).

In order to develop a bioprocess for the production of amino acids, isolates RU-KM1 and RU-OR were cultured in defined and rich media to characterise their growth, resulting in the development of different media for biomass containing active hydantoin-hydrolysing enzymes.

A range of hydantoinsubstrates were synthesised and were subsequently utilised to establish the substrate specificities of crude extracts and whole cells of both hydantoin-hydrolysing isolates. RU-KM1 whole cells displayed a greater selectivities for the 5-

isopropylhydantoin, whilst the crude extract of this organism showed higher activity with *p*-hydroxyphenylhydantoin. Strain RU-OR whole cells exhibited a greater selectivity for the 5-methylhydantoin in the biocatalytic reactions, whilst the crude extract of this organism prefered 5-isopropylhydantoin. In both cases the differences in the selectivities of the crude and a whole cell activities was probably due to mass transfer across cell membranes, resulting in the poor movement of the substrate into the cells or the products out of the cells. Thus, both strains demonstrated relatively broad substrate selectivity. This has distinct advantages for a process designed to synthesis a number of commercially important amino acids. From a process perspective this broad substrate range is advantageous; as the market demands change for different amino acids, the system can be utilised with ver ylittle alteration for the production of different amino acids.

Strain RU-KM1 was shown to contain a highly D-selective hydantoinase. This would result in the ultimate production of D-intermediate and finally D-amino acids. The presence of known racemases or operating the system at elevated temperatures and alkaline conditions (which the enzymes have been shown to withstand) would alleviate the use of pure chiral substrates, as chemical racemisation of chiral hydantoins, would occur (Tsuji *et al.,* 1987, Takahashi *et al.,* 1978). This was shown to be correct for L-methylhydantoin, which racemised over a 24 h period. These results indicate that under similar conditions there would be spontaneous racemisation of the chiral substrate, and the highly enantioselective D-hydantoinase would thus preferentially utilise the D-methylhydantoin. The equilibrium between the D- and L- forms would thus favour the racemisation to the correct isomer, by the continual hydrolysis of that substituted hydantoin.

Increased expression of hydantoin-hydrolysing enzymes within the cell can provide a means of increasing the yield of the final product of the biotransformation. The production of the enzymes is controlled at a genetic level, and may be constitutive or inducible. If the latter case is true, the selection and application of a suitable inducer during culturing is important. Different hydantoin analogues were selected for their ability to induce the hydantoin-hydrolysing activities of the cells. The best inducer of hydantoinase activity in RU-KM1was hydantoin at a concentration of 0.1 %. Dihydrouracil, thiouracil and dimethyl hydantoin were toxic to hydantoinase activity at concentrations greater than 0.01%. *N*-Carbamoylase activity was not affected by the addition of inducers. However, the dihydrouracil, thiouracil and dimethylhydantoin were toxic if added and a significant reduction of *N*-carbamoylase activity was measured if these compounds were present. Strain RU-OR was showed a 2-fold increase in *N*-carbamoylase activity, when induced with 2-thiouracil. This hydantoin analogue had no effects on the growth of RU-OR (Hartley *et al*., 1998, Hartley, 2001).

In a biotransformation reaction, high substrate concentrations may inhibit the enzymes involved in the system. In this study, we investigated the optimal level of hydantoin to utilise within the reaction. The results indicated that the optimal concentration for the RU-KM1 crude extract was between 0.1 and 1 M of hydantoin. At higher levels than this, and in the presence of solid substrate, assay results did not indicate an increase in activity, or resulted in difficulties in assaying for the production of products due to the formation of slurries. Similar results were obtained for strain RU-OR (Harthley *et al*., 1998, Hartley, 2001).

An obvious way to improve the reaction yields would be to operate the biocatalysis system at elevated temperatures and at optimal pH. RU-KM1 hydantoinase showed a wide operating temperature range, with small change in activity between 30 and 60 °C, with the highest hydantoinase activity at 60 °C. The *N*-carbamoylase of RU-KM1 exhibited the same broad temperature profile, but its optima was determined to between 50 - 60 °C. The *N*-carbamoylase of RU-OR showed the same broad temperature profile, with its optima being at 60 °C. This data has positive implications for the process, as it will improve yields of products due to increased rates of reaction, lowering of contamination by other microbial species that could utilise the substrates and possible facilitating in the increase in the solubilities of some of the more highly substituted substrates. No data is reported for the RU-OR microorganism, as the assay for its hydantoinase enzyme is meaningless as the product is rapidly converted to glycine.

The pH profiles of the two organisms also showed a wide range, with the hydantoinase of RU-KM1 showing hydantoin-hydrolysing activity between pH 5 -10. Its optimum was determined at pH 7.0. The *N*-carbamoylase enzyme showed a similar broad pH range with an optimum between pH 8-9. The *N*-carbamoylase activity of RU-OR was optimal between pH 9 and 10. These data are consistent with pH-activity profiles reported for hydantoin-hydrolysing enzyme systems of other bacteria (Hartley *et al*., 1998).

Preliminary results indicated that the hydantoinase enzymes may be associated with the cellular membranes. Experiments were conducted involving the removal of cellular membranes and the subsequent assaying of the different components of the disrupted cells for hydantoinase and *N*-carbamoylase activity. The results indicated that the hydantoinase enzyme was associated with the membranes, and removal of membrane fractions from

French-pressed and protoplasted cells of RU-KM1 resulted in an almost complete loss of activity. The resuspension of cellular debris and membranes from the burst protoplasts resulted in the recovery of hydantoinase activity, thus indicating that the activity was associated with the cellular membranes. *N*-Carbamoylase activity was not associated with the cell membranes, and was only detected in the cytosolic fraction of the protoplasts. T our knowledge, this is the first reported data showing the possible association of the hydantoinase with the membranes and the *N*-carbamoylase associated with the cytosolic fractions.

Surfactants were used in attempts to dissociate the hydantoinase from the membranes of the cells. Only surfactant W-1 was effective in increasing the yield of NCG in both RU-KM1 and RU-OR. However, surfactant W-1 reduced the *N*-carbamylamino acid amidohydrolase activities of both organisms.

