DEVELOPMENT OF A HYDANTOIN-HYDROLYSING BIOCATALYST FOR THE PRODUCTION OF OPTICALLY PURE AMINO ACIDS USING *AGROBACTERIUM TUMEFACIENS* STRAIN RU-ORPN1

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ABSTRACT

A calcium alginate bead-immobilised biocatalyst was developed utilising the D-hydantoinase and D-*N*-carbamoylase from a novel, mutant *Agrobacterium tumefaciens* strain RU-ORPN1. The growth conditions for the inducer-independent strain were optimised for production of hydantoinase and *N*-carbamoylase activities. Methods for the preparation of crude enzyme extracts were evaluated in terms of hydantoinase and *N*-carbamoylase activities produced. After comparison of the enzyme activities and stabilities in various extracts from fresh and frozen cells, sonication of frozen cells for 5 minutes was found to be the best method for the production of the enzyme extract. The optimal pH and temperature for the hydantoinase activity were pH 10 and 30^0C, respectively, while pH 9 and 40^0C were optimal for *N*-carbamoylase activity. The hydantoinase activity was enhanced by the addition of Mg^{2+} ions to the enzyme extract and the *N*-carbamoylase was enhanced by the addition of Mg^{2+}, Mn^{2+} or Zn^{2+} ions to the enzyme extract. The enzyme activities increased in the presence of ATP suggesting that the enzymes may be ATP-dependent. The addition of DTT and PMSF to the enzyme extract enhanced the hydantoinase activity but had no effect on the *N*-carbamoylase activity. The *N*-carbamoylase was unstable at 40^0C and was almost completely inactivated after 24 hours incubation at this temperature.

The hydantoinase and *N*-carbamoylase appeared to be insoluble. Various techniques were investigated for the solubilisation of the enzymes including various cell lysis methods, cell lysis at extremes of pH and ionic strength, addition of a reducing agent and protease inhibitors, and treatment with hydrolysing enzymes and detergents. Treatment with Triton X-100 was most effective for the solubilisation of the enzymes indicating that the enzymes were membrane-bound. Hydropathy and transmembrane prediction plots of the predicted amino acid sequences for two identified *N*-carbamoylase genes from *A. tumefaciens* RU-ORPN1 revealed possible transmembrane regions in the amino acid sequences, and thus supported the hypothesis that the enzymes were membrane-bound.

Various methods were evaluated for the immobilisation of the enzymes in whole cells and enzyme extracts. Immobilisation of the enzyme extract in calcium alginate beads was found to be the best method in terms of enzyme activity retention and stability. The hydantoinase retained 55% activity while the *N*-carbamoylase exhibited a remarkable sevenfold increase in activity after immobilisation by this method.

Furthermore, the hydantoinase activity increased after storage at 4^{0}C for 21 days, while the *N*-carbamoylase retained 30% activity after this storage period. The calcium alginate bead-immobilised enzymes were further biochemically characterised and then applied in a bioreactor system for the production of D-hydroxyphenylglycine (D-HPG) from D,L-5-hydroxyphenylhydantoin (D,L-5-HPH). The pH and temperature optima for the immobilised hydantoinase were pH 7 and 50^{0}C, respectively, while pH 8 and 40^{0}C were optimal for the immobilised *N*-carbamoylase enzyme. The immobilised enzymes showed improved thermostability at 40^{0}C in comparison to the free enzymes and retained high levels of activity after five repeated batch reactions.

Low levels of conversion were obtained in a packed-bed bioreactor containing the *A. tumefaciens* RU-ORPN1 biocatalyst due to the low hydantoinase activity present in the strain, relative to *N*-carbamoylase. A novel, packed-bed bioreactor system was therefore developed for the production of D-HPG from D,L-5-HPH using the *A. tumefaciens* biocatalyst in combination with a *Pseudomonas* sp. biocatalyst having high hydantoinase activity. A conversion yield of 22 to 30% was achieved for the production of D-HPG from D,L-5-HPH over 5 days operation demonstrating that the hydantoin-hydrolysing enzymes from *A. tumefaciens* RU-ORPN1 could be stabilised by immobilisation and, in combination with a biocatalyst with high hydantoinase activity, could be applied to the fully enzymatic conversion of D,L-5-HPH to D-HPG.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
CSIR	Council for Scientific and Industrial Research
DCW	dry cell weight
D-HPG	D-hydroxyphenylglycine
D,L-5-HPH	D,L-5-monosubstituted-hydroxyphenylhydantoin
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride

EDTA	Ethylenediaminetetraacetic acid
HPLC	high performance liquid chromatography
NCG	*N*-carbamylglycine
NC-HPG	*N*-carbamyl-hydroxphenylglycine
OD	optical density
PMSF	phenylmethanesulphonylfluoride
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

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LIST OF PRESENTATIONS

The following is a list of publications, reports and conference proceedings resulting from the research presented in this thesis.

PUBLICATIONS

Foster IM, Dorrington RA and Burton SG (2003) Enhanced hydantoinase and *N*-carbamoylase activity on immobilisation of *Agrobacterium tumefaciens*. Biotechnology Letters **25**:67-72

REPORTS

Biotechnology for the South African Chemical Industry: A New Biocatalysis Process. Interim Progress Reports 1-11.

INTERNATIONAL CONFERENCE PROCEEDINGS

Foster IM, Kirchmann S and Burton SG (2001) Development and optimisation of a bioreactor system for the production of optically pure amino acids using hydantoin-hydrolysing bacterial strains. ASM Conference on Biodegradation, Biotransformation and Biocatalysis, San Juan, Puerto Rico.

LOCAL CONFERENCE PROCEEDINGS

Foster IM and Burton SG (1999) Biochemical characterisation of hydantoin-hydrolysing enzyme systems in *Agrobacterium tumefaciens* RU-ORPN1 for utilisation in production of enantiomerically pure amino acids and related compounds. Biotech SA. BioY2K. Grahamstown

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CHAPTER 1

INTRODUCTION

The studies described in this thesis form part of a broader research project currently being undertaken by the Hydantoinase Research Group at Rhodes University. The group is involved in investigating a number of novel bacterial strains isolated from soil samples in South Africa having hydantoin-hydrolysing enzyme activity (Burton *et al.*, 1998; Buchanan *et al.*, 2001). The ultimate objective of the project is developing and applying biocatalysts in an industrial process for the production of optically pure amino acids. One of the isolated strains, *Agrobacterium tumefaciens* strain RU-OR, was found to produce high levels of hydantoin-hydrolysing activity when grown in the presence of hydantoin and the hydantoin-analogue inducer, 2-thiouracil (Hartley *et al.*, 1998). The strain was later improved by the production of a regulatory mutant, *Agrobacterium tumefaciens* RU-ORPN1, which was able to produce high levels of hydantoin-hydrolysing activity in the absence of 2-thiouracil (Hartley *et al.*, 2001), increasing the potential of the strain as a biocatalyst for the industrial production of amino acids. This study was conducted with the aim of developing a process for amino acid production, utilising the enzymes from this strain, through further biochemical characterisation of the hydantoin-hydrolysing enzymes. A further aim was to investigate various strategies for the production of a stabilised biocatalyst which would be suitable for application in such a process.

1.1 Applications of amino acids

Optically pure amino acids have a wide variety of commercial applications including the pharmaceutical, agro-chemical and food/feed additive industries. D-phenylglycine and D-*p*-hydroxyphenylglycine(D-HPG), for example, are used as intermediates in the synthesis of the broad spectrum $-lactam antibiotics ampicillin and amoxycillin, respectively (Tripathi *et al.*, 2000; Shimizu *et al.*, 1997). D-valine is used as an intermediate in the production of the pyrethroid insecticide, Fluvalinate (Drauz *et al.*, 1991) and L-valine is used as a feedstock (Kamphuis *et al.*, 1990). The increasing demand for non-nutritive sweeteners has resulted in the development of various processes for the production of the constituent amino acids, including L-phenylalanine, the major constituent of aspartame, and D-alanine (Buson *et al.*, 1996). Medicinal chemists have also become increasingly interested in biologically active peptide-analogs containing " -alkyl amino acids (Smith *et al.*, 2001).

1.2 Industrial production of amino acids

Amino acids can be produced by chemical, chemo-enzymatic or enzymatic processes. The bulk amino acids such as L-glutamate, L-lysine and D,L-methionine are produced by biological and chemical syntheses while a number of enzymatic processes have been developed for the production of optically pure speciality amino acids (Syldatk *et al.*, 1990a). Enzymatic processes include the use of acylases, esterases, aminopeptidases or amidases for racemate separation, while hydantoinases and *N*-carbamoylases are used for amino acid production by hydantoin hydrolysis (Syldatk *et al.*, 1990a; Drauz, 1997). The precursors for D,L-" -amino acids are D,L-5-substituted hydantoin derivatives. Hydantoin was first produced by Baeyer in 1861 by the hydrogenation of allantoin, a naturally occurring cyclic amide found in many plants (Syldatk and Pietzsch, 1995). Although a number of processes have been developed for the production of 5-substituted hydantoins (Dressman *et al.*, 1996; Syldatk *et al.*, 1990a), those used in conventional amino acid production are synthesised by the Bucherer-Burg reaction from carbonyl compounds (Yamada *et al.*, 1980). Alkaline chemical hydrolysis of hydantoins produces racemic mixtures of " -amino acids. However, the use of microbes or enzymes which stereospecifically hydrolyse hydantoin substrates allows the production of optically pure products.

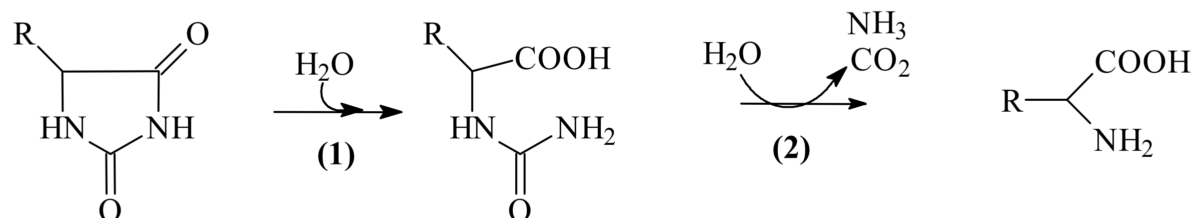
Biocatalytic conversions offer a number of major advantages including stereoselectivity, specificity and mild reaction conditions which can potentially result in cheaper industrial processes (Polastro, 1989). Increasing demands for environmentally benign processes add to the advantages of biocatalytic conversions which are environmentally safe since they make use of low salt, low metal and limited solvent conditions and have decreased by-product formation (Azerad, 2001). Microbial production of amino acids has therefore become increasingly important in industrial biotechnology (Hartley *et al.*, 1998) and several biotechnological processes have been patented (Oliveiri *et al.* 1981; Tramper, 1985, Grifantini *et al.*, 1998a; Neal *et al.*, 1999).

Chemo-enzymatic processes for the production of amino acids involve the hydrolysis of D,L-5-monosubstituted hydantoins by a hydantoinase enzyme to produce an *N*-carbamyl amino acid. The *N*-carbamyl amino acid is then further hydrolysed to the corresponding amino acid by chemical methods such as treatment with nitrous acid (Yamada, 1981, Takahashi *et al.*, 1979, Gokhale *et al.*, 1996). Such

processes are operated on an industrial scale for the production of D-HPG by the Japanese company, Kanegafuchi (Kamphuis *et al.*, 1990) and the Italian company, Snamprogetti (Syldatk and Pietzsch, 1995). Chemical hydrolysis of *N*-carbamyl derivatives requires high reaction temperatures, has characteristically low yields and long reaction times, and requires resolution of enantiomers (Olivieri *et al.*, 1979). Furthermore, the chemical hydrolysis adds a step to the process and has the potential to generate large quantities of unwanted waste (Kim and Kim, 1994; Oh *et al.*, 2002).

The enzymatic production of amino acids from hydantoins involves a two step reaction shown in Figure 1.1, known as the "hydantoinase process". D,L-5-Substituted hydantoins are hydrolysed to their corresponding *N*-carbamylamino acids catalysed by a hydantoinase enzyme which may be D-, L- or non-stereospecific. The *N*-carbamylamino acid is then hydrolysed to its respective amino acid, ammonia and carbon dioxide catalysed by a D- or L-*N*-carbamylamino acid amidohydrolase *(N*-carbamoylase) enzyme. Under alkaline conditions, 5-monosubstituted hydantoins undergo spontaneous racemisation, resulting in a theoretical 100% yield from a racemic mixture of hydantoin substrate (Morin *et al.*, 1987; Keil *et al.*, 1995). A third enzyme known as a hydantoin racemase enzyme is also present in some biological systems, resulting in the enzymatic racemisation of the D- or L-hydantoin substrates (Watabe *et al.*, 1992; Siemann *et al.*, 1993; Wiese *et al.*, 2000). The potential 100% conversion of a hydantoin derivative into the corresponding optically pure amino acid which can be achieved in this way makes this hydantoin-hydrolysing enzyme system highly favourable for use in an industrial process (Altenbuchner *et al.*, 2001). Recordati and Degussa produce D-HPG in a one-step industrial conversion process using an *Agrobacterium tumefaciens* (formerly *radiobacter*) strain having both D-hydantoinase and D-*N*-carbamoylase enzymes (Syldatk and Pietzsch, 1995). Ajinomoto Co. produce D-HPG using a *Pseudomonas* sp. strain having both enzyme activities (Syldatk *et al.*, 1990a).

Figure 1.1 Enzymatic conversion of a 5-monosubstituted hydantoin substrate to its corresponding amino



acid catalysed by hydantoinase (1) and *N*-carbamylamino acid amidohydrolase (2).

Although a number of biotechnological processes have been patented, the efficiency of these processes could be improved by further optimisation. One of the greatest drawbacks of the one-step conversion process is the instability of the *N*-carbamoylase enzyme under process conditions. Microbial hydantoinases exhibit a good overall stability in the bioconversion process while *N*-carbamoylases are more easily deactivated, and thus the re-utilisation of the biocatalyst in multiple successive batches has been hampered (Meyer and Runser, 1993). The efficiency of biocatalysts could be improved by the introduction of resistance to product inhibition, high substrate and salt concentrations, protease inactivation, organic solvents, low or high pH values and higher temperatures, thereby improving the robustness of the bacterial strain used in the process (Kamphuis *et al.*, 1990; Grifantini *et al.*, 1998b). The requirement for inducers to stimulate the production of the enzymes has also elevated the costs of biotechnological processes and variable enzyme activities from batch to batch of biomass have been encountered. As a result, there is continued interest in newly isolated or improved enzymes which could be applied to the industrial production of amino acids, improving the efficiency of the process (Arcuri *et al.*, 2000; Altenbuchner *et al.*, 2001).

Over the last thirty years extensive research has been done on a broad range of bacterial species having hydantoin-hydrolysing activity in an effort to either improve the currently used industrial biocatalysts, or to find more robust strains and enzymes with greater thermal and chemical stability. Many of the hydantoinase and *N*-carbamoylase enzymes have been purified and biochemically characterised and the genes for the enzymes have been isolated, cloned and sequenced. Amino-acid over-producing bacterial strains have been produced by classical mutagenesis and selection methods and more recently, as the

understanding of the molecular genetics of amino acid production in bacteria has increased, recombinant strains with more precisely altered amino acid biosynthetic pathways have been produced with enhanced amino acid production efficiency (Fotheringham, 2000). These techniques have allowed the construction of novel biochemical pathways using genes derived from heterologous sources in single recombinant strains. This has, in turn, paved the way for the construction of biochemical pathways for the synthesis of unnatural amino acids for broader agricultural or pharmaceutical applications (Bommarius *et al.*, 1998; Schulze and Wubbolts, 1999; Fotheringham, 2000). Improved recombinant enzymes, fusion proteins and specially designed recombinant whole cell biocatalysts and the optimization of enzyme properties by directed evolution are the subjects of many of the recent publications on hydantoinase and *N*-carbamoylase enzymes (May *et al.*, 2000; Altenbuchner *et al.*, 2001).

1.3 Hydantoinase enzymes

1.3.1 Distribution and classification

Hydantoin-hydrolysing activities have been reported in the literature since the 1930's (Syldatk *et al.*, 1999). Hydantoinases have been isolated from plants (Eadie *et al.*, 1949; Morin, 1993, Morin *et al.*, 1995a; Morin *et al.*, 1997, Fan and Lee, 2001) and animals (Berheim and Bernheim, 1946) and are also widely distributed in bacteria and fungi (Meyer and Runser, 1993). These enzymes represent a large group of cyclic amidases (E.C. 3.5.2.), all catalyzing the reversible hydrolytic ring cleavage of hydantoin or 5'-monosubstituted hydantoin derivatives (Kim and Kim, 1998; May *et al.*, 1998c, Syldatk *et al.*, 1999). Hydantoinases have been classified as L- (Yokozeki *et al.*, 1987a; Nishida *et al.*, 1987, Siemann *et al.*, 1994), non- (Buchanan *et al.*, 2001) or D-enantiomer specific (Louwrier and Knowles, 1996). For a long time, D-selective hydantoinase was considered a dihydropyrimidinase (E.C. 3.5.2.2) as it catalyses the hydrolysis of 5,6-dihydropyrimidine, 5,6-dihydrouracil and 5,6-dihydrothymine (Drauz and Waldman, 1995) in contrast to the L-selective hydantoinase or dihydro-orotase (E.C. 3.5.2.3). In 1993, however, Runser and Meyer reported the isolation of a hydantoinase enzyme from *Agrobacterium* sp. with no 5,6-dihydropyrimidine amidohydrolase activity. Other, more recent, studies also confirm that the D-hydantoinases are a diverse group of enzymes with a variety of metabolic functions (Ogawa and Shimizu, 1997; May *et al.*, 1998e; Soong *et al.*, 1999; Gojkovic *et al.*, 2000, Kim *et al.*, 2000a). Natural functions for the L- and non-specific hydantoinases remain unclear (May *et al.*, 1996).

1.3.2 Purification and biochemical characterisation of bacterial hydantoinases

Hydantoinase enzymes from a wide variety of bacterial species have been described in the literature. These species include *Arthrobacter* sp. (Syldatk *et al*., 1987; Möller *et al.*, 1988; Siemann *et al*., 1994; Zhang *et al.*, 2001), *Bacillus* sp. (Lee *et al*., 1994; Mukohara *et al*., 1994; Sharma and Vohra, 1997; Kim *et al.*, 1997; Luksa *et al*., 1997), *Agrobacterium* sp. (Runser and Meyer, 1993; Durham and Weber, 1995; Weber and Durham, 1997; Achary *et al*., 1997; Burton *et al*., 1998), *Pseudomonas* sp. (Takahashi *et al*., 1978; Shimizu *et al*., 1980; Kim *et al*., 1994; LaPointe *et al*., 1994; Ogawa *et al.*, 1994a; Xu and West, 1994; Morin *et al*., 1995b; Gokhale *et al*., 1996; Chien *et al*., 1998) and *Blastobacter* sp. (Soong *et al*., 1999). The biochemical characteristics of some of these microbial hydantoinases are summarised in Table1.1. It should be noted that the temperature optima given relate to specifically defined reaction conditions in each case which are not necessarily identical.

Table1.1 Biochemical properties of some microbial hydantoinases.

Species	Specificity	pH optima	pH stability	Temperature optima	Thermostability	Metal dependence/ activation	Inhibition	Molecular weight	Subunits

Pseudomonas striata (Takahashi *et al.*, 1978)	D-specific	8-9	6-7	45-55	<60		metal chelators p-chloromercuribenzoate	190kDa	
Pseudomonas fluorescens DSM 84 (Morin *et al.*, 1986)	D-specific	9	5.5-8.5	55	<40	activated by Fe^{2+}, Mn^{2+}	Cu^{2+} p-hydroxymercuribenzoate 8-hydroxyquinoline	230kDa	4×60kDa
Pseudomonas stutzeri (Xu and West, 1994)	not specified	7.5-9		45	40-45	activated by Mg^{2+}	Zn^{2+}, Cu^{2+}	115kDa	
Pseudomonas putida (Ogawa *et al.*, 1995a)	L-specific	6-10	5.5-9.5	20-50	<60	dependent on Mg^{2+}, Mn^{2+}, Co^{2+}		300kDa	2×70kDa 2×80kDa
Agrobacterium sp. IP I-671 (Runser and Ohleyer, 1990)	D-specific	10	7.5-10.5	60	<65	activated by Ni^{2+}, Mg^{2+}		250kDa	4×62kDa
Bacillus stearothermophilus SD-1 (Lee *et al.*, 1995, 1997a)	D-specific	8		65	<60	dependent on Mn^{2+}		126kDa	2×54kDa
Bacillus sp. AR9 (Sharma and Vohra, 1997)	D-specific	9.5	8-10	65	55-70	activated by Co^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+}			

Species	Specificity	pH optima	pH stability	Temperature optima	Thermostability	Metal dependence/ activation	Inhibition	Molecular weight	Subunits
Bacillus circulans (Luksa *et al.*, 1997)	D-specific	8-10	8.5-9.5	75	<60	dependent on Mn^{2+}, Ni^{2+}, Co^{2+}	thiol reagents	212kDa	4×53kDa
Bacillus thermocatenulatus GH-2 (Park *et al.*, 1998)	D-specific	7.5	6-9.5	65	<75	activated by Mn^{2+}		230kDa	4×56kDa
Arthrobacter crystallopoietes (Siemann *et al.*, 1999)	D-specific	8		50	<50	metals had no effect	8-hydroxyquinoline-5-sulfonic acid	257kDa	4×60kDa
Blastobacter sp., strain A17p-4 (Soong *et al.*, 2001)	D-specific	9-10	5-8.5	60	<60	activated by Ni^{2+}, Co^{2+}, Mn^{2+}	SH-inhibitors, Hg^{2+}	200kDa	4×53kDa

Many bacterial hydantoinases have been purified to homogeneity using methods including ammonium sulphate precipitation, gel filtration and various combinations of ion exchange and hydrophobic interaction chromatography (Runser and Meyer, 1993; Lee *et al.*, 1995; Luksa *et al.*, 1997; Park *et al.*, 1998; May *et al.*, 1998d; Siemann *et al.*, 1999; Abendroth *et al.*, 2000a; Chung *et al.*, 2002). The majority of these reported hydantoinases have been found to be D-specific, homotetramers ranging in size from 115kDa to 300kDa. The enzymes all exhibit optimal activities at alkaline pH and have temperature optima ranging from 20 to 75^{0}C. The catalytic activity of most hydantoinases has been found to be dependent on, or activated by, the presence of divalent metal ions, particularly Mg^{2+} and Mn^{2+} ions. Each mole of the active hydantoinase from *Arthrobacter aurescens* DSM 3745 was found to contain 10 moles of zinc ions by inductive/coupled plasma-atomic emission spectrometry (May *et al.*, 1998a). Furthermore, the group showed that zinc plays an essential role for the catalytic activity and for the stabilisation of the active quarternary structure of the hydantoinase enzyme (May *et al.*, 1998b). This enzyme was also shown to be dependant on cobalt, manganese and zinc ions in metal/chelator-caused inactivation and reactivation studies. Similarly, the hydantoinase from *Arthrobacter crystallopoietes* was shown to require zinc as a cofactor by the same method (Siemann *et al.*, 1999). The crystallisation and three-dimensional structures of the L-hydantoinase from *Arthrobacter aurescens* DSM 3745 (May *et al.*, 1996; Abendroth *et al.*, 2002a) and the D-hydantoinases from *Thermus* sp. (Abendroth *et al.*, 2000b; Abendroth *et al.*, 2002b) and from *Bacillus stearothermophilus* (Cheon *et al.*, 2002) have been published recently.

1.4 *N*-carbamylamino acid amidohydrolase (*N*-carbamoylase)

1.4.1 Classification

The natural role of *N*-D-carbamoylase was thought to be a $-ureidopropionase (E.C. 3.5.1.6) which catalyses the decarbamoylation of $-ureido propionic acid in pyrimidine catabolism (Drauz and Waldman, 1995). However, Ogawa and Shimizu (1997) classify $-ureidopropionase and *N*-D-carbamoylase as separate enzymes since the D-*N*-carbamoylases from *Comomonas* sp. E222c and *Blastobacter* sp. A17

p-4 do not hydrolyse $-ureidopropionate. Furthermore, these authors purified a microbial $-ureidopropionase that showed *N*-carbamyl-L-amino acid hydrolysing activity (Ogawa *et al.*, 1995b). Ikenaka *et al.* (1998b), also described an *N*-D-carbamoylase without ureidopropionase activity. Although *N*-L-carbamoylases have been reported in microorganisms that metabolise D,L-5-monosubstituted hydantoins to L-amino acids (Syldatk *et al.*, 1990b; Gross *et al.*, 1990; Mukohara *et al.*, 1993; Wagner *et al.*, 1996; Buchanan *et al.*, 2001), few have been purified due to the instability of the enzyme (Ishikawa *et al.*, 1996).

1.4.2 Purification and biochemical characterisation of bacterial *N*-carbamoylases

A number of *N*-carbamoylases from different bacterial sources have been described in the literature. Bacterial sources of this enzyme include *Pseudomonas* sp. (Ishikawa *et al*., 1996; Ikenaka *et al.*, 1998b), *Agrobacterium* sp. (Runser *et al.*, 1990; Buson *et al.*, 1996; Louwrier and Knowles, 1997; Nanba *et* al., 1998a; Ikenaka *et al*., 1999, Sareen *et al.*, 2001), *Bacillus* sp. (Mukohara *et al.*, 1993), *Comomonas* sp. (Ogawa *et al*., 1993), *Alcaligenes* sp. (Ogawa *et al*., 1995b), *Blastobacter* sp. (Ogawa *et al*., 1994b) and *Arthrobacter* sp. (Moller *et al*., 1988). The biochemical characteristics of some of these microbial *N*-carbamoylases are summarised in Table1.2. As previously mentioned for Table 1.1, the temperature optima given relate to specifically defined conditions in each case which are not necessarily identical.

In contrast to hydantoinases, *N*-carbamoylases are generally unstable, even in bacterial resting cells, and therefore have not been widely employed in practical processes (Ogawa *et al.,* 1994b). D- and L-specific *N*-carbamoylases from a number of bacterial species have been purified to homogeneity using a variety of procedures involving heat shock, ammonium sulphate precipitation, ion exchange, gel filtration and affinity chromatography (Ogawa *et al.,*1993; Ogawa *et al*., 1994b; Ogawa *et al.*, 1995a; Ishikawa *et al.*, 1996). Most purification procedures were carried out in the presence of dithiothreitol since *N*-carbamoylases are highly susceptible to oxidation (Ogawa *et al.*, 1993; Grifantini *et al.*, 1996; Nanba *et al.*, 1998a; Sareen *et al.*, 2001).

Table 1.2 Biochemical properties of some microbial *N*-carbamoylases.

Species	Specificity	pH optima	pH stability	Temperature optima (^0C)	Thermostability (^0C)	Metal dependence/ requirements	Inhibition	Molecular weight	Subunits
*Agrobacterium radiobacter (*Oliveiri *et al*., 1979)	D-specific	7	7-9	60	<40	none	*p*-chloromercuribenzoate NH_4Cl		
Agrobacterium sp. IP-I 671 (Kim and Kim, 1995)	D-specific	7	7-7.5	50		none	ammonia, sulfhydral agents, Co^{2+}, Zn^{2+}, Ni^{2+},		
Agrobacterium sp. 80/44-2A (Louwrier and Knowles, 1996)	D-specific	7.3-7.4	5.8-9	70		none	iodoaceticacid, iodoacetamide	73.2kDa	2×38.1kDa
Agrobacterium sp. KNK712 (Nanba *et al*., 1998a)	D-specific	7	7	65	<55	not specified	thiol reagents, ammonia, Cu^{2+}, Ag^{2+}, Hg^{2+}		34kDa
Comomonas sp. E222c (Ogawa *et al*., 1993)	D-specific	8	7-9	40	<40	none	thiol reagents, Cu^{2+}, Zn^{2+}, Ag^{2+}, Hg^{2+}, Cd^{2+}	120kDa	3×40kDa
Blastobacter sp. A17p-4 (Ogawa *et al*., 1994b)	D-specific	8-9	6-9	55	<50	not specified	*p*-chloromercuribenzoate Cu^{2+}, Ag^{2+}, Hg^{2+}, Cd^{2+}	120kDa	3×40kDa
Alcaligenes xylosoxidans (Ogawa *et al*., 1995b)	L-specific	8-8.3	6-9.5	35	<35	required Mn^{2+}, Ni^{2+} or Co^{2+}	sulfhydral reagents	135kDa	2×65kDa
Pseudomonas sp. NS671 (Ishikawa *et al*., 1996)	L-specific	7.5		40	<25	required Mn^{2+}	*p*-chloromercuribenzoate ATP		2×45kDa

The majority of the purified *N*-carbamoylases were found to be dimers with subunit sizes ranging from 34 to 65kDa. The D-carbamoylases did not appear to require metal ions for activity while the two L-specific enzymes required Mn^{2+} for activity. Almost all the *N*-carbamoylases were inactivated by sulfhydryl reagents (Table

1.2). Progress has been made in elucidating the molecular structure of *N*-carbamoylase in order to further understand the catalytic mechanism of this enzyme in detail (Altenbuchner *et al.*, 2001). The first three-dimensional structure of a D-carbamoylase (from an *Agrobacterium* sp.) has been published (Nakai *et al.*, 2000).

1.5 Hydantoin racemase

The rate of chemical racemisation of 5-monosubstituted hydantoin substrates under alkaline conditions depends on the nature of the substituent in the 5-position. However, in the presence of a hydantoin racemase, fast and total conversion of hydantoins which racemize chemically very slowly can be achieved (Drauz and Waldman, 1995). These enzymes therefore have potential for the optimisation of industrial processes, and a number of racemases have been described in the literature. Hydantoin racemases have been purified and characterised from *Arthrobacter aurescens* strains DSM 3747 (Siemann *et al.*, 1994) and DSM 3745 (Drauz and Waldman, 1995), and *Pseudomonas* sp. strain NS671(Watabe *et al.*, 1992).

1.6 Strain and enzyme improvement through genetic engineering of hydantoinase and *N*-carbamoylase genes

DNA technology has allowed the production of many recombinant hydantoinase and *N*-carbamoylase enzymes. One of the advantages of heterologous expression of these genes is the increased enzyme yield which can be obtained compared with the wild-type cells and therefore lower cost of these catalysts (Lee *et al.*, 1997b; Altenbuchner, 2001; Hartley *et al.*, 2001, Kim and Kim, 2002). In addition, cloning and manipulation of the genes has resulted in easier purification of the enzymes (Nanba *et al.*, 1998a; Pietzsch *et al.*, 2000; Abendroth *et al.*, 2000a) and has allowed improved enzyme properties such as activity (Chao *et al.*, 2000a), thermostability (Ikenaka *et al.*, 1998a) and stereoselectivity (May *et al.*, 2000) by random mutagenesis and directed evolution.

1.6.1 Improved enzyme activities and yield by over-expression of hydantoinase and *N*-carbamoylase genes

The gene coding for the D-hydantoinase enzyme from *Pseudomonas putida* CCRC 12857 has been cloned into *Escherichia coli*. The activity of the hydantoinase enzyme in the transformed strain was found to be 20-fold higher than in the wild-type strain (Chien *et al.*, 1998). The gene coding for the thermostable D-hydantoinase from the thermophilic bacterium *Bacillus stearothermophilus* SD1 has been cloned and over-expressed in *E. coli*. The recombinant strain showed a 30-fold increase in enzyme production compared with the wild-type (Lee *et al.*, 1996a). The *N*-carbamoylase activity of recombinant *E. coli* expressing the *N*-carbamoylase gene from *Pseudomonas* sp. strain KNK003A was 40-fold that of native strain (Ikenaka *et al.*, 1998b). The homologous expression of the D-hydantoinase and D-*N*-carbamoylase genes from *Agrobacterium* sp. IP I-671 in *E.coli* resulted in increased hydantoinase and D-*N*-carbamoylase activity 2.5-fold and 10-fold, respectively. The genes encoding the L-hydantoinase, L-*N*-carbamoylase and the hydantoin racemase from *Arthrobacter aurescens* have been co-expressed in *E. coli* resulting in a 6-fold increase in the productivity for L-tryptophan from the corresponding hydantoin in comparison to the wild-type strain (Wilms *et al.*, 2001).

The genes encoding the hydantoinase and *N*-carbamoylase enzymes from the industrial *Agrobacterium tumefaciens* strain NRRL B 11291 (formerly *Agrobacterium radiobacter*) have been cloned and expressed in *E. coli* by a number of research groups (Buson *et al.*, 1996; Grifantini *et al.*, 1998b, Chao *et al.*, 2000a; Park *et al.*, 2000). Buson and co-workers achieved increased enzyme stability by expression of the *N*-carbamoylase gene in *E. coli*. Grifantini and co-workers cloned both the hydantoinase and *N*-carbamoylase genes in *E. coli* and the recombinant strain was found to produce D-amino acid twice as efficiently as the industrial *A. tumefaciens* strain (Grifantini *et al.*, 1996, 1998b). Chao and co-workers have achieved high expression of the *A. tumefaciens* NRRL B11291 hydantoinase and *N*-carbamoylase genes in *E. coli* resulting in a 97% conversion yield of D,L-5-hydroxyphenylhydantoin (D,L-5-HPH) to D-HPG, five times the productivity obtained with the industrial strain (Chao *et al.*, 1999a and b, Chao *et al.*, 2000a). Kim and co-workers used the *N*-carbamoylase gene from *Agrobacterium tumefaciens* NRRL B11291 and the D-hydantoinase gene from *Bacillus stearothermophilus* SD1 to construct a bifunctional *N*-carbamoylase/D-hydantoinase fusion enzyme. The group used directed evolution by DNA shuffling in order to increase the structural stability of the fusion enzyme which was subjected to extensive proteolysis *in vivo.* The evolved fusion enzyme had enhanced structural stability and showed a six-fold increase in the production of D-

amino acid (Kim *et al.*, 2000b). The conversion of hydantoin derivatives by the fusion enzyme was much higher than the separately expressed enzymes and was comparable to that by the coexpressed enzymes (Kim *et al.*, 2000c).

1.6.2 Improved thermostability of hydantoinase and *N*-carbamoylase enzymes

Thermostable enzymes are of interest in basic studies concerning protein stability as well as in the development of industrial processes. Limited substrate solubility and enzyme stability have posed problems in the industrial production of optically active D-amino acids using microbial D-hydantoinases (Kim *et al.*, 1999). One of the advantages of using thermostable enzymes is an enhanced production rate when slightly insoluble substrates are used. By elevating the reaction temperature, the solubility of these substrates is increased thereby enhancing productivity (Lee *et al.*, 1996a). The thermostability of the *N*-carbamoylase enzyme from *Agrobacterium* species KNK712 was improved by 5^0C through mutation and selection based on thermotolerance (Ikenaka *et al.*, 1998a; Nanba *et al.*, 1999). The highest temperature at which this *N*-carbamoylase remained stable was further improved by 19^0C, to 75^0C, through various combinations of thermostability-related mutations. The mutant enzyme also showed enhanced pH stability (Ikenaka *et al.*, 1999). Three amino acids were shown to be related to the thermostability of the enzyme (Ikenaka *et al.*, 1998c).

Truncated mutant enzymes, constructed using the genes of two thermostable D-hydantoinases from *Bacillus stearothermophilus* SD1 and *Bacillus thermocatenulatus* GH2 have been engineered having significantly enhanced thermostability in comparison to the wild-type enzymes (Kim *et al.*, 1999). Recently, Oh *et al.* (2002) achieved an 18-fold increase in the oxidative stability and an 8-fold increase in the thermostability of the *N*-carbamoylase from *Agrobacterium tumefaciens* NRRL B11291 by directed evolution of the enzyme.

1.6.3 Inducer-independent expression of enzymes

Currently-used industrial strains require induction for optimal production of hydantoin-hydrolysing activity, thereby increasing the costs of industrial processes. Inducer-independent strains, therefore, have enhanced potential for industrial use. A regulatory mutant strain, *A. tumefaciens* RU-ORPN1, which

constitutively produces both D-hydantoinase and D-*N*-carbamoylase enzymes, was isolated in our laboratory by random mutagenesis of the wild-type organism (Hartley *et al.*, 1998). No inducer was therefore required for expression of the hydantoinase and *N*-carbamoylase enzymes. Constitutive expression of hydantoinase and *N*-carbamoylase genes was achieved in a recombinant *E. coli* strain expressing the genes from the industrial *Agrobacterium tumefaciens* NRRL B11291 strain (Grifantini *et al.*, 1998b). Inducer-independent mutant strains of *Arthrobacter* sp. DSM 7330 (Wagner *et al.*, 1996) and *Pseudomonas putida* DSM 84 (LaPointe *et al.*, 1994) have also been isolated.

1.7 Stabilisation of hydantoinase and *N*-carbamoylase enzymes by immobilisation

One of the greatest constraints in the application of enzymes in industrial processes is the relative lack of stability during storage and use (Govardhan, 1999). As a result, the development of methods for the stabilisation of enzymes has been an important goal in enzyme technology (Fernandez-Lafuente *et al.*, 1995). A number of molecular mechanisms are involved in the inactivation of enzymes or proteins. The physical processes can be separated into two phases. The first phase involves reversible conformational changes while the second phase involves irreversible unfolding of the protein polypeptide chains resulting in a permanent loss of activity (Cheon *et al.*, 2000). Procedures that have been particularly successful for the stabilisation of enzymes are those that are aimed at the prevention of the unfolding of the protein tertiary structure. These include the use of immobilisation techniques or the addition of certain stabilizing agents to the enzyme solutions such as certain salts, sucrose, glycerol, thiol reducing agents or the addition of the enzyme substrate (O'Fagain *et al.*, 1988; Lee and Vasmatzis, 1997).