The addition of protease inhibitors increased the hydantoinase activity of RU-KM1, but had no effect on the activity in RU-OR. The *N*-carbamoylase activity of RU-KM1 was also improved, although this might be due to the increased NCG available for conversion. The *N*-carbamoylase activity of RU-OR was unaffected by addition of protease inhibitors. Thus from a process perspective, proteolytic degradation of the biocatalyst would not be a consideration that could affect the choice of whole cells or crude extract. This option alleviates the problems associated of mass-transfer of substrates and products across the membranes.

The presence of the reducing agent DTT also enhanced the hydantoinase activity of RU-KM1. This suggests that the enzyme contains –SH groups that under normal reaction

conditions are oxidised, lowering the hydantoinase activity. The presence of DTT inhibited the oxidation of these –SH groups thus promoting activity. The increase in glycine produced under these conditions can be attributed to the increased amounts of NCG available for conversion. In the case of RU-OR, addition of DTT had no effect on hydantoinase activity, but *N*-carbamoylase activity was inhibited.

The addition of ATP significantly enhanced hydantoinase activity of RU-KM1 extracts at concentrations below 1mM. Beyond this concentration, the activity declined in a concentration dependent manner until it reached initial levels. A similar result was reported where the addition of low concentrations of ATP to a Pseudomonad crude extract resulted in increased activity (Kim *et al*., 1987; Ogawa *et al*., 1995b,c; Ishikawa *et al*., 1993). This suggests that the hydantoinase of RU-KM1 is ATP dependant, but is inhibited at high concentrations. Addition of ATP did not have as large an effect on the *N*-carbamoylase activity, but there was a small increase in glycine production in the lower concentrations. RU-OR hydantoin-hydrolysing activity was not affected by ATP, suggesting no ATP dependence.

The effects of various metal ions were investigated over a range of concentrations. The metal ions, $MnSO_4$, $CuSO_4$, $FeSO_4$ and $CoSO_4$ all displayed differing effects on the enzyme activities. The $MnSO_4$ had an activating role in the hydantoinase activity of RU-KM1 at a concentration of 1-4 mM. At levels greater than 4 mM, there was a decrease in measured activity. The $FeSO_4$ and the $CuSO_4$ at a concentration of 0.1 mM, increased hydantoinase activity slightly, but this increase was not considered significant. At concentrations greater than this, the activity decreased significantly. Thus it appears that the RU-KM1 hydantoinase is a metalloenzyme containing a catlytically important Mn^{2+}

ion. This is a similar result to other reports where Mn^{2+} and Cu^{2+} were important for catalytic activity of of a *Bascillus stearothermophilus* purified extract (Mukohara *et al*., 1994). Other reports have various different metal ions to important for hydanoin-hydrolsing activity (May *et al*., 1998b, Syldatk *et al*., 1987). Thus it appears that there are different hydantoinase that contain a number of divalent metal ions that are important for activity, and the activity of the RU-KM1 hydantoinase is based in Mn^{2+} ions.

Both whole cells and crude extract showed a significant increase in hydantoinase activity (26.8 and 12.4 % respectively), when they were reacted under the most favourable conditions as indicated by the collective results of the optimisation experiments. The addition of protease inhibitors, reducing agents and ATP together increased the hydantoinase activity in the crude extract. Along with increased temperature (50 °C) for the assay, an overall increase in productivity of the system resulted. The addition of protease inhibitors and anti-oxidants, resulted in the protection of the enzyme from protelytic attack and oxidation. Coupled to this, the additon of ATP and Mn^{2+}, resulted in activation of the catalytic site. Best activity was obtained in the crude extract, which showed an increase in product produced from 18.8 $\mu mol.h^{-1}.mL^{-1}$ to 25 $\mu mol.h^{-1}.mL^{-1}$. This represents a 50 % conversion of substrate to product per hour. From these results, the difference between the final conversions, when using crude extract or whole cells, is small (< 9%). From a process perspective it may be better to use whole cells and not crude extract as the whole cells require fewer additions to the reaction mix, to obtain high activities. However, this would need to be further investigated on an economic basis, bearing in mind that there are mass transfer problems associated with whole cells that are not experienced with crude extract.

Strain RU-KM1 produced extracellular polysaccharide (EPS) when cultured with glucose as a carbon source. Not only is this EPS a waste of carbon that may be utilised for cellular metabolism and reproduction, it also has serious implications in the utilisation of this organism or its enzymes for the biocatalytic production of amino acids. EPS can only be readibly removed using harsh conditions that would denature proteins or kill the cells. In addition, EPS capsules limit the transfer of substrates and products across the medium: cellular interface. The use of a carbon source that is not so readably available to the organism, viz. mannitol, resulted in a reduction of EPS production. There was a small loss in biomass yield and the fermentation time was substantially longer (72 h) using this carbon source. However, no adverse effects were detected in terms of activity of the hydantoin hydrolysing enzymes. This result is an important finding as it shows an alternative option as a carbon source, that eliminates the production of polysaccharide, and has little or no effect on the biomass production and activity respectively. This carbon source could possibly be used in a step in the inoculation train for a production media for the culture of RU-KM1. For downstream processing (DSP) if the cells are mechanically or chemically disrupted, the polysaccharide is not normally affected, and this itself may have cost implications in a process design as the polysaccharide would need to be removed during the recovery of the products. Thus the medium will need to be optimised for biomass production and reduction in EPS. However for purification studies the use of the manitol-based medium will overcame the production of EPS without incurring any loss of activity.

The use of cellulytic enzymes to digest cell wall components has been widely reported and documented (Cull and McHenry, 1990). These results indicate that the use of these lytic enzymes has potential for facilitating production of crude extracts and purification of

hydantoin-hydrolysing enzymes. Of the cellulytic enzymes tested in this study, lysozyme was shown to assist in the release of the enzymes from the cells. This could lead to the production of cell extracts on a large-scale as lysozyme is available in bulk quantities for industrially purpose. Thus production of crude extracts from large-scale fermentation would be possible using lysozyme.