Immobilisation of enzymes on solid supports has not only been successful for the stabilisation of enzymes but has allowed reusability, which has introduced the potential for continuous production processes and allowed the more economical use of biocatalysts (Boshoff, 2001). The stabilisation of immobilised enzymes results from the multipoint attachment of the enzyme structure to a support thereby preventing the unfolding of the enzyme structure (Klibanov, 1979). Enzymes can be immobilised by (1) cross-linking, (2) adsorption to a solid support, (3) adsorption to a solid support followed by cross-linking, (4) covalent attachment to a solid support, (5) entrapment and (6) enzyme crystallization (Katchalski-Katzir and

Kraemer, 2000). Entrapment is by far the most widely used method in the literature for the immobilisation of whole cells and enzymes (Busto *et al.*, 1995).

For industrial processes, immobilisation techniques have also been applied to whole cells which avoids laborious and costly enzyme purification procedures and the need for cofactor addition to the system (if required), and allows the reuse of the biocatalyst. In addition, immobilisation of whole cells by entrapment results in protection against microbial attack, exogenous proteases, extremes of pH and temperature, organic solvents and leakage of enzymes from the matrix is avoided (Klibanov, 1979; Klibanov, 1983; Park and Chang, 2000).

1.7.1 Immobilisation of hydantoin-hydrolysing bacterial cells

The immobilisation of whole bacterial cells containing hydantoinase and *N*-carbamoylase enzymes for the production of amino acids has been described by numerous researchers. Olivieri *et al.* (1979) reported the immobilisation of whole cells of *Agrobacterium radiobacter* having both D-hydantoinase and D-*N*-carbamoylase activities, in fibres. The effect of the immobilisation on the enzyme stabilities and productivities were not reported. Yamada *et al.* (1980) described the immobilisation of alkalophilic *Bacillus* sp. 121-3 whole cells with high D-hydantoinase activity using a polyacrylamide gel entrapment method and observed no significant change in activity or substrate specificity. The immobilised cells completely converted D,L-5-HPH to *N*-carbamyl-hydroxyphenylglycine (NC-HPG) for ten repeated batch reactions, while only 50% conversion was achieved for the free enzyme after 10 batch reactions.

Recombinant *E. coli* cells expressing the D-hydantoinase and *N*-carbamoylase genes from *Agrobacterium tumefaciens* NRRL B11291 were immobilised by entrapment in κ-carrageenan beads. The immobilisedcells achieved a 93% conversion of D,L-5-HPH to D-HPG in comparison to a 20% conversion rate with *A. tumefaciens* NRRL B11291 after the same reaction time. Free cells could be recycled six times while immobilised cells could only be used twice due to fouling on the gel surface (Chao *et al.*, 1999b).

One of the major disadvantages of using immobilised whole cells as biocatalysts has been the diffusional limitation of the substrate. Yin and co-workers achieved a 60% increase in productivity of recombinant

E.coli cells expressing the D-hydantoinase gene from *Pseudomonas putida* by permeabilization of the cell wall and membrane with *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB). However, the permeabilised cells had reduced enzyme stability toward thermal and organic denaturation. After immobilisation of the recombinant cells in calcium alginate beads, the immobilised D-hydantoinase exhibited enhanced thermal stability but conversion of D,L-5-HPH to NC-HPG was low (Yin *et al.*, 2000). The immobilised D-hydantoinase enzyme retained over 95% of its activity after being reused three times in comparison to 22% by the free cells (Chen *et al.*, 1999).

Immobilisation of *Agrobacterium tumefaciens* strain 47C cells, having D-hydantoinase activity, has been reported. The cells, immobilised in Hypol foam, were shown to be suitable for biocatalyst reuse, with 60% of the initial conversion rate remaining after 8 reactions (Durham and Weber, 1995).

Pseudomonas putida DSM 84 cells, having hydantoinase activity, were immobilised in calcium alginate beads and in a microporous hollow fibre bioreactor. The effect of the immobilisation on the biochemical characteristics of the enzyme were not reported but the highest productivity of *N*-carbamyl-$-alanine was obtained with the alginate bead immobilised cells in a packed-bed bioreactor (Chevalier *et al.*, 1989).

A *Pseudomonas* sp. strain KBEL 101 isolated from soil was immobilised in polyacrylamide gel and used for the synthesis of D-HPG from D,L-5-HPH in the presence of an organic solvent, dimethylsulfoxide (DMSO). The optimum reaction conditions for the immobilised whole cells were pH 8 and 30^0C. The stability of the immobilised whole cell hydantoinase was significantly reduced with increasing pH and temperature. Conversion yields of 99% were obtained for four repeated batch reactions (Kim and Kim, 1993, Kim *et al.*, 1994).

Whole cells of *Peptococcus anaerobius* with D-hydantoinase activity were immobilised by adsorption on activated charcoal. The immobilised cells were applied to various continuously operated bioreactors for the production of *N*-carbamyl-D-alanine. Conversion yields of 20-30% were obtained continuously over 7 days (Morin and Lafond, 1992).

1.7.2 Immobilisation of isolated enzymes

Although the use of resting free or immobilised whole cells in industrial processes has been more economically viable than isolated enzymes in most cases, whole cell biocatalysts are now being replaced by immobilised enzymes, because whole cell biotransformations often result in products that are coloured or contaminated with metabolites derived from cell lysis. This necessitates downstream processing of the products, which is often a tedious procedure (Pietzsch *et al.*, 2000). Also, *N*-carbamylamino acid intermediates are easily transported out of cells but are not as easily taken up, resulting in poor yields (Olivieri *et al.*, 1981; Yin *et al*, 2000).

1.7.2.1 Immobilisation of hydantoinases

The production of NC-HPG from D,L-5-HPH using immobilised thermostable D-hydantoinase from *Bacillus stearothermophilus* SD-1 has been described. The enzyme, in crude extract form, was immobilised by adsorption on a variety of supports including activated carbon, silica gel, celite and zeolite Y. DEAE-cellulose resin was most effective in terms of amount of protein bound and activity recovery. The enzyme retained more than 90% activity after immobilisation and the optimal reaction conditions were pH9 and 55^0C. The production rate of NC-HPG from D,L-5-HPH was maintained over nine successive batch operations (Lee *et al.*, 1996b).

An immobilised D-hydantoinase biocatalyst (produced from a recombinant *E. coli* strain) has been produced by covalent attachment of the enzyme on a granulated organic polymer. The half-life of the biocatalyst under process conditions of 50^0C and pH 8.5 was eight batches (Lee and Lin, 1996). The biocatalyst, purchased from Boeringer Mannheim (Mannheim, Germany), was applied effectively to various bioreactor systems for the production of NC-HPG from D,L-5-HPH. High concentrations of D,L-5-HPH (4% w/v) were converted to NC-HPG using this biocatalyst in bioreactors (Lee and Fan, 1999).

A partially purified D-hydantoinase from lentil seeds has been immobilised by adsorption and cross-linking on DEAE-cellulose. The enzyme retained 80% activity after immobilisation and exhibited increased shelf-life, thermostability and stability in various organic solvents (Rai and Taneja, 1998). A D-hydantoinase purified from adzuki bean was immobilised by adsorption on fine polyglutaraldehyde particles followed by

entrapment in calcium alginate beads. The immobilised hydantoinase converted more than 95% D-phenylhydantoin to *N*-carbamoyl-D-phenylglycine for five repeated batch reactions at 40^0C without loss of activity (Fan and Lee, 2001).

The partially purified hydantoinase from *Arthrobacter aurescens* DSM 3747 was immobilised by covalent attachment on EAH Sepharose 4B, Eupergit C(-NH_2) and Eupergit C 250 L (-NH_2) with activity yields up to 60%. Immobilisation on more hydrophobic supports such as Eupergit C and Eupergit C 250 L resulted in lower yields of activity (Ragnitz *et al.*, 2001a). The enzyme immobilised on Eupergit C 250 L (-NH_2) showed enhanced stability in comparison to the free enzyme, with a half-life of 14000 hours. The temperature optimum for the immobilised hydantoinase increased from 40 to 60^0C and the pH optimum ranged from 8 to 9.5 (Ragnitz *et al.*, 2001b).

The D-hydantoinase from *Bacillus stearothermophilus* SD1, purified from recombinant *E. coli* cells expressing the hydantoinase gene, was stabilised by intersubunit cross-linking with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride. The stability of the cross-linked enzyme was significantly increased in comparison to the free enzyme which was shown to lose catalytic activity due to the dissociation of its subunits under operational conditions. The cross-linked enzyme showed a four-fold increase in half life and was stable in the presence of 20% methanol and under acidic conditions. The cross-linked enzyme was also able to convert D,L-5-HPH to NC-HPG more efficiently than the free enzyme (Cheon *et al.*, 2000).

1.7.2.2 Immobilisation of *N*-carbamoylases

Relatively few reports have been made of the immobilisation of *N*-carbamoylases in comparison to immobilised hydantoinases. The D-*N*-carbamoylase from a recombinant *E. coli* strain expressing the cloned gene from *Agrobacterium* sp. strain KNK712 was immobilised on various porous polymers for use in the production of amino acids. The best supports for the enzyme in terms of activity and stability were found to be Duolite A-568 and Chitopearl 3003. The remaining activity of the enzyme, immobilised on Duolite A-568 in the presence of dithiothreitol and cross-linked with 0.2% glutaraldehyde, was 63% after

14 repeated reactions. The immobilisation of two thermotolerant *Pseudomonas N*-carbamoylase enzymes immobilised under the same conditions was shown to be far superior in terms of enzyme stability, although the activity of these enzymes was lower (Nanba *et al.*, 1998b). The thermostability of the *Agrobacterium* sp. strain KNK712 *N*-carbamoylase was subsequently improved through mutation. The stability of the immobilised mutant enzyme was significantly improved in comparison to the immobilised native enzyme (Nanba *et al.*, 1999).

The immobilisation of crude L-*N*-carbamoylase from *Arthrobacter aurescens* DSM 3747 gave low activity yields. However, the highly purified enzyme, obtained from cultivation of recombinant *E. coli*, was immobilised by covalent attachment to EAH Sepharose 4B with an activity yield of almost 100% (Ragnitz *et al.*, 2001a). The enzyme immobilised on EAH-Sepharose 4B showed enhanced stability in comparison to the free enzyme, with a half-life of 900 hours. The temperature optimum for the immobilised L-*N*-carbamoylase increased from 50 to 60^0C and the pH optimum increased from 8 to 9.5 (Ragnitz *et al.*, 2001b).

1. 8 Application of immobilised hydantoin-hydrolysing enzymes in bioreactor systems

Despite the large number of hydantoinase and *N*-carbamoylase enzymes that have been characterised and immobilised in the literature, very few reports on the development of bioreactor systems for the production of amino acids have been published. In most cases, the bioreactors that have been reported use immobilised hydantoinase enzymes for the production of *N*-carbamyl intermediates. Very few reports on the use of *N*-carbamoylases in bioreactor systems have been made, presumably due to the instability of this enzyme.

Partially purified D-hydantoinase from *Pseudomonas fluorescens* strain DSM 84 was used in a laboratory-scale membrane bioreactor for the conversion of 50mM isopropylhydantoin to *N*-carbamyl-D-valine. A 10 to 30% conversion yield was obtained over 10 days continuous operation (Morin *et al.*, 1986).

Pseudomonas putida DSM 84 cells immobilised in calcium alginate beads have been used in packed-bed columns and in a hollow fibre membrane bioreactor for the continuous conversion of dihydrouracil into *N*-carbamyl-$-alanine. A conversion yield of 40% for 50mM dihydrouracil was obtained for the packed-bed bioreactor over four days continuous operation. The conversion yields in the hollow fibre membrane bioreactor ranged from 8 to 16% with different dilution rates (Chevalier *et al.*, 1989).

A D-hydantoinase from the anaerobic bacterial strain, *Peptococcus anaerobius*, was immobilised on activated charcoal and used for the conversion of 100mM D,L-5-methylhydantoin to *N*-carbamyl-D-valine in continuously operated bioreactors. A bioreactor operated by percolation continuously produced *N*-carbamyl-D-valine with a conversion yield of 30% for 5 days, while a bioreactor operated by fluidisation gave a conversion yield of 20% over 8 days (Morin and Lafond, 1992).

A D-hydantoinase extracted from legumes has been used for the continuous conversion of 100mM D,L-methyl-mercaptoethylhydantoin to *N*-carbamyl-D-methionine in a membrane bioreactor for 10 days at 50^{0}C with a conversion yield ranging from 45 to 95% (Morin, 1993).

An immobilised whole cell D-hydantoinase from *Pseudomonas* sp. KBEL 10, having a half-life of only 50 hours, was applied in a continuous stirred tank reactor (Kim *et al.*, 1994). The activity of the immobilised whole cell biocatalyst was maintained in the reactor for 7 days by the addition of 0.1% (w/v) glycerol to the production medium, and the addition of 0.1% yeast extract prolonged the half-life of the enzyme to 25 days.

Pseudomonas sp. M-38 cells were immobilised in calcium alginate beads and used in a packed-bed bioreactor system for the continuous production of L-cysteine from D,L-2-amino-thiazoline-4-carboxylic acid. The bioreactor system consisted of two packed-bed columns containing immobilised cells, and two ion-exchange resin Dowex 50W columns, for L-cysteine extraction, developed to reduce product inhibition. The overall productivity of the system with the extractors was 43% higher than without them (Ryu *et al.*, 1997).

A pressure swing bioreactor having two filter-separated stirred compartments for the conversion of high concentrations of D,L-5-HPH to NC-HPG has been developed. Due to the insoluble nature of D,L-5-HPH, high concentrations take the form of solid substrate suspensions. By separating the immobilised D-hydantoinase from the substrate suspensions, fouling of the biocatalyst was avoided. Applying pressure swing resulted in cross-flow through the filter between the compartments and a conversion yield of about 80% for concentrations as high as 15% w/v D,L-5-HPH was achieved in 10 hours with fed-batch operation (Lee and Fan, 1999, Lee *et al.*, 2001b).

The hydantoinase and *N*-L-carbamoylase from *Arthrobacter aurescens* DSM 3747 have been used in a packed-bed bioreactor and continuously stirred tank reactor for the production of L-tryptophan. The hydantoinase, immobilised on Eupergit C 250 L (NH_2), and *N*-L-carbamoylase, immobilised on EAH-Sepharose, had half-life times of approximately 14000 and 900 hours, respectively, in a packed-bed bioreactor. The immobilised hydantoinase maintained conversion rates of over 80% for 5000 hours in the packed-bed reactor and for 1200 hours in the continuous stirred tank. The conversion rates of the immobilised *N*-L-carbamoylase decreased to near 10% after 33 days in both bioreactors (Ragnitz *et al.*, 2001b).

1.9 Production of D-HPG from D,L-5-HPH by hydantoin-hydrolysing bacterial strains

Although hydantoinase and *N*-carbamoylase enzymes co-existing in the same bacterial strain are rare, a few strains have been reported in the literature. These strains have the ability to transform racemic hydantoins to the corresponding amino acids in a one-step process. (Olivieri *et al.*, 1979; Yokozeki *et al.*, 1987b; Möller *et al.*, 1988; Runser *et al.*, 1990, Hartley *et al.*, 1998). The process for the preparation of D-HPG from D,L-5-HPH using the hydantoin-hydrolysing enzymes from *Agrobacterium tumefaciens* (formerly *radiobacter)* NRRL B11291 was developed in the 1980's (Olivieri *et al.*, 1981). The process was simple but showed room for improvement in terms of productivity. Since the 1980's, a number of researchers have reported on optimisation of the production of D-HPG from D,L-5-HPH using *Agrobacterium* species and other bacterial strains.

The isolation of *Agrobacterium tumefaciens* NRRL B11291 was reported in 1979 (Olivieri *et al.*, 1979). Crude enzyme extracts from this strain were used by this group of researchers to prepare D-amino acids of industrial interest such as D-phenylglycine, D-HPG and D-thienylglycine by the stereospecific hydrolysis of D,L-5-substituted hydantoins (Olivieri *et al.*, 1981). The spontaneous racemisation of the hydantoin substrates allowed for the complete conversion of the racemic substrate to the D-amino acid in one reactor and therefore the production of D-amino acids using this strain was patented (Deepa *et al.*, 1993). A detailed process for the biotransformation of D,L-5-HPH using whole cells of *Agrobacterium tumefaciens* NRRL B 11291 was described by Deepa and co-workers in 1993. The growth medium composition was optimised for maximal production of the hydantoin-hydrolysing enzymes resulting in 1.5 times more enzyme being produced than in the previously used media. The bacterium produced the enzymes constitutively and substrate analogues did not induce enzyme production (Deepa *et al.*, 1993). A simple and economically viable soya meal medium for the growth of *Agrobacterium tumefaciens* NRRL B 11291 for the production of D-amino acids was described by George and Sadhukhan (1996). Biomass and enzyme production were further optimised by making use of an empirical modelling technique (Achary *et al.*, 1997). In 1998, Grifantini and co-workers identified *Agrobacterium radiobacter* NRRL B11291 as *Agrobacterium tumefaciens* NRRL B11291 (Grifantini *et al.*, 1998b). The productivity of the strain for D,L-5-HPH conversion to D-HPG was increased five-fold by Chao *et al.* (1999b) by overexpression of the hydantoinase and *N*-carbamoylase genes in *E. coli*. The gene for the *N*-carbamoylase from *Agrobacterium tumefaciens* NRRL B11291 has been expressed in *E. coli* for the production of D-HPG from NC-HPG (Fan *et al.*, 2000). This gene has also been co-expressed with the gene for the D-hydantoinase from *Bacillus stearothermophilus* SD1 in *E. coli* for the production of D-HPG from D,L-5-HPH (Park *et al.*, 2000) and the kinetics of the reaction system was recently modelled using whole cells with the separately and co-expressed enzymes (Park *et al.*, 2002). Lee *et al.* (2001a) observed an interesting phenomenon when examining the effect of the cell membrane of this strain on the production of D-amino acids. They found that the presence of the intact cell membrane could enhance the production of D-amino acid when whole cells were used for the hydrolysis of D,L-5-substituted hydantoins. This was attributed to the lower permeability of the cell membrane to the carbamoyl derivatives produced as intermediates in the reaction.

An *Agrobacterium* sp., IP-671, able to convert of D,L-5-HPH to D-HPG has been described. The hydantoinase and *N*-carbamoylase enzymes involved were strictly D-stereospecific and the biosynthesis of the enzymes was inducible by the addition of 2-thiouracil to the growth medium (Runser *et al.*, 1990). Inhibition studies revealed the inhibition of the *N*-carbamoylase enzyme by the ammonium ions co-produced with D-HPG. The conversion yield of D-HPG from D,L-5-HPH was increased from 50% to 98% by the addition of specific adsorbents for ammonium ions (Kim and Kim, 1994). Numerical simulation of the enzymatic production of D-HPG allowed these researchers to further optimise the reaction conditions by finding the optimal ratio of D-hydantoinase to *N*-carbamoylase which was identified as 3:1 (Kim and Kim, 1995). The induction of the biosynthesis of the hydantoin-hydrolysing enzymes in *Agrobacterium* sp. IP I-671 was further improved by the addition of non-metabolizable thiolated hydantoins or pyrimidines to the growth medium. Addition of 2,4-thiouracil to the cell cultures resulted in a 40-fold increase in the hydantoinase activity and a 15-fold increase in the *N*-carbamoylase activity in comparison to inducer-free cultures (Meyer and Runser, 1993).

Table 1.3 Summary of the optimised conversion yields of D-HPG from D,L-5-HPH for reported hydantoin-hydrolysing bacterial strains.

Organism	**Biocatalyst configuration**	**Biocatalyst concentration**	**D,L-5-HPH concentration**	**Molar conversion yield (%)**	**Reaction time (h)**	**Temperature (^{0}C)**	**pH**
A. radiobacter NRRL B11291 (Olivieri *et al.*, 1981)	resting cells	3% (DCW/v)	4% (w/v)	90	28	40	8
A. radiobacter NRRL B11291 (Deepa *et al.*, 1993)	resting cells	not specified	3% (w/v)	94	7-8	50	9
A. radiobacter NRRL B11291 (Chao *et al.*, 2000b)	recombinant *E. coli* cells	1.9% (DCW/v)	2% (w/v)	97	5	40	7.5
A. radiobacter NRRL B11291 and *B. stearothermophilus* SD1 (Park *et al.*, 2000)	recombinant *E. coli* cells	not specified	0.4% (w/v)	98	15	45	7
A. radiobacter NRRL B11291 (Lee *et al.*, 2001a)	resting cells	2% (DCW/v)	3% (w/v)	86	6	40	8

Agrobacterium sp. I-671 (Runser *et al.*, 1990)	resting cells	1% (DCW/v)	3% (w/v)	96	20	45	6.7
Agrobacterium sp. I-671 (Kim and Kim, 1994)	resting cells	1% (DCW/v)	1.5% (w/v)	98	27	30	8
Agrobacterium sp. I-671 (Kim and Kim, 1995)	partially purified enzymes (hyd:nca =1:3)	0.1% hyd (DCW/v) 0.3% nca (DCW/v)	0.5% (w/v)	100	15	30	8
Blastobacter sp. A17p-4 (Ogawa *et al.*, 1994b)	resting cells	1% (wet mass/v)	0.5% (w/v)	13	2	45	8
Pseudomonas sp. AJ-11220 (Yokozeki *et al.*, 1987c)	resting cells	5% (wet mass/v)	1% (w/v)	94	30	30	8

A large number of stock cultures and soil samples were screened for microorganisms able to produce D-HPG from D,L-5-HPH by Yokozeki *et al.* (1987c). A *Pseudomonas* species, *Pseudomonas* sp. AJ-11220, isolated from soil, had the highest productivity and optimisation of the biotransformation conditions for the production of D-HPG from D,L-HPH was reported. Under optimal conditions D,L-5-HPH was converted to D-HPG with a molar conversion yield of 92%.

The D,L-5-HPH conversion yields obtained from the various bacterial species reported in the literature able to convert D,L-5-HPH to D-HPG are summarised in Table 1.3.

1.10 Project objectives

A. tumefaciens RU-ORPN1 was found to produce high hydantoinase and *N*-carbamoylase activity in the absence of an inducer (Hartley *et al.*, 2001). The addition of inducers to the growth medium of industrial strains increases the costs of these processes, therefore, inducer-independent strains are favoured. Furthermore, the high levels of both hydantoinase and *N*-carbamoylase activity produced by *A. tumefaciens* RU-ORPN1 and the ability of the strain to efficiently convert D,L-5-HPH to D-HPG, made it potentially an ideal strain for the development of a biocatalyst for the one-step production of D-HPG. The high levels of *N*-carbamoylase activity also made this an ideal strain for further research on the stabilisation of this enzyme.

The aim of this research was the development of a hydantoin-hydrolysing biocatalyst utilising the hydantoinase and *N*-carbamoylase from *A. tumefaciens* RU-ORPN1. This objective was based on the hypothesis that the hydantoin-hydrolysing enzyme activity in this strain could be optimised, isolated, purified and stabilised for industrial application as biocatalyst(s) for the production of enantiomerically pure amino acids from 5-monosubstituted hydantoin substrates. The objectives set to test this hypothesis were as follows:

10.1 The evaluation and optimisation of the growth conditions for *A. tumefaciens* RU-ORPN1 for optimal hydantoin-hydrolysing enzyme activity and biomass production would allow for a source of cells with consistently high levels of hydantoinase and *N*-carbamoylase activity.

10.2 The optimisation of the conditions for optimal hydantoin-hydrolysing activity in the enzyme extract would be required to consistently produce an enzyme extract with high activities. This would include investigation of methods for the production and storage of the enzyme extract, and identifying the optimal enzyme reaction conditions for maximum hydantoinase and *N*-carbamoylase activities.

10.3 Purification and characterisation of the hydantoinase and *N*-carbamoylase enzymes would be required for the biochemical characterisation of the enzymes. This would include investigation of the protein quarternary structures, molecular weights and enzyme reaction kinetics. The

investigation of the enzyme kinetics would allow modelling of the enzyme reaction which would provide important information for the application of the enzymes in an industrial process.

10.4 Stabilisation of the hydantoinase and *N*-carbamoylase activities by immobilisation of whole cells and enzyme extracts would be required for the successful application of the biocatalysts in bioreactor systems. Different immobilisation methods would be compared in order to select the most suitable biocatalyst in terms of biocatalytic activity and stability.

10.5 Characterisation of the immobilised biocatalyst, with the most potential, in terms of optimal enzyme reaction conditions, substrate specificity, thermostability and reusability would be required for the identification of the optimal operating conditions for the application of the biocatalyst in bioreactor systems.

10.6 Development of a bioreactor system suitable for the application of the biocatalyst, and evaluation of the productivity of the bioreactor, with a view to industrial process development, would be required in order to assess the suitability of the hydantoinase and *N*-carbamoylase from *A. tumefaciens* RU-ORPN1 for application in an industrial process for the production of D-HPG.

CHAPTER 2

GROWTH OF *AGROBACTERIUM TUMEFACIENS* RU-ORPN1 AND ASSAY OF HYDANTOIN-HYDROLYSING ACTIVITY

2.1 INTRODUCTION

This chapter describes the investigation into the conditions under which *Agrobacterium tumefaciens* RU-ORPN1 was cultured and maintained most efficiently. Initially, the bacterium was grown on a small-scale in flask cultures according to the methods developed by Hartley *et al.* (1998), who isolated the wild-type, *Agrobacterium tumefaciens* RU-OR, locally from soil samples, and later, the inducer-independent mutant, *A. tumefaciens* RU-ORPN1, which was produced by mutagenesis with ethylmethane sulfonate (Hartley

et al., 2001). Demands for larger quantities of biomass required for later experimental protocols, resulted in a change to larger-scale fermentations, initially in a 10L culture flask, and finally in a 5L benchtop fermenter. The growth media used, the biomass yields and enzyme activities obtained under these culturing conditions are described.

In earlier work carried out on these strains it was shown that the wild-type *A. tumefaciens* RU-OR produced no hydantoinase activity without the presence of an inducer (Hartley *et al.*, 1998), while significant levels of hydantoinase and *N*-carbamoylase activities were detected in the mutant, *A. tumefaciens* RU-ORPN1, in the absence of an inducer (Hartley *et al.*, 2001). The inducer-independence of this mutant strain made it a suitable candidate for the development of an industrial biocatalyst and this was one of the reasons why it was chosen for this work. An initial experiment compared the hydantoin-hydrolysing activity of the wild-type strain, grown in the presence of an inducer, 2-thiouracil, and the mutant strain, without an inducer, in order to compare the hydantoin-hydrolysing activities of the two strains.

Some preliminary experiments were conducted in order to optimise the growth conditions for *A. tumefaciens* RU-ORPN1 in flask cultures. The hydantoin-hydrolysing activities measured in cells grown in various growth media were compared, and the effect of the addition of high concentrations of metal ions to the growth medium was investigated. Further optimisation of the growth media was carried out by collaborators on the project at the Council for Scientific and Industrial Research (CSIR), Modderfontein, South Africa, who developed the PP2 growth medium described for use in the fermentation vessel. Finally, the accuracy of the colorimetric enzyme assays was evaluated by comparing the enzyme activities measured using the colorimetric enzyme assays with those determined using high performance liquid chromatography (HPLC).

2.2 MATERIALS AND METHODS

2.2.1 Materials

Hydantoin was purchased from Aldrich Chemical Company. *N*-carbamylglycine (NCG), D-HPG, 2-thiouracil and 5-fluorouracil were purchased from Sigma Chemical Co. D,L-5-HPH and NC-HPG were synthesized by CSIR, South Africa. All reagents were analytical grade.

2.2.2 Methods

2.2.2.1 Culture media and growth conditions for *A. tumefaciens* RU-OR and inducer-independent mutant *A. tumefaciens* RU-ORPN1

A. tumefaciens RU-ORPN1 was grown on hydantoin minimal medium (HMM) agar plates containing, per litre (pH 7): 60g Na_2HPO_4, 30g KH_2PO_4, 5g NaCl, 10g glucose, 10g hydantoin, 0.02g $MgCl_2$, 0.011g $CaCl_2$, 0.04g boric acid, 0.04g $MnSO_4$, 0.02g $(NH_4)_6Mo_2O_{24}.4H_2O$, 0.01g KI and 0.004g $CuSO_4$. A single colony was inoculated into HMM broth (50mL) and grown for 3-4 days to stationary phase with shaking at 200rpm. The OD_{600nm} of the starter culture was measured and diluted to 0.02 in minimal medium containing 0.01% casamino acids. The cells were then grown for 18 -24 h to mid-log growth phase with shaking at 200rpm (approximately OD_{600nm}=0.5-0.8). Cells were harvested by centrifugation at 7 000rpm for 10 minutes and washed twice with half the original volume of potassium phosphate buffer (0.1M, pH 9). Cell pellets were stored at -20^{0}C until required. For growth curves, OD_{600nm} readings were taken every hour over 35 h and plotted against enzyme activities assayed in samples (200mL) taken at various stages of growth in order to determine at which stage of growth maximum enzyme activities were obtained. *A. tumefaciens* RU-OR was grown as described for *A. tumefaciens* RU-ORPN1, except that the growth medium contained 0.1% 2-thiouracil as inducer.

For the 10L flask culture, *A. tumefaciens* RU-ORPN1 was grown exactly as described above, except that the HMM starter culture was diluted with minimal medium containing 0.01% casamino acids to an OD of 0.02 in a final volume of 10L. The cells were then grown to mid-log growth phase with sterile air as a source of aeration and agitation.

2.2.2.2 Growth conditions for *A. tumefaciens* RU-ORPN1 in a 5L benchtop fermenter

A. tumefaciens RU-ORPN1 was cultured in a 5L Bioflow 3000 benchtop fermenter using PP2 medium (Appendix A) which was developed and optimised by collaborators on the project at CSIR for growth and production of hydantoin-hydrolysing activity. *A. tumefaciens* RU-ORPN1 was streaked from a hydantoin minimal medium agar plate onto a nutrient agar plate and grown overnight at 28^{0}C. Cells from the nutrient agar plate were then inoculated into a 500mL nutrient broth starter culture containing 0.1% hydantoin and incubated at 28^{0}C overnight with shaking at 200rpm. The starter culture was diluted with PP2 medium (pH 7) to a final OD_{600nm} of 0.1 in a final volume of 4.5L. The fermentation conditions were maintained at 29^{0}C and minimum aeration of 40%. The pH was maintained by the addition of 1M sodium hydroxide solution.

The cells were harvested at early stationary phase after approximately 18-21 h growth. The cell pellets were washed in two volumes of potassium phosphate buffer (0.1M, pH 9) and assayed for hydantoinase and *N*-carbamoylase activities. For growth curves, cells from 100mL samples were harvested every 4 h hours over 48 h and assayed for hydantoinase and *N*-carbamoylase activities. Cells from samples (1mL), also taken every 4 h, were pelleted by centrifugation, washed twice in 1% HCl and dried in an oven in pre-weighed centrifuge tubes. Dry cell weights were then measured and plotted against enzyme activities in order to determine the stage of growth producing maximum enzyme activities.

2.2.2.3 Determination of resting cell hydantoinase and *N*-carbamoylase activities

Cells were resuspended at a concentration of 20mg/mL (wet mass) in potassium phosphate buffer (0.1M, pH 9) containing final concentrations of 50mM hydantoin or 25mM NCG in a final volume of 5mL. Triplicate reactions were carried out for each substrate at 40^0C for 6 h at 200rpm, together with controls containing cells or substrate only. Following incubation, cells were pelleted by centrifugation and the concentrations of NCG and glycine in the reaction supernatants were determined using colorimetric assays. Hydantoinase activities are expressed as the sum of the NCG (: mol/mL) and glycine (: mol/mL) concentrations produced from 50mM hydantoin after incubation at 40^0C for 6 h at 200rpm in a 5mL volume. *N*-carbamoylase activities are expressed as the concentration of glycine (: mol/mL) produced from 25mM NCG after incubation at 40^0C for 6 h at 200rpm in a 5mL volume.

NCG was detected using Ehrlich's reagent, 10% (w/v) 4-dimethylaminobenzaldehyde in 6M HCl according to the method described by Morin *et al.* (1987). Reaction supernatants (1mL) or NCG standard solutions ranging from 0-25mM (1mL) were added to 0.5mL 12% (w/v) trichloroacetic acid. Ehrlich's reagent (0.5mL) and 3mL distilled water were added to each sample and the absorbance at 420nm was read after 15 minutes. NCG concentrations in the supernatants were then interpolated from the NCG standard curve (Appendix B).

Glycine was detected using the Ninhydrin assay adapted from Plummer (1987). Reaction supernatants were diluted 1/50 in potassium phosphate buffer (0.1M, pH 9). Ninhydrin reagent (1mL) was added to 1mL diluted reaction supernatant or standard glycine solution (0-0.5mM) and the samples were boiled for 15minutes. After cooling, 3mL 50% ethanol was added to each tube and the absorbance at 570nm was

read. The glycine concentrations in the supernatants were interpolated from the glycine standard curve (Appendix C).

In some experiments, D,L-5-HPH and NC-HPG were used as substrates for the enzymes. For these assays, cells were resuspended to give a final concentration of 20mg/mL (wet mass) in potassium phosphate buffer (0.1M, pH 9) containing final concentrations of 12.5mM D,L-5-HPH or NC-HPG in a final volume of 5mL. Triplicate reactions were carried out for each substrate at 40^0C for 6 hours at 200rpm, together with controls containing cells or substrate only. Following incubation, cells were pelleted by centrifugation and the concentrations of NC-HPG and D-HPG in the reaction supernatants were determined using Ehrlich's and Ninhydrin assays, respectively, as described above or by HPLC (section 2.2.2.4). The standard curves generated for NC-HPG and D-HPG using the colorimetric assays are shown in Appendices B and C, respectively. Where D,L-5-HPH and NC-HPG were used as substrates, hydantoinase activity is expressed as the sum of the NC-HPG (: mol/mL) and D-HPG (: mol/mL) concentrations produced from 12.5mM D,L-5-HPH after incubation at 40^0C for 6 h at 200rpm in a 5mL volume. *N*-carbamoylase activity is expressed as the concentration of D-HPG (: mol/mL) produced from 12.5mM NC-HPG after incubation at 40^0C for 6 h at 200rpm in a 5mL volume.

All enzyme activities are reported as the mean of triplicate reactions and error bars represent SEM (n = 3, unless otherwise stated). All spectrophotometric measurements were made using a Shimadzu UV-160A spectrophotometer.

2.2.2.4 Determination of resting cell hydantoinase and *N*-carbamoylase activities by HPLC

Whole cells were reacted with 12.5mM D,L-5-HPH or 12.5mM NC-HPG under exactly the same assay conditions as described in section 2.2.2.3. Following the reaction, the concentrations of D,L-5-HPH, NC-HPG and D-HPG in the reaction supernatants were determined using HPLC according to a method adapted from Louwrier and Knowles (1997). Samples (20µL) were injected on a Waters C_{18} HPLC column (46mm×50mm). The mobile phase consisted of 9% (v/v) methanol (HPLC grade), 2mM ammonium acetate, pH3.2 in acetic acid. The flow rate was 0.7mL/min and the compounds were detected by their absorbance at 280nm. The retention times of D,L-5-HPH, NC-HPG and D-HPG were 7.27, 6.13 and 4.31

minutes, respectively, and the concentrations of each were interpolated from calibration curves generated for each substrate. All solutions were filtered through 0.22µm filters before use.

2.2.2.5 Effects of various growth media on the production of hydantoin-hydrolysing enzyme activity in *A. tumefaciens* RU-ORPN1

The effects of various growth media on the production of hydantoin-hydrolysing activity was determined by inoculating a HMM starter culture of *A. tumefaciens* RU-ORPN1 into hydantoin minimal medium, minimal medium containing 0.01% casamino acids, nutrient broth and nutrient broth containing 0.1% hydantoin to give a final OD_{600nm} of 0.02. Cells were then grown to mid-log phase, harvested as described in section 2.2.2.1 and assayed for hydantoinase and *N*-carbamoylase activity.

2.2.2.6 Effect of the addition of metal ions to the growth medium on the production of hydantoin-hydrolysing enzyme activity in *A. tumefaciens* RU-ORPN1

A. tumefaciens RU-ORPN1 was grown in HMM containing 0.01% casamino acids in the presence of 0.1% $MgSO_{4}.7H_{2}0$, 0.002% $MnCl_{2}.4H_{2}0$ and 0.001% $FeS0_{4}.7H_{2}0$. The cells were harvested and assayed for hydantoinase and *N*-carbamoylase activity.

2.2.2.7 Culture maintenance and storage of stock cultures of *A. tumefaciens* RU-ORPN1

Stock cultures (1.5mL) of *A. tumefaciens* RU-ORPN1 in HMM (OD_{600nm} = 1) containing 20% glycerol were stored at -80^{0}C. The strain was re-cultured from glycerol stocks biannually by streaking crystals of glycerol stock onto a minimal medium agar plate selective for *A. tumefaciens* RU-ORPN1 containing 0.1% ammonium sulphate and 0.1% 5-fluorouracil. The wild-type strain is unable to grow in the presence of the toxic hydantoin analogue, 5-fluorouracil, while the mutant strain is able to cleave the analogue to produce a non-toxic product, allowing growth (Hartley *et al.*, 1998). Single colonies were then inoculated into HMM starter cultures and cultured as described in section 2.2.2.1.