One method of mechanical disruption is the use of sonication. This method makes use of liquid shear and cavitation to disrupt the cells within the liquid. The use of sonication as a mechanical means of disrupting RU-KM1 cells was successful in disrupting the cells and releasing the proteins into the supernatant. Hydantoinase was initially only detected in the pellet, but gradually released from the pelleted fraction over the sonication period. When using whole cells of RU-KM1 no *N*-carbamoylase activity was detected. After sonication commenced, there was a rapid increase in the *N*-carbamoylase activity in both the pellet and the supernatant, as the cells were ruptured releasing the enzyme. This occurred as more cells were disrupted with time. This method of releasing protein was effective in disrupting the cells and releasing the membrane bound hydantoinase from the RU-KM1 cells. However, it must be mentioned that this method of disruption of the cells is not a technique that could be employed on a large scale. Further development of a mechnical process would need to be investigated for the complete disruption of the cells. If extract was chosen as a better system over the whole cells, for the production of amino acids.

The use of surfactants and solubilising agents for the enhanced release of the hydantoinase and *N*-carbamoylase from the crude extract of the cells met with limited success. The only surfactants that had significant effects were W1 and CHAPS, which showed the ability to disrupt membranes of the RU-KM1 cells and hence to increase the activity. Again these

surfactants could not be used on an industrial scale as they are cost inhibitive, and thus this technique is only suitable for a small scale preparation of protein.

A protocol was developed for the partial purification of the hydantoinase of RU-KM1, involving ammonium sulphate precipitation, and hydrophobic chromatography. This process proved successful in reducing the number of proteins present, but low enzyme yields were obtained. A further purification process involving the precipitation of solubilised proteins was developed. Proteins were liberated by disrupting RU-KM1 protoplast. The removal of the cellular debris resulted in the removal of a large proportion of contaminating proteins. Subsequent precipitation of protein from the supernatant yielded a semi-pure hydantoinase preparation, with a molecular weight of between 48.5 and 66 kDa, which is similar to other hydantoinases from Psedomonad species that have been reported (Morin *et al*., 1986a, 1990; LaPointe *et al*., 1994; Chein *et al*., 1998; Chen and Tsai, 1990).

The effects of multiple freeze-thaw cycles was investigated in order to determine the storage stability of the enzyme extract. The results obtained indicate that the hydantoinase enzyme is very durable in terms of its ability to withstand multiple freeze-thaw cycles. This durability may be because of the association of the enzyme with the membrane. The *N*-carbamoylase enzyme was not as stable as the hydantoinase. There was an initial increase in activity with a single freeze-thaw cycle, after which the activity decreased with subsequent freezing cycles. It appears that the *N*-carbamoylase was not associated with the membranes.

The stability of the enzymes was investigated in terms of storage times at different temperatures. Whole cells and the crude extract showed similar trends. The hydantoinase and *N*-carbamoylase enzymes were more active in the crude extract than the whole cells, probably due to the greater availability of the substrate to the enzyme in the disrupted cells. The whole cells and the crude extract both showed no deleterious effects on activity for a 24 h duration. After this period, there was a significant decrease in activity in the cells and the extract due to the instability of the *N*-carbamoylase. Samples stored at 25 °C showed an increase in activity with an increase in the length of storage. This was attributed to breakdown of the cellular membranes and liberation of more enzyme. Storage periods greater than 24 h resulted in significant decrease in activity in the whole cells and the crude extracts. These are important results with relevance in process development, as as it gives an indication of the robustness of the biocatalyst, which is a useful characteristic, in production of amino acids. The robustness of the biocatalyst would allow a simpler process design, as no complicated cooling systems would need to be developed to protect the protein from denaturation during storage or transport.

Product inhibition is a cellular regulatory mechanism. Ammonium ions inhibit hydantoin-hydrolysing activity (Runser *et al.*, 1990; Kim and Kim, 1994; Ogawa and Shimizu, 1995), particularly the *N*-carbamoylase enzyme. Product inhibition by ammonium ions was detected at concentrations greater than 1 mM in the biocatalytic reactions with RU-KM1 crude extracts. Hydantoin-hydrolysing activity in whole cells was also affected by the presence of ammonium. Thus the process design would need to take into account the production of ammonia as a bi-product of hydantoin hydrolysis, and be able to remove the ammonia from the system, or separate the biocatalyst from the products immediately, thus removing the inhibitory effect of the ammonia.

The standard method for the cultivation of RU-KM1 involved HMM and incubating for between 3 and 5 days depending on the carbon source. This seed culture was then transferred to a fermentation vessel containing HMM, where it was cultured until it had reached stationary phase. This process would was very slow and yielded approximately 3.0 $g.L^{-1}$ DCW with hydantoinase and *N*-carbamoylase activities ranging from 8 to 12 $\mu mol.h^{-1}.mL^{-1}$ and 2 to 3 $\mu mol.h^{-1}.mL^{-1}$ respectively. From a large-scale production point of view, this protocol is not suitable, as the fermentation time was in excess of seven days and the yields of biomass and activity low. A growth medium designed specifically for the fermentation of *Pseudomonas putida* has been reported (PP1) (Lee *et al*., 1999). This medium was investigated in terms of DCW yields and hydantoin-hydrolysing activity. This production medium showed biomass yields of 9.2 $g.L^{-1}$, and hydantoin-hydrolysing activity of 6.5 to 7.9 $\mu mol.min^{-1}mg^{-1}$. However, the culture took ten hours to reach stationary phase.

Different nitrogen and carbon sources were assessed in order to reduce the fermentation time. A medium containing yeast extract and ammonium sulphate showed a reduced fermentation time to 4 h, although the biomass yield was reduced and poor activity was obtained. The medium was further developed with changes in carbon sources; glucose, oleic acid, succinic acid or sucrose. The fermentations all reached stationary phase after 4 h, although biomass yields were still low.

The results from PP1 optimisation experiments were combined and a new optimised media was proposed for the fermentation of RU-KM1. The medium, PP2 (Appendix A2), was developed and scaled-up to a 15 litre fermenter. The biomass yields were 16.6 $g.L^{-1}$

and 18.7 $g.L^{-1}$ in the PP2 and PP1 media respectively. Enzyme activities also showed significant increases with the hydantoinase activity reaching 12.1 and 20.6 $\mu mol.h^{-1}mL^{-1}$ in PP1 and PP2, respectively. The *N*-carbamoylase activity measured in RU-KM1 cells from PP2 medium showed the highest activity recorded during this research project, (8.1 $\mu mol.h^{-1}mL^{-1}$). Following the optimisation of the production medium, all subsequent fermntations were carried out with the optimised medium using 10 $g.L^{-1}$ YE and oleic acid. Hydantoin-hydrolysing activity was shown to be directly linked to the duration the culture. The optimal time for harvesting the culture was 12 to 14 h into the stationary phase. This represents a total 18 hour fermentation time, which is a huge reduction in fermentatiom time in HMM.