2.3 RESULTS AND DISCUSSION

2.3.1 Comparison of the enzyme activities in the wild-type *A. tumefaciens* RU-OR and the inducer-independent mutant *A. tumefaciens* RU-ORPN1

The hydantoin-hydrolysing activities in the wild-type *A. tumefaciens* RU-OR and the inducer-independent mutant *A. tumefaciens* RU-ORPN1 were compared in order to confirm the earlier findings of Hartley and co-workers, who isolated the strains (Hartley *et al.*, 1998; Hartley *et al.*, 2001). These researchers found that the mutant strain was able to produce similar levels of hydantoinase and *N*-carbamoylase activity without an inducer to those produced by the wild-type strain in the presence of an inducer. Figure 2.1 shows similar enzyme activities produced by the mutant (without an inducer) and the wild-type strain (in the presence of 2-thiouracil) confirming the findings of Hartley *et al.* (2001).

While this experiment confirmed the ability of strain RU-ORPN1 to produce similar activity levels to the induced wild-type strain, the high levels of hydantoinase and *N*-carbamoylase activity reported by Hartley *et al.* (2001) for both the wild-type and mutant strains could not be reproduced. In particular, the levels of hydantoinase activity shown in Figure 2.1 were considerably lower than those reported by Hartley *et al.* (2001) for *A. tumefaciens* RU-ORPN1. These researchers report a three-fold increase in the hydantoinase activity of *A. tumefaciens* RU-ORPN1 in comparison to the wild-type cells under these conditions. However, the levels of hydantoinase and *N*-carbamoylase activities shown in Figure 2.1 are similar to those reported for the wild-type *Agrobacterium radiobacter*, *Agrobacterium tumefaciens* 47C and *Agrobacterium* IP-I 671 strains reported in the literature (Olivieri *et al.*, 1981; Runser *et al.*, 1990; Durham and Weber, 1995). Furthermore, the high *N*-carbamoylase activity produced by *A.tumefaciens* RU-ORPN1 was of interest to the project and it was decided to continue the research using this strain.

Generally, bacterial strains possessing both enzyme activities have higher D-hydantoinase than *N*-carbamoylase activity. The *N*-carbamoylase is therefore the rate-limiting factor in the conversion of hydantoin substrates to the corresponding amino acids (Kim and Kim, 1995). This, together with the general instability of the *N*-carbamoylases, is considered one of the most serious drawbacks in process development using these strains. This was not the case for *A. tumefaciens* RU-ORPN1 which produced lower hydantoinase activity than *N*-carbamoylase activity, indicating that the hydantoinase reaction would be rate-limiting in this strain. The *N*-carbamoylase activity was significantly increased in later experiments by altering the growth conditions of the strain, resulting in an even larger difference in the activities of the two enzymes. It therefore became apparent over the course of the research that in order to develop an efficient, one-step process for the production of amino acids, the *N*-carbamoylase enzyme from *A.*

tumefaciens RU-ORPN1 would need to be combined with a strain having higher levels of hydantoinase activity. The use of the *A. tumefaciens* RU-ORPN1 enzymes in combination with such a strain is described in Chapter 6. Despite the relatively low hydantoinase activities obtained, both the hydantoinase and *N*-carbamoylase activities were assayed throughout the research, as originally proposed.

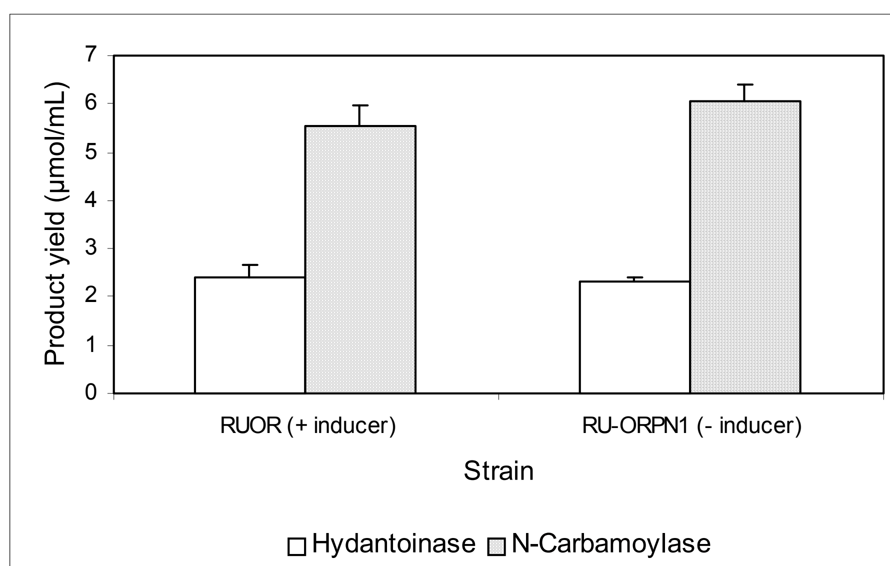


Figure 2.1 Comparison of the hydantoin-hydrolysing activity in the wild-type *A. tumefaciens* RU-OR (in the presence of an inducer, 2-thiouracil) and the mutant, *A. tumefaciens* RU-ORPN1 (without an inducer).

2.3.2 Comparison of the growth curves, biomass yields and hydantoin-hydrolysing enzyme activities for *A. tumefaciens* RU-ORPN1 grown in flask culture and in a 5L benchtop fermenter

A growth curve of *A. tumefaciens* RU-ORPN1 in minimal medium containing 0.01% casamino acids (flask cultures) was generated in order to determine at which stage of the growth curve the highest hydantoinase and *N*-carbamoylase activities were produced (Figure 2.2). Both the hydantoinase and *N*-carbamoylase activities were highest during late-exponential growth phase and cells were therefore harvested at this stage of growth for subsequent experiments. This is consistent with reports in the literature for other hydantoin-hydrolysing strains (Runser and Ohleyer, 1990; Ogawa *et al.*, 1994a; Luksa *et al.*, 1997).

The growth curve generated for *A. tumefaciens* RU-ORPN1 in the 5L benchtop fermenter in PP2 medium is shown in Figure 2.3. As was the case for the minimal medium containing 0.01% casamino acids, the hydantoinase and *N*-carbamoylase activities were highest during late-exponential growth phase. Hydantoinase activities measured were similar for both growth media, while a significant increase in the *N*-carbamoylase activity was observed in the cells grown in the PP2 medium. The highest product yield for the *N*-carbamoylase in cells grown in minimal medium containing 0.01% casamino acids was 6.5: mol/mL compared with 17.2: mol/mL for the *N*-carbamoylase in cells grown in the PP2 medium. The levels of *N*-carbamoylase activity produced in the PP2 medium were double those reported for the *N*-carbamoylase in minimal medium with 0.01% casamino acids by Hartley *et al.* (2001). The very high *N*-carbamoylase activity produced throughout the growth of the strain in this medium indicated that the enzyme was produced constitutively by the strain and that the carbon and nitrogen source in the medium favoured the production of this enzyme. The hydantoinase activity levels remained low during the growth in PP2 medium indicating that the nitrogen in the medium may not have been sufficiently limiting for the production of high levels of the enzyme and/or the carbon source may have been too limiting for the production of high levels of the enzyme. The more controlled conditions mediated by the use of the benchtop fermenter may also have contributed to the increased *N*-carbamoylase levels.

The biomass yields and enzyme activities obtained for each of the growth conditions are given in Table 2.1. Low biomass yields of 1.5g wet cell mass/L and 1.1g wet cell mass/L were obtained in the 400mL and 10L flask cultures, respectively. In wild-type cells, low biomass yields are often encountered since

maximum enzyme activity is obtained when cells are grown in media with growth-limiting nitrogen sources (Hartley *et al.*, 2001). The biomass yields of *A. tumefaciens* RU-ORPN1 obtained in the flask cultures, however, were lower than those reported in the literature for other flask cultures, which range from 6.8g/L for wet cells (Xu and West, 1994) to 4g/L for dry cells (Olivieri *et al.*, 1979). The biomass yields obtained with the PP2 growth medium in the benchtop fermenter were much higher than those obtained in the flask cultures, increasing to an average of 15g wet cell weight/L. This is in the range of biomass yields reported in the literature for cells grown in similar fementation vessels, which range from 6g/L (Morin *et al.*, 1995b) to 24g/L (George and Sadhukhan, 1996) for wet cells, and from 7.2g/L (Meyer and Runser, 1993) to 28g/L (Syldatk *et al.*, 1990b) for dry cells. A dry cell weight yield of 4g/L was obtained in the PP2 medium (Figure 2.3). Although this was lower than the dry cell weights reported in the literature for *Agrobacterium* sp., the high yields reported in the literature were only combined with high enzyme activities by continuous feeding of inducers throughout the growth of the organism (Meyer and Runser, 1993).

The enhanced biomass yields and enzyme activities obtained with the use of the PP2 medium and fermenter made this the best system for biomass and enzyme production. The development of the PP2 medium, however, occurred during the later stages of the project and therefore the majority of research covered in this thesis was conducted on cells grown in flask cultures, in minimal medium containing 0.01% casamino acids.

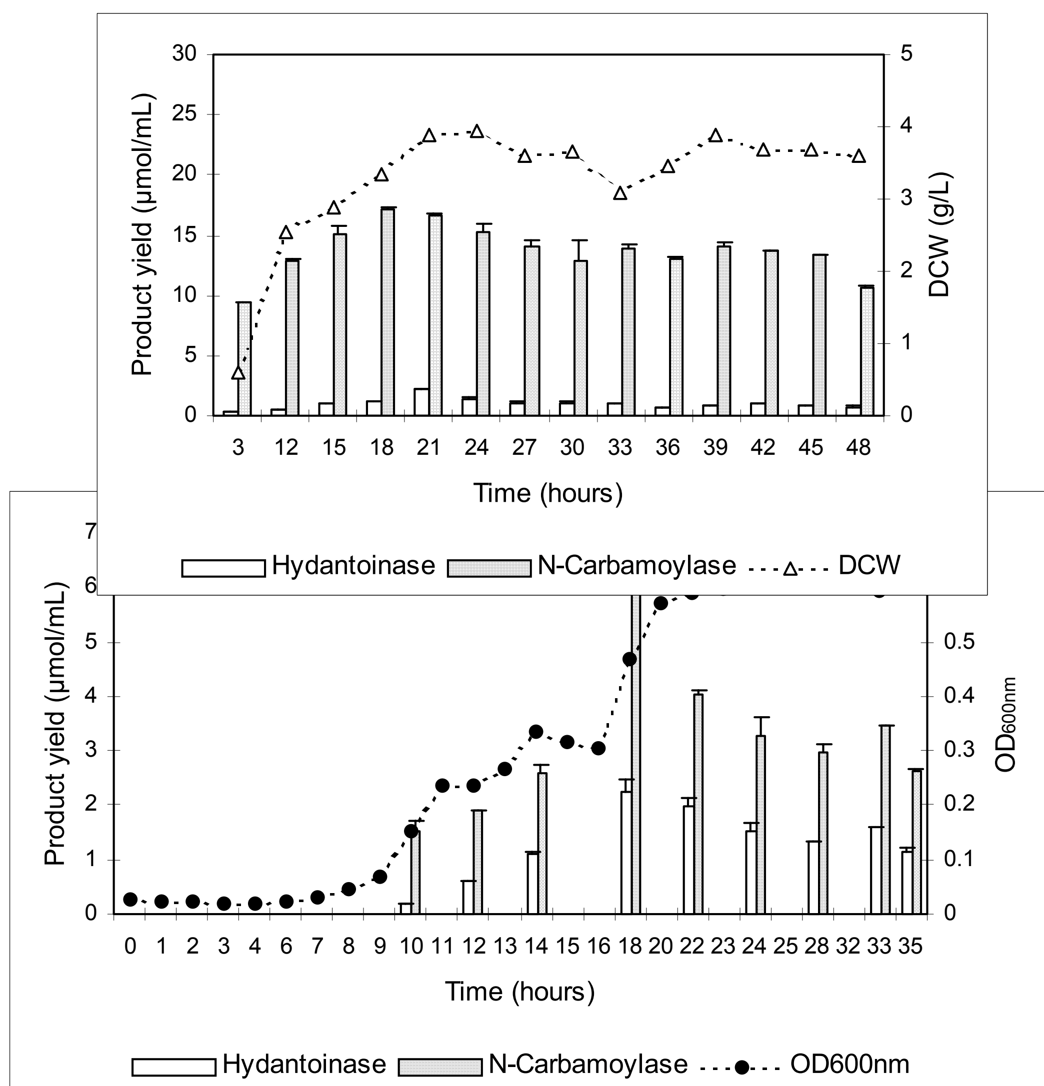


Figure 2.2 Growth curve of *A. tumefaciens* RU-ORPN1 in minimal medium containing 0.01% casamino acids in flask cultures.

Figure 2.3 Growth curve of *A. tumefaciens* RU-ORPN1 in PP2 growth medium cultivated in a 5L benchtop fermenter.

Table 2.1 *A. tumefaciens* RU-ORPN1 biomass yields and enzyme activities obtained under various growth conditions.

Growth conditions	Biomass yield (g cells (wet weight)/L)*	Hydantoinase product yield (: mol/mL)*	*N*-carbamoylase product yield (: mol/mL)*
400 mL flask cultures, MM + 0.01% CA	1.49 ± 0.21	2.05 ± 0.29	5.67 ± 0.73
10 L flask cultures, MM + 0.01% CA	1.08 ± 0.13	1.39 ± 0.78	4.80 ± 1.02
5 L fermenter, PP2 medium	15.00 ± 2.40	3.72 ± 1.74	16.48 ± 2.16

* Errors represent the SEM (n =9)

2.3.3 The effects of various growth media on the hydantoin-hydrolysing activity of *A. tumefaciens* RU-ORPN1

Some preliminary experiments were conducted at the start of the project to optimise the growth conditions of *A. tumefaciens* RU-ORPN1 for the production of hydantoin-hydrolysing activity. *A. tumefaciens* RU-ORPN1 flask cultures were grown in a number of different growth media to assess the effects of the growth media on the production of hydantoin-hydrolysing enzyme activity in the cells. The cells were grown in

minimal medium containing 1% hydantoin, minimal medium containing 0.01% casamino acids, nutrient broth, and nutrient broth containing 1% hydantoin. The hydantoinase and *N*-carbamoylase activities measured in the cells harvested from the various growth media are shown in Figure 2.4. Cells grown in the minimal media produced higher hydantoinase and *N*-carbamoylase activities in comparison to the complete media. This was expected, since hydantoin-hydrolysing cells grown in nitrogen limiting conditions generally produce higher enzyme activities (Hartley *et al.*, 2001). Furthermore, higher hydantoinase and *N*-carbamoylase activities were measured when 0.01% casamino acids was used as the nitrogen source in comparison to the use of hydantoin. This data is consistent with the results obtained for the wild-type strain, *A. tumefaciens* RU-OR, which also produced highest enzyme activity in minimal medium containing 0.01% casamino acids (Hartley *et al.*, 2001). This medium was therefore used for the growth of *A. tumefaciens* RU-ORPN1 in subsequent flask cultures.

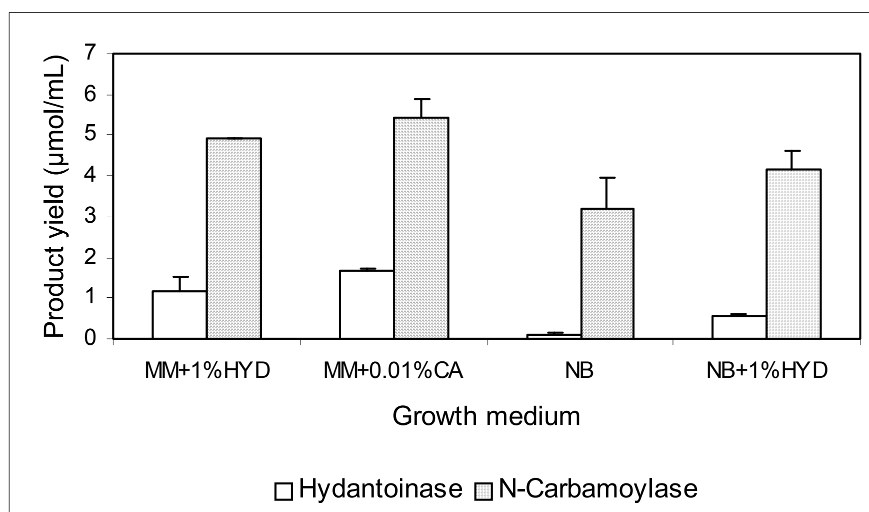


Figure 2.4 Effect of various growth media on the production of hydantoin-hydrolysing activity in *A. tumefaciens* strain RU-ORPN1(MM = minimal medium, CA = casamino acids, NB = nutrient broth, HYD = hydantoin).

2.3.4 Effect of the addition of metal ions to the growth medium on the hydantoin-hydrolysing activity in *A. tumefaciens* RU-ORPN1 flask cultures

The effect of the addition of metal ions to the growth medium on the production of hydantoin-hydrolysing enzyme activity was investigated as a further preliminary experiment for the optimisation of the hydantoinase and *N*-carbamoylase activities in *A. tumefaciens* RU-ORPN1 flask cultures. Magnesium and manganese sulphate were added to the growth medium at 1000ppm and 20ppm, respectively, since generally, hydantoinase enzymes have been shown to be activated by these metal ions. Similar levels of these metal ions added to growth media was shown to significantly promote the formation of hydantoin-hydrolysing enzymes in a *Pseudomonas* sp. (Yokozeki *et al.,* 1987c). The effect of the metal ions on the hydantoin-hydrolysing activity in *A. tumefaciens* RU-ORPN1 is shown in Figure 2.5. The hydantoinase product yield was slightly increased from 1.64: mol/mL to 1.93: mol/mL after growth in the presence of the elevated metal ion concentrations. The *N*-carbamoylase also showed higher activity after growth in the presence of the metals, with the product yield increasing from 4.56: mol/mL to 4.80: mol/mL. The levels of these metal ions in the growth medium were therefore adjusted accordingly in subsequent flask cultures. The effects of the addition of a broader range of metal ions to the enzyme extract on the enzyme activities was later investigated in more detail (Chapter 3, section 3.3.8).

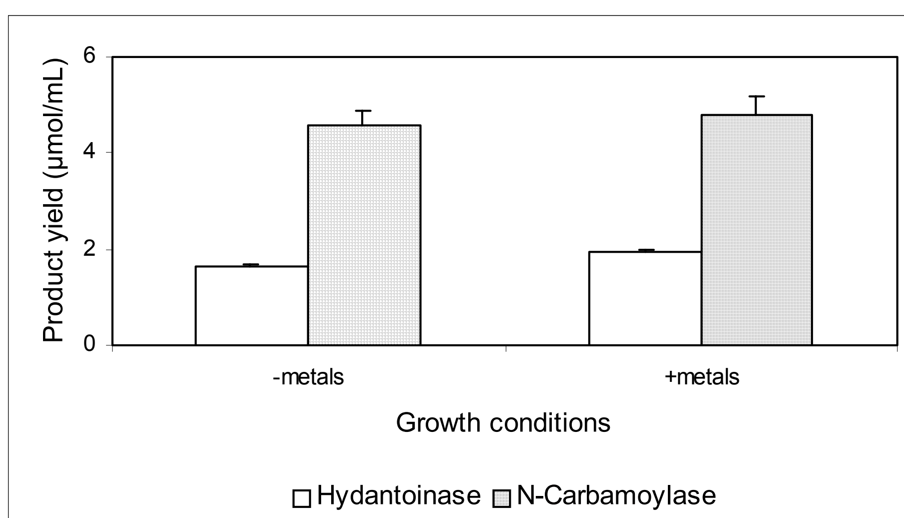


Figure 2.5 Investigation of the effect of the addition of Mg^{2+} and Mn^{2+} ions to the growth medium on the hydantoin-hydrolysing activity in *A. tumefaciens* RU-ORPN1.

2.3.5 Comparison of colorimetric and HPLC determinations of hydantoin-hydrolysing enzyme activities

The enzyme activities measured using the colorimetric Ehrlich's and Ninhydrin assays were verified by comparing the enzyme product yields measured colorimetrically with those obtained using HPLC. D,L-5-HPH (12.5mM) and NC-HPG (12.5mM) were used as substrates in resting cell assays and the hydantoinase and *N*-carbamoylase enzyme activities were determined using the two assay techniques. The enzyme activities measured are shown in Table 2.2. Some discrepancy was revealed by the data generated, with the HPLC determinations indicating higher enzyme activities. The enzyme activity measured for the *N*-carbamoylase by HPLC was three times higher than the activity measured using the Ninhydrin assay. This discrepancy can only be attributed to a difference in the sensitivity of the two techniques. Despite this discrepancy, each method was consistent. Hydantoin and NCG were much more readily available and economical than the substrates required for the HPLC method and therefore, the colorimetric assay was used throughout the research for relative activity measurement. Furthermore, it

should be noted that the HPLC determination gave the higher HPG measurement and therefore the colorimetric method was at least not an overestimation of the concentrations present.

Table 2.2 Comparison of HPLC and colorimetric determinations of hydantoinase and *N*-carbamoylase activities.

Enzyme	Colorimetric determination	HPLC determination
Hydantoinase product yield (: mol/mL)*	0.23 ± 0.03	0.39 ± 0.05
N*-Carbamoylase product yield (: mol/mL)	1.40 ± 0.10	4.20 ± 0.12

* Errors represent SEM (n=3)

2.4 CONCLUSIONS

The inducer-independence of *A. tumefaciens* RU-ORPN1 was one of the primary aspects of interest for the development of an industrial biocatalyst and it was shown that this strain could produce levels of enzyme activity similar to the wild-type strain without the presence of an inducer. In particular, the high levels of *N*-carbamoylase activity produced by the strain in the absence of an inducer are of industrial importance, since the induction of bacterial strains in industrial processes adds an additional step to the process, and elevates costs. Furthermore, the relatively low levels of *N*-carbamoylase activity in strains possessing both hydantoinase and *N*-carbamoylase enzymes (Kim and Kim, 1995) and the relative instability of *N*-carbamoylases has hampered the introduction of these strains into industrial processes for the production of amino acids. The high levels of *N*-carbamoylase produced by *A. tumefaciens* RU-ORPN1 therefore make it, potentially, an ideal strain for the development of a biocatalyst for the one-step industrial production of amino acids.

Preliminary experiments, conducted for the optimisation of the hydantoin-hydrolysing enzyme activity in *A. tumefaciens* RU-ORPN1 flask cultures, indicated that minimal medium containing 0.01% casamino acids and high concentrations of magnesium and manganese sulphate gave the best enzyme activities. The biomass yields obtained were significantly increased by fermentation of the strain in PP2 growth medium. Growth of *A. tumefaciens* RU-ORPN1 in this medium also resulted in the doubling of the *N*-carbamoylase activity in the cells. The high *N*-carbamoylase activity produced relative to the hydantoinase

activity, suggested that the *N*-carbamoylase could be combined with a hydantoinase having higher activity, in order to develop an efficient one-step process for the production of amino acids.

Yields of cells and cellular enzyme activity levels of cells grown in flask cultures and by fermentation were found to vary considerably over the course of this work. Variability in enzyme activities has been experienced by other researchers working with hydantoin-hydrolysing enzymes, as well as by those working with other biological systems, and variability in enzyme activity is one of the draw-backs of using biological systems in industrial processes as apposed to chemical methods. Furthermore, growth of the cells during the long enzyme reaction times may have resulted in changes in the catalyst concentration. The OD of the assay could have been measured in order to exclude the effect of growth during the reaction. However, over the course of this research, experiments were conducted using single batches of cells, and treated cells, exposed to various experimental conditions, were always compared with untreated control cells from the same batch. The relative effects of the various experimental conditions could therefore be accurately compared despite batchwise variability in enzyme activity.

CHAPTER 3

PRODUCTION OF ENZYME EXTRACT AND OPTIMISATION OF ENZYME REACTION CONDITIONS

3.1 INTRODUCTION

One of the objectives of this research was to purify and biochemically characterise the hydantoinase and *N*-carbamoylase enzymes from *A. tumefaciens* RU-ORPN1.Since the purification of the enzymes was not achieved, as described in Chapter 4, biochemical characterisation of the enzymes was not possible. This chapter focussed on firstly, the development of a set of conditions under which an enzyme extract having high hydantoin-hydrolysing activity could be consistently produced, and secondly, the enzyme reaction conditions giving maximum product yield had to be identified. It should be noted here that the hydantoinase and *N*-carbamoylase activities described in this chapter are simply measured by the quantity of product yielded by each enzyme under specific reaction conditions and the optimal enzyme reaction conditions were those achieving maximum conversion of the substrates. Therefore the use of the term "enzyme activity" does not refer to the measurement of an amount of enzyme but to a quantity of product yielded by the enzyme, with the aim of process development.

The methods evaluated for the production of the enzyme extract were sonication and french-pressing, used both separately and combined. Most enzyme extracts reported in the literature are produced using one of these methods, followed by removal of the cell debris by centrifugation. The resulting supernatant is then designated as the enzyme extract. In this work, as will be discussed in more detail in Chapter 4, the major portion of the hydantoin-hydrolysing activity was found in the cell debris. For this reason, the debris was not removed and the enzyme extract comprised the supernatant and cell debris. Initially, a number of experiments were carried out to compare the enzyme product yields obtained using extracts produced from freshly harvested cells and frozen cells. Various enzyme reaction conditions were then investigated in order to establish the conditions for optimal hydantoinase and *N*-carbamoylase product yields. The apparent pH and temperature optima of the enzymes under the optimised reaction conditions were identified and the metal dependence of the enzymes was evaluated. The effects of various additives to the enzyme extract on the hydantoinase and *N*-carbamoylase product yields were also determined in order to further optimise the reaction conditions. The substrate specificities and thermostabilities of the enzymes in the enzyme extract were assessed. Finally, the stability of the enzymes under various storage conditions was investigated to establish the conditions under which the hydantoinase and *N*-carbamoylase activities could be most effectively conserved.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma Chemical Co. Dithiothreitol (DTT), phenylmethanesulphonylfluoride (PMSF) and adenosine triphosphate (ATP) were purchased from Boeringer Mannheim. All reagents were analytical grade.

3.2.2 Methods

3.2.2.1 Comparison of hydantoin-hydrolysing enzyme activities in freshly harvested cells and in frozen cells

Hydantoinase and *N*-carbamoylase activities were assayed in freshly harvested cells and in cells frozen overnight using resting whole cell assays as described in Chapter 2, section 2.2.2.3.

3.2.2.2 Storage stability of the hydantoin-hydrolysing activity in frozen cells

Freshly harvested cells were aliquoted into five cell pellets, frozen and stored at -20^{0}C. The cells were assayed for residual hydantoinase and *N*-carbamoylase activities after 0, 7, 14, 21 and 28 days using resting whole cells assays as described in Chapter 2, section 2.2.2.3.

3.2.2.3 Preparation of the enzyme extract by french-pressing of fresh and frozen cells

Freshly harvested cells and frozen cells were resuspended (40mg/mL) in cold potassium phosphate buffer (0.1M, pH 9) and disrupted by passing the cell suspensions through a Yeda-Press (LINCA Lamon Instrumentations Co., Israel) at 15kPa and 4^{0}C. The hydantoinase and *N*-carbamoylase activities in the resulting enzyme extracts were compared with those in the non-disrupted cells.

3.2.2.4 Preparation of enzyme extracts by sonication of fresh and frozen cells

Freshly harvested cells and frozen cells were resuspended (40mg/mL) in cold potassium phosphate buffer (0.1M, pH 9) and disrupted on ice by sonication (10sec blasts, followed by 10 sec rests) for 5, 10 and 15 minutes using a Vibria cell sonicator (Sonics and Materials Inc., USA). The hydantoinase and *N*-carbamoylase enzyme activities in the resulting enzyme extracts were compared with those in the non-disrupted cells.

3.2.2.5 Preparation of enzyme extracts by french-pressing and sonication of fresh and frozen cells

Freshly harvested cells and frozen cells were resuspended (40mg/mL) in cold potassium phosphate buffer (0.1M, pH 9) and disrupted by passing the cell suspensions through an ice cold french-press at 15kPa, followed by sonication for 5, 10 and 15 minutes. The hydantoinase and *N*-carbamoylase activities in the resulting extracts were compared with those in the non-disrupted cells.

3.2.2.6 Determination of hydantoinase and *N*-carbamoylase product yields in enzyme extracts

Enzyme extract (1mL) was diluted 1/2 in potassium phosphate buffer (0.1M, pH 9) containing hydantoin or NCG as substrates to give final substrate concentrations of 50mM and 25mM, respectively, in a 2mL volume. Triplicate reactions were carried out for each substrate at 40^0C for 6 h at 200rpm, together with controls containing enzyme extract or substrate only. Following incubation, reaction supernatants were collected by centrifugation. The supernatants were assayed for NCG and glycine concentrations using colorimetric assays as described in Chapter 2, section 2.2.2.3. Hydantoinase product yield is expressed as the sum of the NCG (: mol/mL) and glycine (: mol/mL) concentrations produced from 50mM hydantoin after incubation at 40^0C for 6 h at 200rpm in a 2mL volume. *N*-carbamoylase product yield is expressed as the glycine concentration (: mol/mL) produced from 25mM NCG after incubation at 40^0C for 6 h at 200rpm in a 2mL volume. All enzyme activities are reported as mean values from triplicate reactions and error bars represent SEM (n=3, except where otherwise stated). All spectrophotometric measurements were made using a Shimadzu UV-160A spectrophotometer.

3.2.2.7 Comparison of optimal enzyme reaction times for the hydantoin-hydrolysing enzymes in extracts from fresh and frozen cells

Freshly harvested cells and frozen cells were resuspended (40mg/mL) in cold potassium phosphate buffer (0.1M, pH 9) and disrupted on ice by sonication (10sec blasts, followed by 10 sec rests) for 5 minutes. The hydantoinase and *N*-carbamoylase product yields in the enzyme extracts were then measured as described in section 3.2.2.6, except that the NCG and glycine concentrations in the reaction supernatants were measured after 2, 4 and 6 h.

3.2.2.8 Comparison of the storage stabilities of the enzyme extracts produced from fresh and frozen cells

Freshly harvested cells and frozen cells were resuspended (40mg/mL) in cold potassium phosphate buffer (0.1M, pH 9) and disrupted on ice by sonication (10sec blasts, followed by 10 sec rests) for 5 minutes. The extracts were then stored at 5^0C, -20^0C and -80^0C for 7 days. Following storage, the residual hydantoinase and *N*-carbamoylase activities in the extracts were assayed.

3.2.2.9 Determination of the apparent pH optima for the hydantoin-hydrolysing enzymes in the enzyme extract

Frozen cells were resuspended (40mg/mL) in cold potassium phosphate buffer (0.1M, pH 9) and disrupted on ice by sonication (10sec blasts, followed by 10sec rests) for 5 minutes. All enzyme extracts were prepared by this method in subsequent experiments. The hydantoinase and *N*-carbamoylase product yields in the enzyme extract were measured as described in section 3.2.2.6, except that the enzyme reactions were carried out at various pH levels by making use of the following buffers: 100mM potassium phosphate buffer, pH 5-8; 100mM Tris-HCl buffer, pH 8-9 and 100mM sodium carbonate buffer pH 9-10.

3.2.2.10 Determination of the apparent temperature optima for the hydantoin-hydrolysing enzymes in the enzyme extract

The hydantoinase and *N*-carbamoylase product yields in the enzyme extract were measured as described in section 3.2.2.6, except the enzyme reactions with the substrates were carried out at 30^0C, 40^0C, 50^0C and 60^0C.

3.2.2.11 Effect of metal ions on the hydantoin-hydrolysing activity in the enzyme extract

EDTA was added to the enzyme extract (50mL) to give a final concentration of 1 mM EDTA. The enzyme/EDTA solution was stirred slowly on ice for 1 h. A sample (7mL) was removed for measurement of enzyme product yields. The solution was then dialysed for 3 h against cold potassium phosphate buffer (0.1M, pH 9) at 4^0C. A further sample (7mL) was removed for measurement of enzyme product yields. The hydantoinase and *N*-carbamoylase productivities in the remaining dialysed enzyme extract were then measured in the presence of cobalt, magnesium, manganese, copper and zinc sulphates, added separately, to give final metal ion concentrations of 2.5mM.

3.2.2.12 Effects of ATP, DTT and PMSF on the enzyme activity in the enzyme extract

The effects of the addition of ATP, DTT and PMSF on the enzyme product yields in the enzyme extract were determined by measuring the product concentrations yielded in the presence of each of the additives. The product yields were measured in the presence of 0, 0.1, 0.5, 1.0, 5.0 and 10mM DTT; 0, 0.1, 0.5, 1.0, 5.0 and 10mM PMSF and 0, 0.1, 0.5, 1.0, 5.0, 10 and 20mM ATP.

3.2.2.13 Comparison of hydantoin and NCG, and D,L-5-HPH and NC-HPG, as substrates for the hydantoinase and *N*-carbamoylase in the enzyme extract

The enzyme extract was assayed for hydantoinase and *N*-carbamoylase activities as described in section 3.2.2.6, except that final hydantoin and NCG concentrations of 12.5mM were used in the enzyme reactions. Under these conditions, the hydantoinase product yield is expressed as the sum of the NCG (: mol/mL) and glycine (: mol/mL) concentrations produced from 12.5mM hydantoin after incubation at 40^{0}C for 6 h at 200rpm in a 2mL volume. The *N*-carbamoylase product yield is expressed as the concentration of glycine (: mol/mL) produced from 12.5mM NCG after incubation at 40^{0}C for 6 h at 200rpm in a 2mL volume. The same enzyme extract was also assayed as above, except final D,L-5-HPH and NC-HPG concentrations of 12.5mM were used as substrates. Under these conditions, hydantoinase product yield is expressed as the sum of the NC-HPG (: mol/mL) and D-HPG (: mol/mL) concentrations produced from 12.5mM D,L-5-HPH after incubation at 40^{0}C for 6 h at 200rpm in a 2mL volume. The *N*-carbamoylase product yield is expressed as the concentration of D-HPG (: mol/mL) produced from 12.5mM NC-HPG after incubation at 40^{0}C for 6 h at 200rpm in a 2mL volume.

3.2.2.14 Evaluation of the stability of the hydantoin-hydrolysing activity in the enzyme extract at 25^{0}C and at 40^{0}C over 24 hours

Enzyme extracts were incubated at 25^{0}C and 40^{0}C. The residual hydantoinase and *N*-carbamoylase productivities in each extract were measured after 0, 2, 4, 6, 8 and 24 h.

3.2.2.15 Evaluation of the effect of freeze-drying on the activity in the enzyme extract

The hydantoinase and *N*-carbamoylase product yields in a freshly prepared enzyme extract were determined, after which, a sample of the extract was subjected to freeze-drying. The freeze-dried extract was then resuspended in the original volume of potassium phosphate buffer (0.1M, pH 9) and the

hydantoinase and *N*-carbamoylase productivities were measured. The effect of freeze-drying was determined by comparing the enzyme reaction product yields before and after freeze-drying.

3.2.2.16 Storage stability of the hydantoin-hydrolysing activity in freeze-dried extracts at -20^{0}C and at 25^{0}C

Samples of freeze-dried extract were stored at 25^{0}C and at -20^{0}C for 28 days. The extracts were resuspended in potassium phosphate buffer (0.1M, pH 9) at the original concentrations, after 0, 7, 14, 21 and 28 days, and the residual hydantoinase and *N*-carbamoylase activities were assayed.

3.3 RESULTS AND DISCUSSION

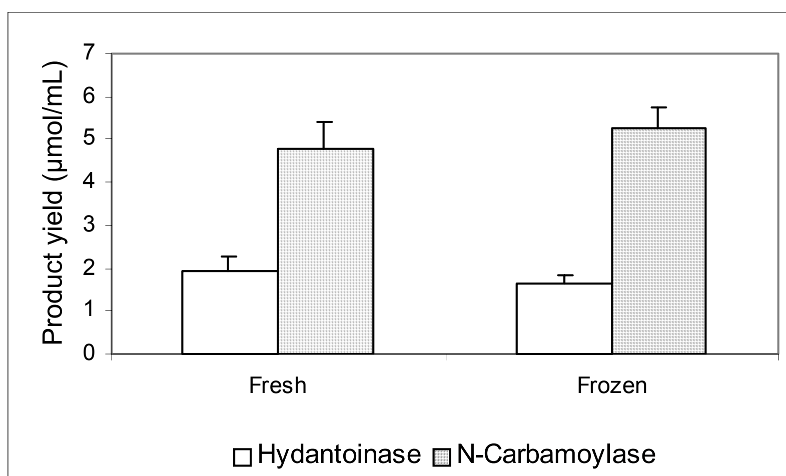
3.3.1 Comparison of the hydantoin-hydrolysing enzyme activity in freshly harvested cells and in frozen cells

The hydantoinase and *N*-carbamoylase activities were measured in freshly harvested cells and in the same cells after freezing at -20^{0}C overnight in order to determine the effect of freezing on the enzyme activities (Figure 3.1). The hydantoinase product yield appeared to decrease slightly after freezing showing a possible sensitivity to the freeze-thaw treatment. The *N*-carbamoylase product yield was found to increase after freezing cells. This could have been due to the possible breakdown of the bacterial cell wall structure during freezing and thawing, resulting in improved substrate transport in and out of the cell. This is consistent with the findings of Lee *et al.* (2001a), who have reported that cell membranes have a very low permeability to *N*-carbamyl derivatives and therefore, when these derivatives are used as substrates for the *N*-carbamoylase enzyme, the enzyme activity is higher when disrupted cells are used. With part of the focus in this project being on the optimisation of the *N*-carbamoylase product yield, in particular, the use of frozen cells which had enhanced *N*-carbamoylase activity was the preferred choice.