An investigation of immobilisation procedures and their evaluation as potential means for the further development of a bioprocess was carried out. Initial immobilisation studies were conducted using four flat sheet membranes, composed of polysulphone, polypropylene, nylon and polycarbonate. The use of a pressure cell to force the biocatalysts into the macrovoids proved successful, with biomass adsorbed to the membrane surfaces and detectable activity from the immobilised biomass. Immobilised whole cells showed similar activity to free whole cells assayed under the same conditions. Their biocatalytic activities showed similar activities over time. The crude extract showed increased activity in comparison to the whole cells. This was attributed to different mass transfer behaviourpotentials of the crude extract and whole cells.

The addition of the cross-linking agent glutaraldehyde resulted in significant decreases in activity of the immobilised and free biocatalysts. For whole cells and crude extracts, the reduction in activity was about 60 % and 90 % respectively. This loss of activity was

attributed to a possible inhibitory or toxicity effect of the glutaraldehyde. It was concluded that an immobilisation procedure requiring glutaraldehyde was not an option for bioprocess development.

A different configuration of the adsorption onto the membranes was considered a further possibility. As an alternative immobilisation matrix, a skinless, polysulphone, hollow-fibre, membrane bioreactor configuration was used. Using a pressurised system for forcing cells into the macrovoids of the membrane, biofilm formation was observed under scanning electron microscopy. The activity detected from samples of the hollow-fibre mini-reactors was comparable to that detected with the flat sheet membranes and free whole cells. This technology was further developed using a larger bioreactor with a greater membrane surface area, namely a transverse-flow hollow-fibre membrane bioreactor. While this bioreactor proved to be capable of hydantoin hydrolysis, biocatalytic activities that were obtained were very low in comparison to the mini-reactors. This membrane bioreactor has to our knowledge not been used for a bacterial based biotransformation, and the low activities could be overcome by increasing the biomass concentration within the bioreactor. This system has shown great potential in the production of amino acids.

The need for a simple bioreactor led to the investigation of alginate beads for entrapment of the biocatalyst. Alginate beads proved to be very stable over a range of temperatures and time periods. The beads showed no adverse effects at temperatures up to 70 °C. The effect of the immobilisation stabilised the enzymes and activity was detected after 4 weeks of storage at 4 °C. Comparative assays of the free extract showed no activity after 2 weeks. There was a significant increase in *N*-carbamoylase activity with the immobilisation. This could be linked to some form of steric stabilisation. The pH optima

of the immobilised enzyme were unchanged. Thus immobilisation of the biocatalyst would not result in different operating parameters of the biocatalyst in an immobilised form in a bioreactor from those already investigated in the whole cells and crude extract.

A fluidised bed bioreactor was assembled using RU-KM1 crude extracts encapsulated in alginate beads. This bioreactor was operated continuously for 15 days and a 95 % conversion of hydantoin to NCG and glycine was achieved after 10 days. A further modification led to the development of the combined bioreactor. This consisted of alternating columns of immobilised RU-KM1 and RU-OR crude extracts, based on a rationale that RU-KM1 hydantoinase would produce NCG and RU-OR *N*-carbamoylase would convert the NCG to glycine. This bioreactor was operated for 14 days and showed a 72 % conversion of hydantoin to NCG and glycine.

The outcomes of this research have included the preliminary isolation and partial characterisation of hydantoin-hydrolysing enzymes from two bacterial strains. These organisms and their enzymes were utilised to produce amino acids from different hydantoins, indicating some potential for the future development of a bioprocess. The development of the bench-top reactor for the biological conversion of hydantoins to amino acids has significant novelty and might ultimately lead to the replacement of the existing chemo-enzymatic process. This process is being considered for further scale-up for the production of amino acids.

Chapter 9

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Appendices

Appendix 1: List of materials

Standard Chemicals

Chemical	Source
Acetic acid (glacial)	SaarChem (South Africa)
Acrylamide	Sigma (South Africa)
bis-acrylamide	Sigma (South Africa)
boric acid	SaarChem (South Africa)
Chloroform	SaarChem (South Africa)
copper (II) acetate	SaarChem (South Africa)
4-dimethylaminobenzaldehyde	Merck (South Africa)
Ethylenediaminetetra-acetic acid (EDTA)	Merck (South Africa)
Hydantoin	Sigma, Aldrich (South Africa, Germany)
hydantoic acid (N-carbamoylglycine)	Sigma (South Africa)
Hydrindantin	Fluka (South Africa, Germany)
hydrochloric acid	NT, Merck (South Africa)
Isoamylalcohol	SaarChem (South Africa)
D-methylhydantoin	Toronto Research Chemicals (Canada)
L-methylhydantoin	Toronto Research Chemicals (Canada)
Methanol	SaarChem (South Africa)
2-methoxyethanol (methylcellusolve)	SaarChem (South Africa)
N,N-dimethyl-L-pheynylalanine	Sigma, Aldrich (South Africa, Germany)
Ninhydrin	Merck (South Africa)
sodium dodecyl sulphate	Merck (South Africa)
thiamine hydrochloride	SaarChem (South Africa)
Tris(hydroxymethyl)aminomethane	Merck (South Africa, Germany)
Tris (hydroxymethyl)aminomethane	Merck (South Africa, Germany)

Enzymes

Phospholipase A_2	Sigma-Aldrich
Chymotrypsin	Sigma-Aldrich
Lyticase	Sigma-Aldrich
Lysozyme	Sigma-Aldrich

Appendix 2: Media

A2.1 Minimal Medium (MM)

Basic minimal medium (MM), based on the M9 minimal medium recipe from Sambrook *et al.* (1989) was composed of the following:

per litre: 10 g glucose; 0.0011 g $CaCl_2$; 0.002 g $MgCl_2$; 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 0.00 4g boric acid, 0.004 g $MnSO_4$, 0.004 g $ZnSO_4$, 0.002 g $(NH_4)_6Mo_2O_{24}.4H_2O$, 0.001 g KI, 0.0004 g $CuSO_4$.