Figure 3.1 Hydantoinase and *N*-carbamoylase product yields in freshly harvested cells and in frozen cells.

3.3.2 Storage stability of the hydantoin-hydrolysing activity in frozen cells

During the later stages of the project, fermentations resulted in large yields of cells which at times had to be stored before use. Storage of cells at -20^{0}C is generally used amongst researchers working with hydantoin-hydrolysing bacterial strains (Chen *et al.*, 1999; Luksa *et al.*, 1997; Xu and West, 1994; Morin *et al.*, 1986) . The length of the storage periods and the effect of storage on the hydantoinase and *N*-carbamoylase activities, however, have not been reported. The residual hydantoinase and *N*-carbamoylase activities in frozen cells were determined over a 28-day period in order to assess the storage stability of the enzymes at -20^{0}C (Figure 3.2).



The hydantoinase product yield showed some variation over the storage period, increasing initially to 172% of the original value over the first 14 days and then decreasing to 80% of the original value over the

following 14 days. The *N*-carbamoylase product yield remained relatively stable over the first 21 days of storage and then decreased to 51% of the original value over the last 7 days. Both enzymes therefore retained almost 100% activity after 3 weeks storage, and accordingly frozen cells were not stored beyond this length of time in subsequent experiments.

The increase in the hydantoinase activity during the storage period could be attributed to the possible sensitivity of an inhibitor of the enzyme to the storage conditions resulting in enhanced activity of the enzyme after storage. Alternatively, the activity increase may have been due to the breakdown of the cell wall due to the freeze-thaw process, resulting in improved mass transfer of the substrate into the cell. However, Lee *et al.* (2001a) reported that when hydantoin was used as substrate, higher hydantoinase activities were obtained with intact cells due to the concentration of the *N*-carbamyl intermediate in the cell, and disrupted cells produced lower hydantoinase activity due to the dilution of the *N*-carbamyl intermediates into the bulk phase. The reasons for the increased hydantoinase activity in this study are therefore not obvious. Increased hydantoinase activities in cells and extracts after storage have been observed by other researchers working with these enzymes from a *Pseudomonas* sp. (S. Kirchmann, *pers. comm.*).

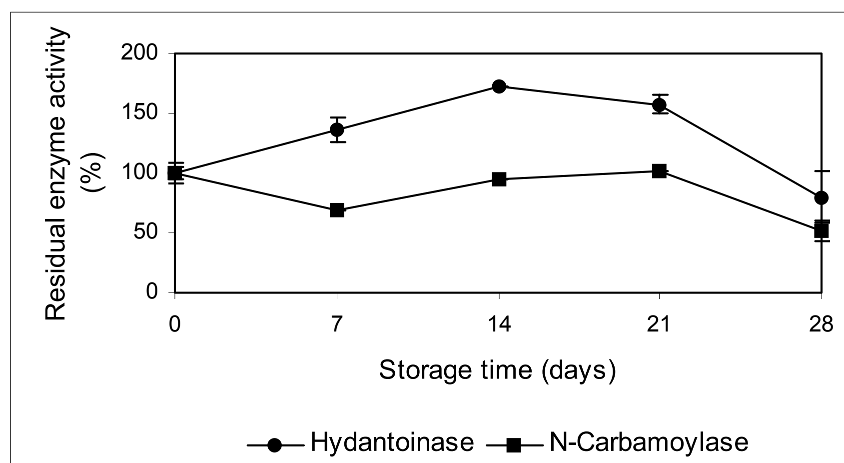


Figure 3.2 Storage stability of the hydantoin-hydrolysing enzyme activity in frozen cells at -20^{0}C.

3.3.3 Effects of sonication and french-pressing on the hydantoin-hydrolysing activity in enzyme extracts from fresh and frozen cells

The hydantoinase and *N*-carbamoylase product yields were measured in enzyme extracts produced from freshly harvested cells and from frozen cells to determine whether the condition of the cells would affect the enzyme activity levels in the extracts produced. French-pressing, sonication and the two techniques combined were evaluated as methods for cell lysis in order to determine which method would provide the enzyme extract with the highest enzyme activity. The enzyme product yields measured in the various enzyme extracts, expressed as percentages of the original product yields in the untreated cells, are shown in Figure 3.3. French-pressing resulted in a 34% decrease in the hydantoinase product yield in fresh cells without affecting the *N*-carbamoylase product yield. However, after french-pressing of frozen cells, the *N*-carbamoylase product yield dropped by 16% with no apparent effect on the hydantoinase product yield. The use of french-pressing for the lysis of *Agrobacterium* sp. (Olivieri *et al.*, 1981; Runser and Meyer, 1993; Durham and Weber, 1995) and *Bacillus* sp. (Lee *et al.*, 1995) having hydantoin-hydrolysing activity has been reported in the literature, but the effect of french-pressing on the enzyme activities in these strains was not reported.

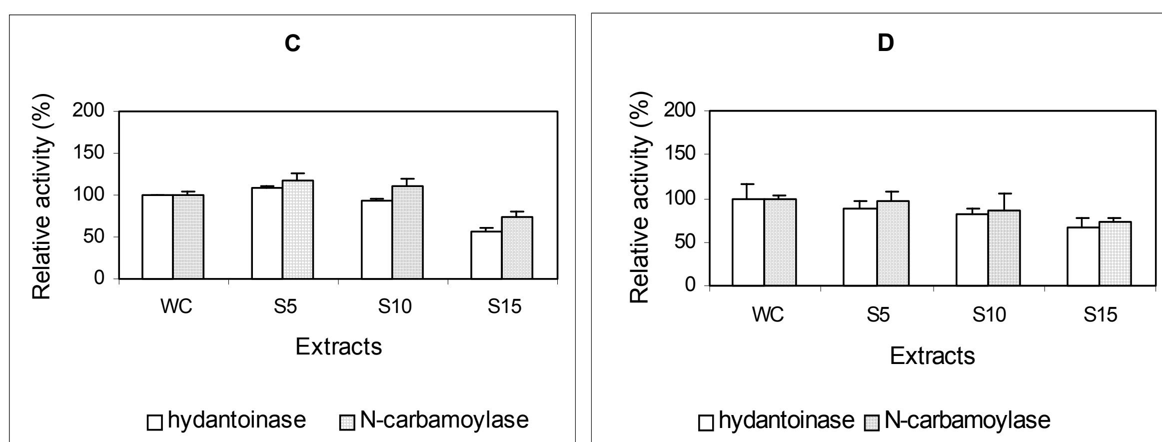
After 5 minutes sonication, slightly increased product yields for both hydantoinase and *N*-carbamoylase enzymes were observed in the extract from fresh cells. The extract produced by the same treatment from frozen cells exhibited a slightly lowered hydantoinase product yield and no apparent change in the *N*-carbamoylase product yield. Sonication has been used for the lysis of *Agrobacterium* sp. (Sareen *et al.*, 2001), *Bacillus* sp. (Sharma and Vohra, 1997; Luksa *et al.*, 1997), *Pseudomonas* sp. (Morin *et al.*, 1986; Xu and West, 1994; Ogawa *et al.*, 1995a;), *Comamonas* sp. (Ogawa *et al.*, 1993), *Blastobacter* sp. (Ogawa *et al.*, 1994b) and *Arthrobacter* sp. (Moller *et al.*, 1988), but the effect of sonication on the enzyme activities in these strains was not reported. The sonication times reported ranged from 5 minutes for a *Peudomonas* sp. (400mg cells/mL) and *Bacillus* sp. (100mg cells/mL) to 2 hours for the *Comamonas* sp. (330mg cells/mL).

The combination of french-pressing and sonication was too denaturing for the effective release of active enzymes from the cells. The hydantoinase and *N*-carbamoylase product yields decreased by 40% and 18%, respectively, after french-pressing and 5 minutes sonication of fresh cells, while the same treatment of frozen cells resulted in a 30% decrease in the *N*-carbamoylase product yield. The combination of french-pressing and sonication for the lysis of hydantoin-hydrolysing cells has not been reported in the literature. This method was tried here in order to investigate whether the combined effect of the methods would release more enzyme activity from the cells.

For all three of the cell lysis methods tested, the use of frozen cells generally appeared to result in lower *N*-carbamoylase product yields, in particular, than the fresh cells. This could have been due to the partial disruption of the frozen cells by the freeze-thaw process, resulting in more rapid disruption of these cells and therefore longer exposure of the enzymes to the adverse effects of the various cell lysis treatments. The relative instability of *N*-carbamoylases in comparison to hydantoinases may also have resulted in the lower enzyme activities.

Sonication produced extracts with the highest enzyme product yields and was therefore chosen as the best method for the production of the enzyme extract. Although 5-minute sonication of fresh cells resulted in slightly increased enzyme activity levels (Figure 3.3), the activity produced in non-disrupted frozen cells was higher than in non-disrupted fresh cells (Figure 3.1). Disruption of frozen cells by 5 minutes sonication

therefore produced the enzyme extract with the highest activity. In addition, the use of frozen cells did not adversely affect the rate of the enzyme reaction or the storage stability of the enzymes, which will be discussed in sections 3.3.4 and 3.3.5 and therefore, cell lysis by 5-minute sonication of frozen cells was chosen as the standard method for the production of the enzyme extract throughout.



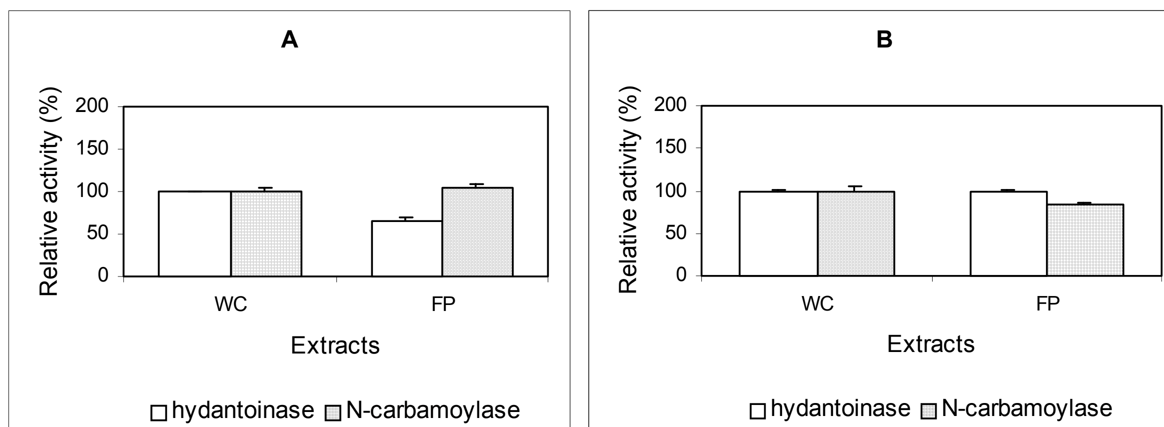
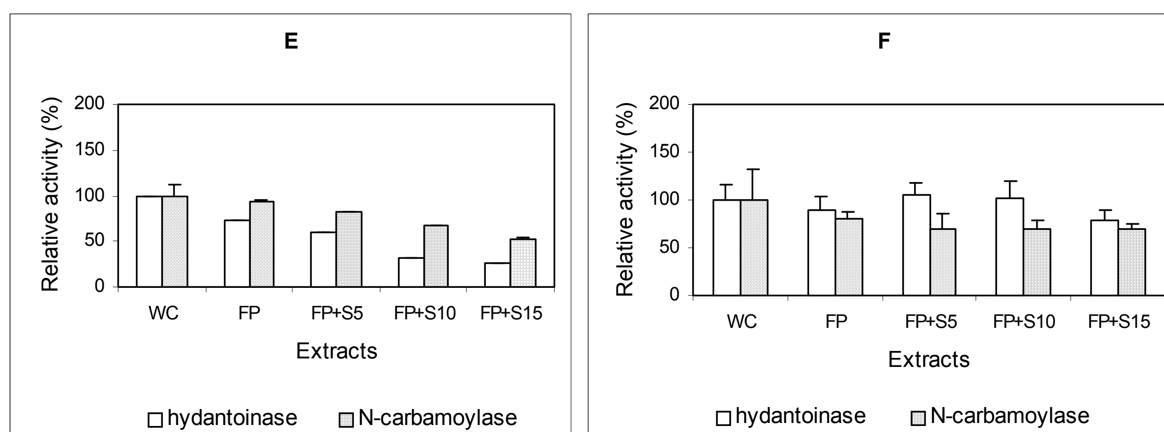


Figure 3.3 Comparison of enzyme extracts produced by french-pressing (**A** and **B**), sonication (**C** and **D**) and combined french-pressing and sonication (**E** and **F**) from fresh (**A**, **C** and **E**) and frozen cells (**B**, **D** and **F**). WC = whole cells; FP = french-pressed; S5 = sonicated 5 minutes; S10 = sonicated 10 minutes; S15 = sonicated 15 minutes.

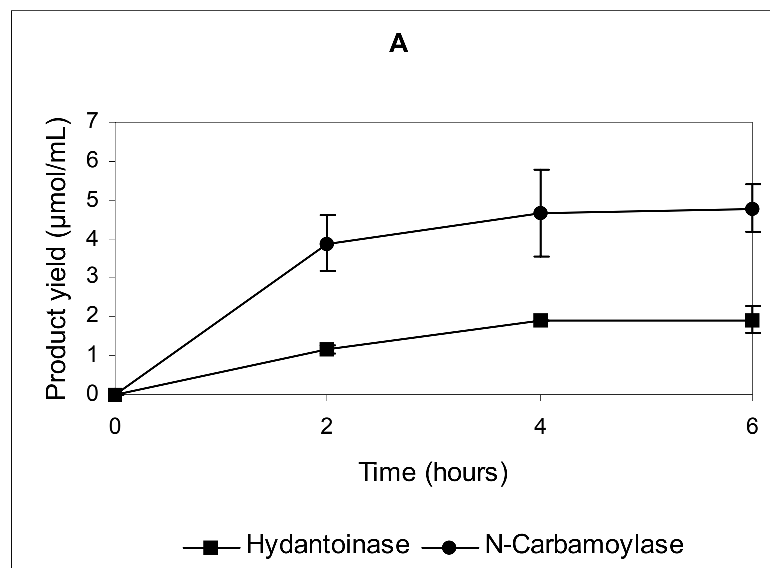
3.3.4 Optimal reaction time for the hydantoin-hydrolysing enzymes in the enzyme extracts from fresh and frozen cells

The optimal enzyme reaction time for maximum hydantoinase and *N*-carbamoylase product yields in *A.*



tumefaciens RU-OR resting cells was determined to be 6 hours by Hartley *et al.* (1998). The product yields for the hydantoinase and *N*-carbamoylase in the enzyme extracts from fresh and frozen cells were determined over shorter time intervals in order to assess the relative product yields of the enzyme reactions in the two extracts, and to determine the optimum enzyme reaction times for the extracts produced. The product yields measured for the enzymes in extracts, produced by 5-minute sonication of freshly harvested cells and of frozen cells, were determined after reacting the extracts with the substrates for 2, 4 and 6 h (Figure 3.4). The hydantoinase and *N*-carbamoylase product yields in the extract from fresh

cells were highest after 4 and 6 hours, respectively, although very little increase in the product yield was observed after 4 hours for both enzymes. Similarly, maximum product yields were measured after 6 hours for the hydantoinase and *N*-carbamoylase in the extract from frozen cells, although very little increase in product yields were observed after 4 hours. Enzyme reactions were therefore conducted over 6 h reaction times and extracts from frozen cells were used throughout the duration of this project unless stated otherwise.



The product yields obtained from the enzyme extracts after 6 h did not reflect a complete conversion of the substrates, indicating that the enzyme titre in the reaction may have been insufficient for complete conversion of the substrate within the 6 hours. Alternatively, the enzymes were unstable under the reaction conditions and denaturing of the enzymes resulted in a progressive loss of the enzyme activities during the reaction, and therefore, the thermostabilities of the enzymes were assessed later (section 3.3.12).

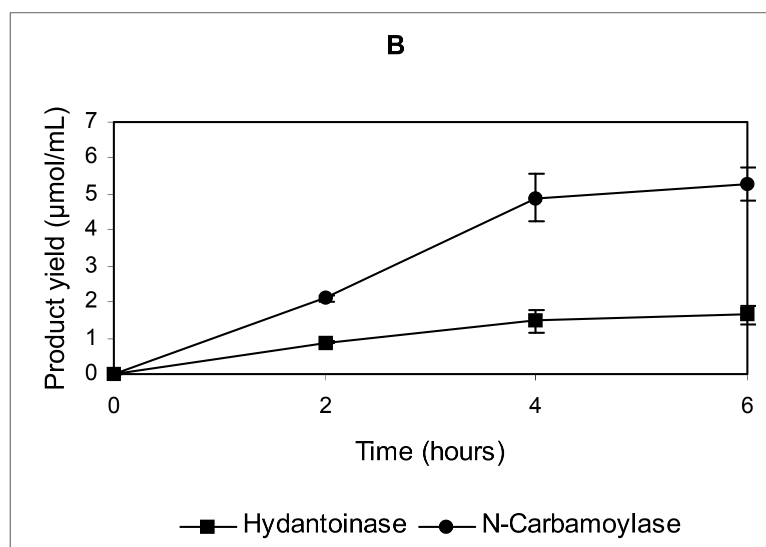


Figure 3.4 Hydantoinase and *N*-carbamoylase product yields assayed in enzyme extracts from fresh (**A**) and frozen (**B**) cells over 6 h.

3.3.5 Comparison of the storage stability of the enzyme extracts from fresh and frozen cells

The hydantoinase and *N*-carbamoylase reaction product yields measured in enzyme extracts produced by 5-minute sonication of freshly harvested cells and frozen cells were compared after storage in order to compare the storage stabilities of the hydantoin-hydrolysing enzymes in the extracts. The relative product yields measured in the enzyme extracts after storage at various temperatures are shown in Figure 3.5. The enzymes in both extracts stored at 4^0C retained high levels of activity after the 7-day storage period with the enzymes in the extract from fresh cells retaining slightly higher activity than those from frozen cells, probably due to a slight sensitivity of the enzymes to the freeze-thaw process. In particular, the hydantoinase enzyme appeared to be sensitive to freezing, since this enzyme from frozen cells showed the largest drop in activity, decreasing to 74%. The sensitivity of the hydantoinase enzyme to freezing was even more pronounced in the extracts stored at -20^0C and at -80^0C. The hydantoinase activity in both extracts decreased significantly after storage at these temperatures, particularly at -20^0C.