MM medium was supplemented with 20 g agar per litre for agar plates. Appropriate amounts of nitrogen sources and inducer were added as required. Several commonly used variations are listed below:

Table A2.1: Commonly used variations of the basic MM medium

Medium Name	**MM medium supplemented with**	
	Nitrogen Source	**Inducer**
HMM	1% hydantoin	-
CAAMM	0.01% casamino acids	-
CAAMM + 2-TU	0.01% casamino acids	0.1% 2-thiouracil
ASM	0.1% $(NH_4)_2SO_4$	-
NH_4MM + 2-TU	0.1% $(NH_4)_2SO_4$	0.1% 2-thiouracil

A typical recipe for making 1 litre of hydantoin minimal medium is described below.

Hydantoin Minimal Medium (HMM)

(for liquid culture or plates)

Initial charge:	Amount per L	Final Concentration
40% Glucose	25 ml	1 %
10 x M9 salts†	100 ml	1X
Trace elements *	10 ml	
1M $MgCl_2$	200 µl	100 µM
1M $CaCl_2$	200 µl	100 µM
4% hydantoin	250 ml	1 %
dH_2O		
agar (if plates)	20 g	2 %

†10 x M9 Salts:	per litre Stock solution	
Na_2HPO_4	60	g
KH_2PO_4	30	g
NaCl	5	g

* Trace elements:	per litre Stock solution	
Boric Acid	50	mg
$MnSO_4 \cdot 7H_2O$	40	mg
$ZnSO_4 \cdot 7H_2O$	40	mg
$(NH_4)MO_7O_{24} \cdot 4H_2O$	20	mg
KI	10	mg
$CuSO_4 \cdot 5H_2O$	4	mg
$FeCl_3$	10	mg

A2.2 Medium "A"

10g glucose, 40g hydantoin, 6g Na_2HPO_4, 3g KH_2PO_4, 0.5 g NaCl, 10 mL trace elements (Sambrook *et al*, 1987), 0.2mM $MgCl_2$, 0.2 mM $CaCl_2$ per litre

A2.3 PP1

Initial charge:	Amount per L	Unit
Citric acid	0.8	g
$(NH_4)_2HPO_4$	3.0	g
$MgSO_4 \cdot 7H_2O$	1.4	g
KH_2PO_4	4.0	g
Yeast extract	10.0	g
Hydantoin	1.0	g
Trace element solution*	10.0	ml
Antifoam	1.0	ml
Glucose	10	g

* Trace elements:	per litre Stock solution	
$FeSO_4 \cdot 7H_2O$	10	g
$CaCl_2 \cdot 2H_2O$	2.6	g
$ZnSO_4 \cdot 7H_2O$	2.2	g
$MnSO_4 \cdot 4H_2O$	0.5	g
$CuSO_4 \cdot 5H_2O$	1	g
$(NH_4)MO_7O_{24} \cdot 4H_2O$	0.1	g
$Na_2B_4O_7 \cdot 10H_2O$	0.02	g
HCl	160	ml
dH_20	840	ml

A2.4 PP2

Table 2: PP2 media.

Initial charge:	Amount per L	Unit
Citric acid	0.8	g
$MgSO_4.7H_2O$	1.4	g
KH_2PO_4	4.0	g
Hydantoin	1	g
Trace element solution*	10.0	mL
Sunflower Oil	10	g
Antifoam (Durapol)	1	mL
Yeast extract	10	g

* Trace elements:	per litre Stock solution	
$FeSO_4 \cdot 7H_2O$	10	G
$CaCl_2 \cdot 2H_2O$	2.6	G
$ZnSO_4 \cdot 7H_2O$	2.2	G
$MnSO_4 \cdot 4H_2O$	0.5	G
$CuSO_4 \cdot 5H_2O$	1	G
$(NH_4)Mo_7O_{24} \cdot 4H_2O$	0.1	G
$Na_2B_4O_7 \cdot 10H_2O$	0.02	G
HCl	160	Ml
dH_20	840	Ml

A2.5 HNB

Table 3: HNB media.

Initial charge:	Amount per L	Unit
Nutrient Broth	16	g
Hydantoin	1.0	g

Appendix 3: Standard procedure for growth, harvesting and resting cell biocatalytic reactions in HMM

Day 1: Starter Culture Inoculation
Inoculate a single colony from HMM (Appendix A2) agar plate into HMM broth (50 ml) and grow 3-4 days to stationary phase at 25 – 30°C.
Day 4/5: Growth in Required Culture Medium
Measure OD_{600} of starter culture – dilute to OD_{600nm} ~ 0.02 in required medium for RU-KM1 (see appendix 2). Grow to late-log (or other required) growth phase
Day 6/7: Harvesting and Resting Cell Biocatalytic Assays
Harvest cells by centrifugation in pre-weighed centrifuge tubes. (7000 rpm, 10 min). Empty off supernatant into biocide container, wash in ~ half volume of 0.1 M potassium phosphate buffer pH 8.0 (unless otherwise required) – vortex or shake vigorously to resuspend. Whilst centrifuging, organise small bottles with a cell blank; substrate blank and at least 3 replicates of reaction (cells + substrate). Centrifuge again (7000 rpm, 10 min). Weigh drained tubes on same balance as before. Calculate the wet cell mass (X mg = final mass- pre-weight of tube) and resuspend the pellet in the required volume (Y ml) of 0.1M potassium phosphate buffer pH 8.0 to give 100mg/2.5ml reaction (40mg/ml): *e.g.* (X mg /100)*2.5 = Y ml or X mg /40 = Y ml Add 2.5 ml of cells in buffer to each reaction bottle, and the cell blank bottle. Cap and incubate for 6 h @ 40°C, 200rpm.

Day 6/7: Colourimetric Assay of Reaction Supernatant
Collect supernatant from reaction bottles by microfuging an eppendorf-ful for 3 min at maximum speed. Use this for analysis to determine the amount of *N*-carbamoyl amino acid and amino acid in the sample. Analyse immediately – do not freeze overnight.

Appendix 4: Analytical methods

A4.1 Colourimetric assays for detection of *N*-carbamoyl amino acids and amino acids

The presence of *N*-carbamoylamino acids and amino acids in resting cell reaction supernatant was quantified colorimetrically with Ehrlich's reagent based on the method of Morin *et al*. (1986), using the procedure outlined below.

Ehrlich's Assay procedure

For each sample prepare a reaction as follows :

0.5ml 12% trichloro-acetic acid

1.0 ml reaction supernatant

0.5 ml Ehrlich's reagent (10% dimethylaminobenzaldehyde in 6N HCl)

3.0 ml dH_2O

Vortex, leave to react for 15minutes, and read versus a standard curve of 0-50mM NCG (see appendix 5) at $OD_{420\ nm}$. Use a spreadsheet with standard curve regression (Quattro Pro, Excel) to calculate the µmol/ml NCG produced for each sample, average and calculate SEM. Typical standards, absorbance values and a standard curve are illustrated in Tabl1e A41 and Figure A4.1. Fresh standard curves were prepared for each assay.

Table A4.1: **Standard Curve**

***N*-carbamoyl amino acid (0-50mM) – Final Volume 1 ml**			
Standard (mM)	**0.1M PO_4 Buffer (ml)**	**100mM NCG in 0.1M PO_4 Buffer (ml)**	**OD $_{420\ nm}$ after Ehrlich's reaction**
0	1.0	0	0
10	0.9	0.1	0.253
20	0.8	0.2	0.465
30	0.7	0.3	0.658
40	0.6	0.4	0.824
50	0.5	0.5	1.125

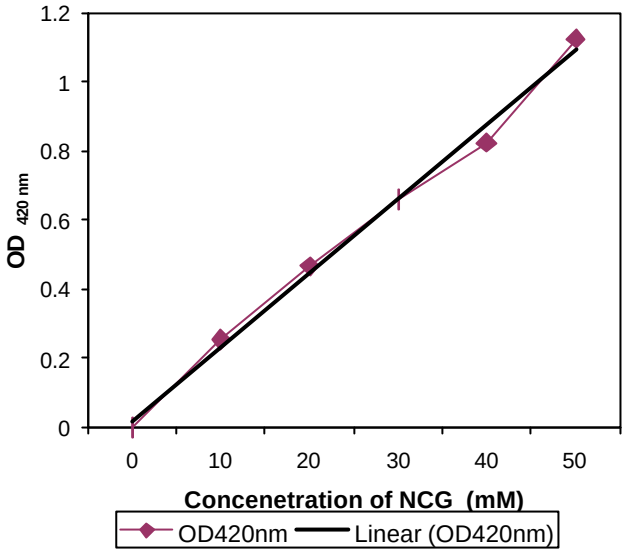


Figure A4.1: Standard curve for reaction of 0-50 mM NCG with Ehrlich's reagent**.**

Ninhydrin Assay Procedure

A standard curve was prepared from 0 to 0.5mM glycine using a 1mM stock.

For each sample test-tube reaction was prepared as follows:

0.998 ml 0.1M potassium phosphate buffer

0.020 ml reaction supernatant

1ml ninhydrin reagent (0.8 g ninhyrin; 0.12g hydrindantin dissolved in 30 ml methylcellusolve, add 10 ml 4M sodium acetate buffer (Plummer, 1987)

Test tubes were then capped, and boiled for 15 min. After cooling, 3.0 ml 50% ethanol was added and the $OD_{595\ nm}$ read versus the standard curve. Spreadsheets with standard curve regression (Quattro Pro, Excel) were used to to calculate the µmol/ml amino acid produced for each sample, average and calculate SEM values. Typical standards, absorbance values and a standard curve are illustrated in Table1 A4.2 and Figure A4.2. Fresh standard curves were prepared for each assay.

Table A4.2: **Standard Curve**

***N*-carbamoyl amino acid (0-50mM) – Final Volume 1 ml**			
Standard (mM)	**0.1M PO_4 Buffer (ml)**	**1mM glycine in 0.1M PO_4 Buffer (ml)**	**OD $_{420\ nm}$ After Ninhydrin reaction**
0	1.0	0	0
0.1	0.9	0.1	0.198
0.2	0.8	0.2	0.412
0.3	0.7	0.3	0.673
0.4	0.6	0.4	0.925
0.5	0.5	0.5	1.143

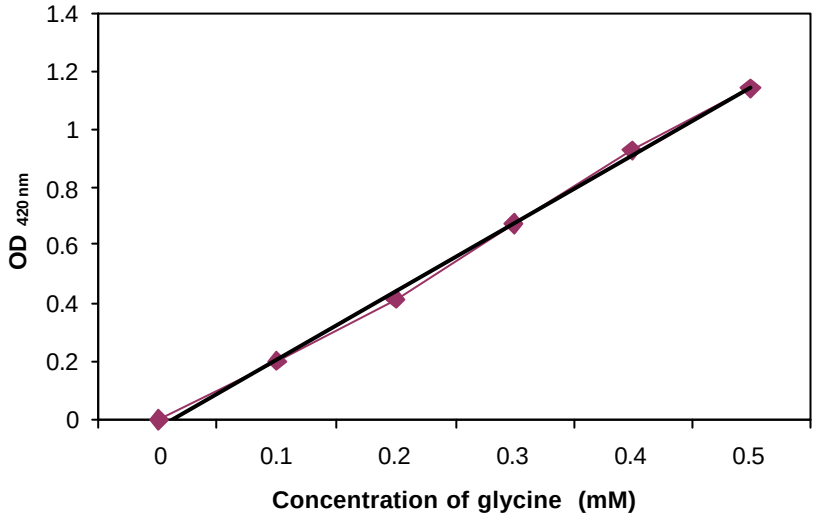


Figure A4.2: Standard curve for reaction of 0-0.5 mM glycine with ninhydrin reagent.

These methods were adapted for the analysis of *N*-carbamoyl-amino acids and amino acids produced from small-scale microtitre resting cell reactions.

A4.2 HPLC detection of *N*-carbamoyl-hydroxyphenylglycine and *p*-hydroxyphenylglycine

N-Carbamoyl-*p*-hydroxyphenylglycine (NCHPG) and *p*-hydroxyphenylglycine (HPG) were analysed by HPLC using a Macherey-Nagel Nucleosil 100-5 C18 column, 0.01% phosphoric acid mobile phase, and a flow rate of 1.1 ml/min. Retention times: D,L-p-hydroxyphenylhydantoin, 4.6 min, *N*-carbamoyl-D,L-*p*-hydroxyphenylglycine, 12.6 min, D,L-p-hydroxyphenylglycine, 17.8 min). The amount of NCHPG and HPG produced was calculated by comparison with a standard curve derived from peak areas produced by known concentrations of the compounds.

A4.3 Chiral Tlc

Chiral TLC with Macherey-Nagel Chiralplate was used to determine the chirality of alanine and p-hydroxyphenylglycine in a mobile phase of acetone/methanol/water (10:5:2), and visualized using ninhydrin reagent as described in Hartley (1995).

A4.4 Chiral HPLC

Chiral HPLC analysis on reaction supernatants was performed using Beckman System Gold with an ODS C18 coulumn. An eluent of 2mM N, N-dimethyl-L-phenylalanine, 1mM copper acetate, 5% (v/v) methanol was used at a flow rate of 1mLmin^{-1}. Retention times: D-HPG, 5.45 minutes; L-HPG, 7.05minutes. Enantiomeric excess of each enantiomer was calculated as a percentage of the total peak area of both two enantiomers.

A4.5 Calibration of dry cell mass and optical density for growth of RU-KM1 in MM medium

In order to calibrate the equivalency between cell growth, increase in optical density and dry cell mass, a calibration curve was produced to demonstrate the relationship between OD_{600nm} and dry cell mass (Figure A4.3).

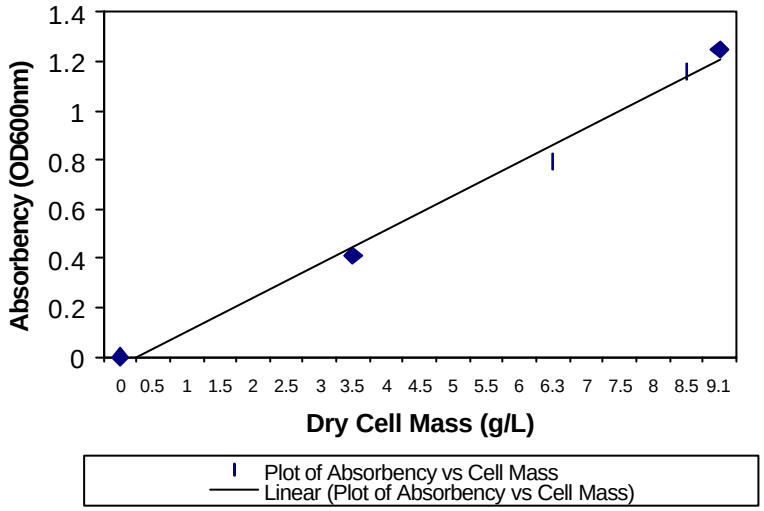
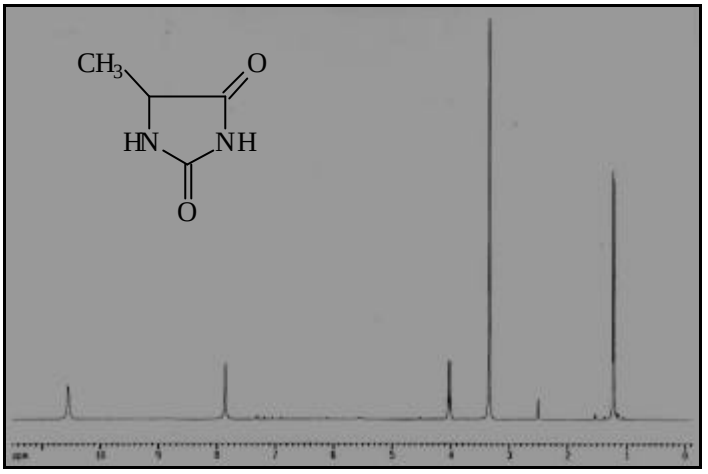


Figure A4.3: Calibration of absorbency with dry cell mass to illustrate OD600nm equates to cell growth. Growth rate average $\mu=0.101\pm0.02$ g/h.

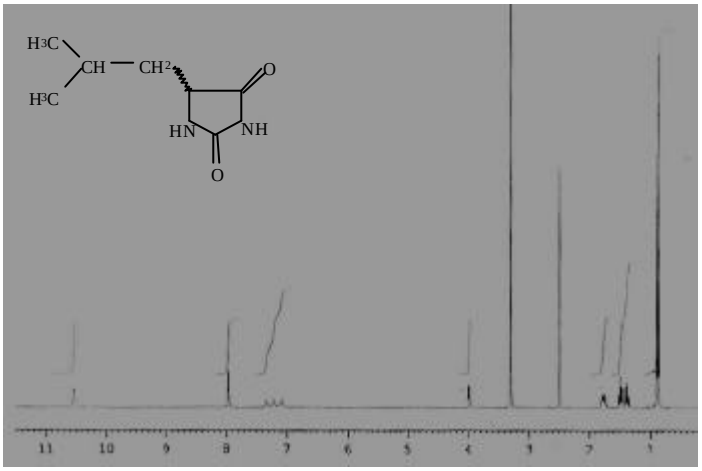
Appendix 5: ^{1H}NMR analysis of synthesised 5-substituted hydantoins



5-methylhydantoin:

(δ 10.5 (O=C-NH-C=O); δ 7.8 (O=C-NH-CH); δ 4.0 (q, H_3C-CH); δ 1.2 (d, H_3C-CH))

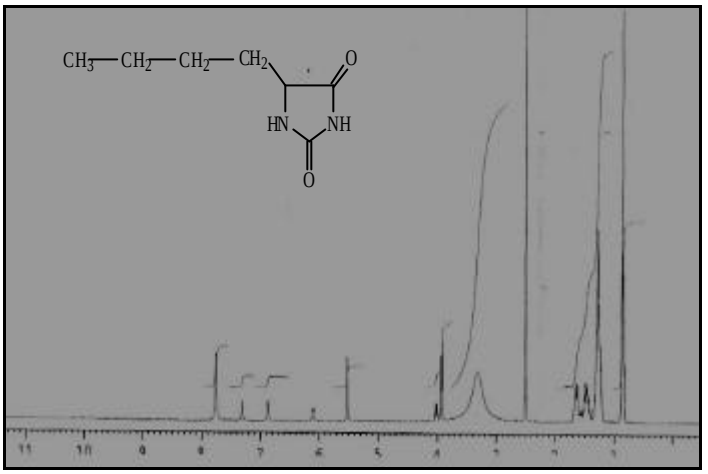
Figure A5.1 ^{1H}NMR spectrum of 5-methylhydantoin



5-isopropylhydantoin:

(δ 10.5 (O=C-NH-C=O); δ 7.9 (O=C-NH-CH); δ 3.9 (NH-CH-C=O); δ 1.O, (m, H_3C-CH-CH_3); δ 0.8 (2xd, H_3C-CH-CH_3))

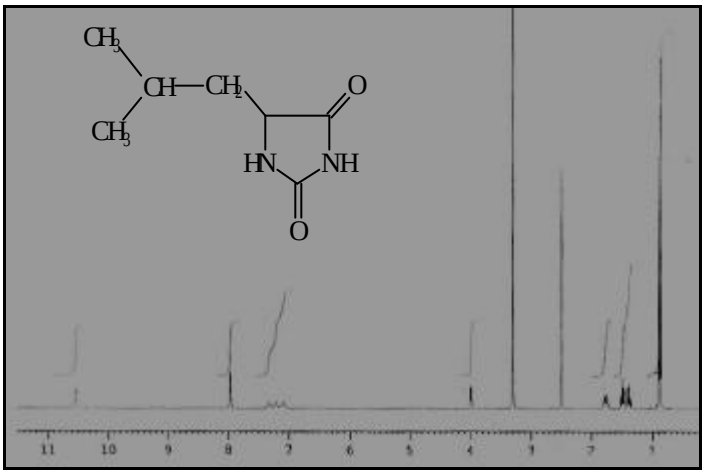
Figure A5.2 ^{1H}NMR spectrum of 5-isopropylhydantoin



5-n-butylhydantoin:

(δ 7.7 (O=C-N<u>H</u>-C=O); δ 5.5 (O=C-N<u>H</u>-CH); δ 3.9 (m, H_2C-C<u>H</u>-C=O); δ 1.4 (m, H_2 C-C<u>H_2</u> -CH); δ 1.2 (q, H_3C-C<u>H_2</u> -C<u>H_2</u>), δ0.8 (t, <u>H_3</u>C-CH_2))

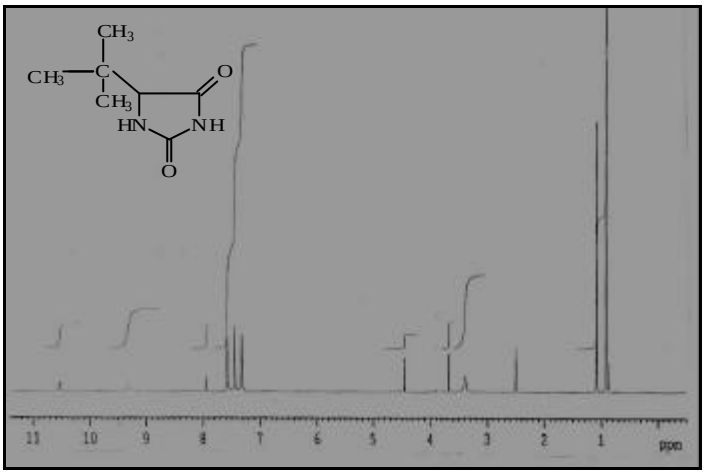
Figure A5.3 ^{1H}NMR spectrum of 5-n-butylhydantoin



5-isobutylhydantoin:

(δ 10.5 (O=C-NH-C=O); δ 8.0 (O=C-NH-CH); δ 4.0 (q, NH-CH-CH_2); δ 1.7 (m, (H_3 C)$_2$-CH-CH_2); δ 1.3 (m, CH-CH_2-CH); δ0.8 (m, H_3C-CH-CH_3))

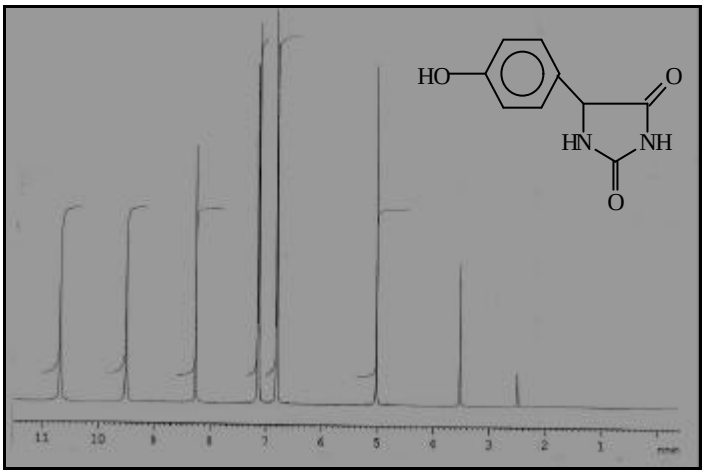
Figure A5.4 ^{1H}NMR spectrum of 5-isobutylhydantoin



5-tert-butylhydantoin:

(δ 10.5 (O=C-N<u>H</u>-C=O); δ7.9 (O=C-N<u>H</u>-CH); δ 3.6 (NH-C<u>H</u>-C(CH_3)$_3$); δ 1.1 ($\underline{H}_3$C-C(CH_3)$_2$); δ 0.9 (H_3C-(C$\underline{H}_3$)$_2$-CH))

Figure A5.5 ^{1H}NMR spectrum of 5-tert-*b*utylhydantoin



5-*p*-hydroxyphenylhydantoin:

(δ 10.6 (O=C-N<u>H</u>-C=O); δ 9.4 (O<u>H</u>-Phe); δ 8.2 (O=C-N<u>H</u>-CH); δ7.1 (d, OH-C(C$\underline{H}_2$)$_2$); δ 6.7 (d, (C$\underline{H}_2$)$_2$ -CH); δ 5.0 (NH-C<u>H</u>-Phe))

Figure A5.6 ^{1H}NMR spectrum of 5-p-hydroxyphenylhydantoin