The decrease in the hydantoinase activity after freezing could be due to a possible sensitivity to the freeze-thaw process, similar to that observed in section 3.2.1, particularly in the case of the extract produced from frozen cells, since these enzymes would have been exposed to freezing twice. Freezing was also reported to result in the inactivation of this enzyme in a *Arthrobacter* sp. (Siemann *et al.*, 1999).

The *N*-carbamoylase activity was relatively stable in comparison to the hydantoinase and other *N*-carbamoylases reported in the literature. The enzyme retained more than 80% activity in both extracts at 4^0C and -20^0C and retained more than 70% activity after storage at -80^0C. Ogawa *et al.* (1993) reported a 50% loss in activity of the *N*-carbamoylase purified from a *Comamonas* sp. after 8 days storage at 4^0C. The *N*-carbamoylase from *A. radiobacter* was also found to be fairly unstable, retaining only 20% of its activity after 14 days storage at 4^0C (Buson *et al.*, 1996). Generally, crude or purified hydantoinases and *N*-carbamoylases reported in the literature are stored at 4^0C (Louwrier and Knowles, 1996; Luksa *et al.*, 1997; Sareen *et al.*, 2001), while resting cells are stored at -20^0C (Morin *et al.*, 1986; Xu and West, 1994; Luksa *et al.*, 1997). Due to the sensitivity of the hydantoinase enzyme in the extracts to freezing, the enzyme extract was prepared immediately prior to use for all subsequent experiments.

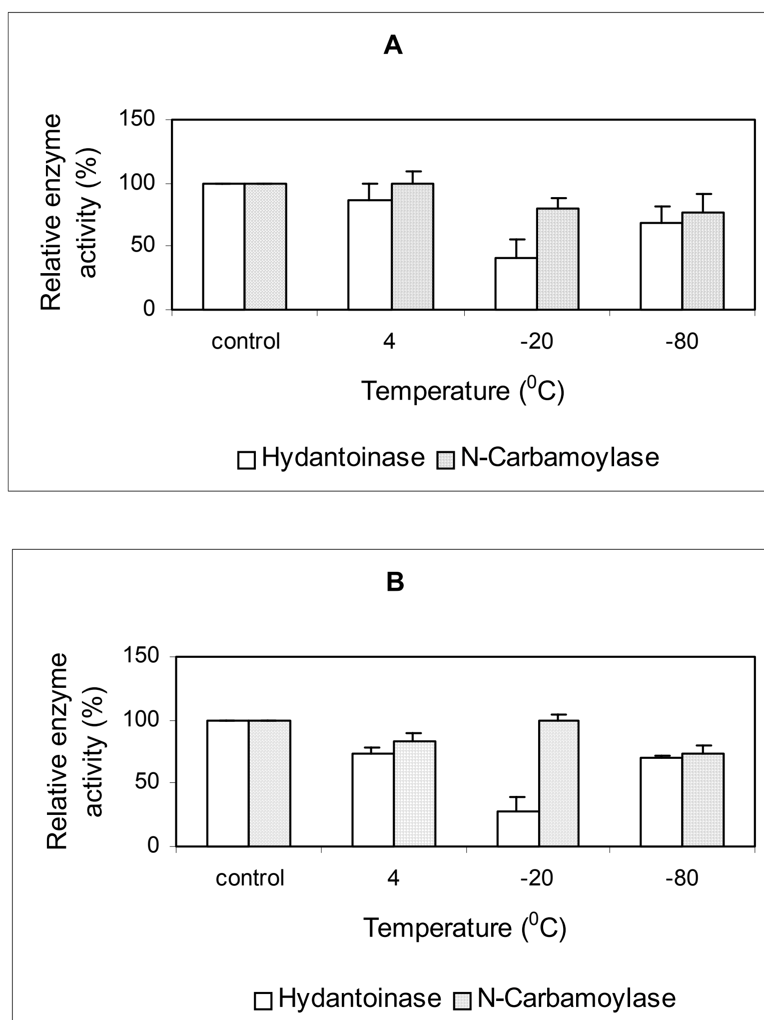


Figure 3.5

Enzyme activities in the enzyme extracts produced from fresh (**A**) and frozen (**B**) cells after storage relative to the enzyme activities in the extracts before storage (control).

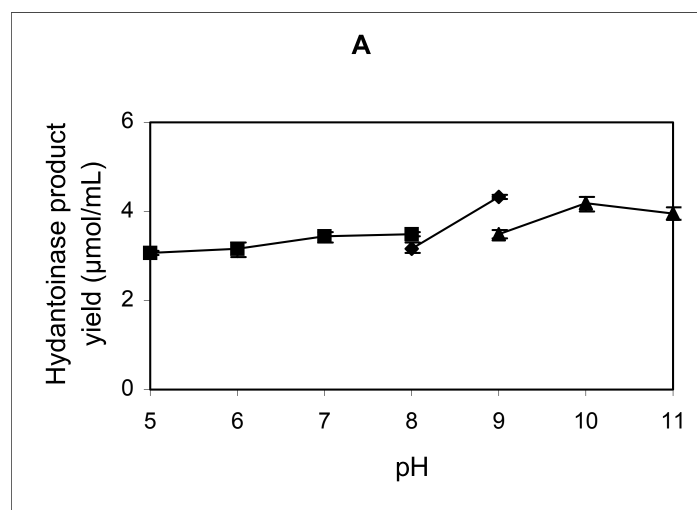
3.3.6 Determination of the apparent pH optima for the hydantoin-hydrolysing enzymes in the enzyme extract

Fresh preparation of the enzyme extract, produced by sonication of frozen cells for 5 minutes, was chosen as the standard method for the production of the enzyme extract as a result of the previous experiments. Highest product yields were produced by the enzymes in this extract after 6 hours incubation with the relevant substrates. The following sections describe the investigation of various conditions for the optimisation of the enzyme reaction yields in the enzyme extract.

This section describes the investigation of the apparent pH optima for the hydantoinase and *N*-carbamoylase enzymes in the enzyme extract. The hydantoinase and *N*-carbamoylase product yields in *A. tumefaciens* RU-OR resting cells were previously both found to be optimal at pH 9 (Burton *et al.*,1998) and this pH was therefore used for the *A. tumefaciens* RU-ORPN1 resting cell assays. The apparent pH optimum of the hydantoinase in the extract was pH 9 to 10 (Figure 3.6), although the product yield of this enzyme was not significantly affected by pH change over a range of pH from pH 5 to pH 11. Similarly, the *N*-carbamoylase product yield was not significantly affected by pH change in reactions carried out from pH 5 to pH10, but was highest at pH 9 (Figure 3.6). The apparent pH optima of the enzymes in the extract therefore remained the same as for the wild-type resting cells. Enzyme reactions were conducted at pH 9 in all subsequent experiments.

A range of pH optima from pH 7.5 to pH 10 are reported in the literature for bacterial hydantoinases and are summarised in Chapter 1, Table 1.1. The pH optima of bacterial *N*-carbamoylases range from pH 7 to pH 9 and are shown in Chapter 1, Table 1.2. The pH optima of two *Agrobacterium* sp. hydantoinases were found to be pH 10 (Durham and Weber, 1995; Runser and Ohleyer, 1990), while the pH optima of *N*-carbamoylases in *Agrobacterium* sp. range from pH 7 to pH 7.4 (Buson *et al.*, 1996; Louwrier and Knowles, 1996, Nanba *et al.*, 1998a; Kim and Kim, 1995). The apparent pH optimum of the *A. tumefaciens* RU-ORPN1 hydantoinase was therefore amongst the most alkaline of the bacterial hydantoinases reported in the literature, while the apparent pH optimum of the *N*-carbamoylase was found to be more alkaline than those reported for other *Agrobacterium* sp. *N*-carbamoylases. The relatively high and matching apparent pH optima of the enzymes are ideal for the development of an industrial biocatalyst utilising these enzymes for the one-step production of amino acids since, firstly, spontaneous racemization of hydantoin substrates

takes place at alkaline pH, potentially allowing the 100% conversion of a hydantoin substrate to an optically pure product, and secondly, the development of an industrial process with two biocatalysts operating under identical conditions would be considerably more cost-effective than a process with separate biocatalysts with different optimal operating conditions.



The hydantoinase and *N*-carbamoylase activities appeared to be affected by the type of buffer used (Figure 3.6). For both the enzymes, slightly higher product yields were detected in the presence of potassium phosphate buffer in comparison to Tris-HCl buffer. Furthermore, the hydantoinase product yield measured in the carbonate buffer was slightly lower than in the Tris-HCl buffer and the *N*-carbamoylase product yield was enhanced by the carbonate buffer in comparison to the Tris-HCl buffer. Similar variations in the hydantoinase activities have been observed with the use of these buffers in the literature (Sudge *et al.*, 1998; Möller *et al.*, 1988; Sharma and Vohra, 1997). The effects of various buffer types on a *Pseudomonas* sp. hydantoinase indicated that Tris-HCl and carbonate buffers were most suitable for substrate conversion, although reactions carried out in Tris-HCl buffer give a higher conversion rate than those in carbonate buffer (Sudge *et al.*, 1998). A pH profile for the *N*-carbamoylase from *Agrobacterium* sp. strain KNK712 using these buffers showed similar trends to those presented in Figure 3.6 for the *A. tumefaciens* RU-ORPN1 enzyme in potassium phosphate and Tris-HCl buffers, but the enzyme in the KNK712 strain exhibited reduced activity in carbonate buffer (Nanba *et al.*, 1998a).

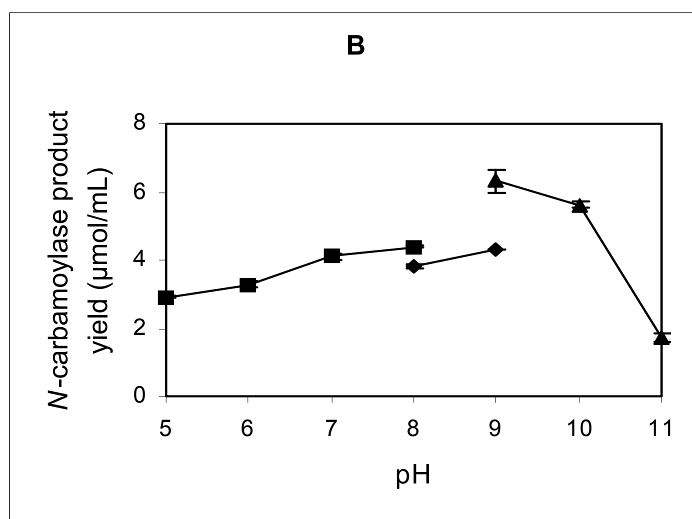


Figure 3.6 Measurement of the apparent pH optima for the hydantoinase (**A**) and *N*-carbamoylase (**B**) enzymes in the enzyme extract. The following buffers were used: 100mM potassium phosphate buffer, pH5.0-8.0 (■); 100mM Tris-HCl buffer, pH8.0-9.0 (●) and 100mM carbonate buffer, pH9.0-11.0 (•).

3.3.7 Determination of the apparent reaction temperature optima for the hydantoin-hydrolysing enzymes in the enzyme extract

The apparent reaction temperature optima for the hydantoinase and *N*-carbamoylase in the enzyme extract were identified to further optimise the reaction conditions for the enzymes. The temperature optimum for the hydantoinase in the wild-type resting cells was between 40^0C and 60^0C, while the optimum temperature for the *N*-carbamoylase was 40^0C (Burton *et al.*, 1998). The effect of temperature on the hydantoinase and *N*-carbamoylase product yields in the enzyme extract are shown in Figure 3.7. The hydantoinase product yield was highest in the extract at 30^0C, although temperatures between 30^0C and 60^0C had very little effect on the product yield measured. The *N*-carbamoylase product yield in the extract was more sensitive to temperature change, being highest at 40^0C with sharp decreases below and above this temperature.

The temperature optima of bacterial hydantoinases reported in the literature range from 20^0C to 75^0C and are listed in Chapter 1, Table 1.1. The temperature optima for bacterial *N*-carbamoylases reported in the literature vary from 35^0C to 70^0C and are listed in Chapter 1, Table 1.2. The temperature optima of the hydantoinases in *Agrobacterium* sp. IP I-671 (Runser and Ohleyer, 1990) and *Agrobacterium tumefaciens* NRRL B11291(Olivieri et al., 1981) were 60^0C while in *Agrobacterium tumefaciens* 47C (Durham and Weber, 1995) optimal activity was detected at 70^0C. The *N*-carbamoylases from *Agrobacterium* sp. had temperature optima ranging from 50^0C to 70^0C (Olivieri *et al.*, 1979; Louwrier and Knowles, 1996; Kim and Kim, 1995; Nanba *et al.*, 1998a). Thus, the *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase apparent temperature optima were considerably lower than those reported for these enzymes from other *Agrobacterium* sp. Higher temperature optima would be favourable for hydantoin-hydrolysing industrial biocatalysts as the substrates are more soluble at higher temperatures.

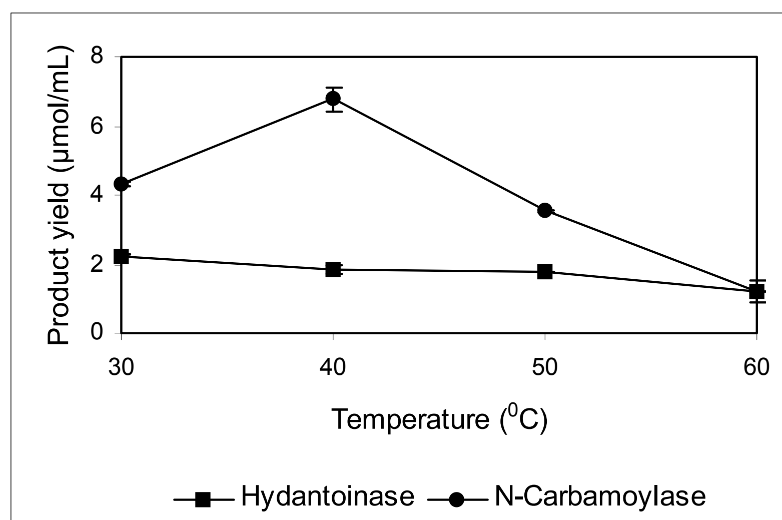


Figure 3.7 Measurement of the apparent temperature optima for the hydantoinase and *N*-carbamoylase enzymes in the enzyme extract.

3.3.8 Effect of metal ions on the hydantoinase and *N*-carbamoylase activities in the enzyme extract

Some reports in the literature have indicated that certain hydantoinases and *N*-carbamoylases require metal ions for their activity. The metal requirements for the microbial hydantoinases and *N*-carbamoylases reported in the literature are shown in Chapter 1, Tables 1.1 and 1.2, respectively. Metal-dependent D-(Lee *et al.*, 1995; Park *et al.*, 1998) and L-hydantoinases (Ogawa *et al.*, 1995a) requiring Mn^{2+}, and Mg^{2+} and K^{+}, respectively, have been reported. Metal-dependent L-*N*-carbamoylases from *Alcaligenes xylosoxidans* requiring Mn^{2+}, Ni^{2+} and Co^{2+} (Ogawa *et al.*, 1995b) and from *Pseudomonas* sp. requiring Mn^{2+} for activity (Ishikawa *et al.*, 1996) have also been reported.

Other hydantoinases, while not dependent on metal ions for activity, are enhanced by the presence of certain metal ions. Hydantoinase activities are most commonly enhanced by the addition of Mg^{2+} and Mn^{2+} ions and these ions have been shown to enhance the hydantoinase activities in a variety of *Pseudomonas* sp. and *Bacillus* sp. strains, and a *Blastobacter* sp. strain (Chapter 1, Table 1.1). Most *N*-carbamoylases reported in the literature have been shown to be unaffected by the removal or addition of metal ions (Chapter 1, Table 1.2). The activity of the hydantoinase in *Agrobacterium* sp. IP I-671has been shown to be enhanced by the addition of Ni^{2+} or Mg^{2+} ions (Runser and Ohleyer, 1990), while the D-carbamoylase from *Agrobacterium radiobacter* was enhanced by the addition of Mg^{2+} and Mn^{2+} (Buson *et al.*, 1996). This

study was therefore conducted to assess the metal dependence of the *A. tumefaciens* RU-ORPN1 enzymes and to investigate whether the enzyme activities in the extract could be enhanced by the addition of metal ions.

In most cases, metal dependence studies of hydantoinases and *N*-carbamoylases have been performed using purified enzymes, and metal-chelating agents have been used to induce metal-caused reactivation of the enzymes (May *et al.*, 1998a). The metals are assumed to be bound to the enzymes and the metal content of the enzymes are therefore not directly measured. Using more sophisticated methods, such as subjecting the purified enzymes to atomic absorption spectrometry (AAS) or inductive coupled plasma-atomic emission spectrometry (ICP-AES), the hydantoinases from *Arthrobacter aurescens* (May *et al.*, 1998a) and *Arthrobacter crystallopoietes* (Siemann *et al.*, 1999) were identified as zinc metalloenzymes.

The metal dependence of the hydantoinase and *N*-carbamoylase in the *A. tumefaciens* RU-ORPN1enzyme extract was investigated by adding EDTA to the enzyme extract as a competing metal ion chelator to remove metal ions bound within the enzymes. The effect of removal of the metal ions from the enzymes in the extract was examined by measuring the enzyme reaction product yields after dialysis of the EDTA-containing extract against buffer. Finally, the enzyme reaction product yields were measured in the above treated extract after the addition of various metal ions (Figure 3.8). The hydantoinase and *N*-carbamoylase activities decreased by 40% and 20%, respectively, after the addition of EDTA to the extract and remained lowered after dialysis. The addition of Mg^{2+} resulted in the restoration of the hydantoinase activity suggesting a possible dependence of the hydantoinase on Mg^{2+}. The addition of Mg^{2+}, Mn^{2+} and Zn^{2+} were all found to restore the *N*-carbamoylase activity. Interestingly, the enzymes did not appear to be inhibited by Cu^{2+} or Zn^{2+} ions which have been reported to inhibit some hydantoinases and *N*-carbamoylases in the literature (Syldatk *et al.*, 1987; Ogawa *et al.*, 1993; Xu and West, 1994).

The results presented in Figure 3.8 indicate that the hydantoinase may be dependent on Mg^{2+} ions for activity while the *N*-carbamoylase appeared to be dependent on the presence of certain divalent metal ions for activity. These results are not conclusive, however, since the biochemical characterisation of the enzymes remained confined to characterisation of the enzymes within the enzyme extract. The presence of other components in the enzyme extract may have shielded some of the effects of the EDTA or metal ions in the extract. Since the level of interference by other components in the extract was unknown, the results presented here would need to be verified by subjecting the purified enzymes to the same analysis.

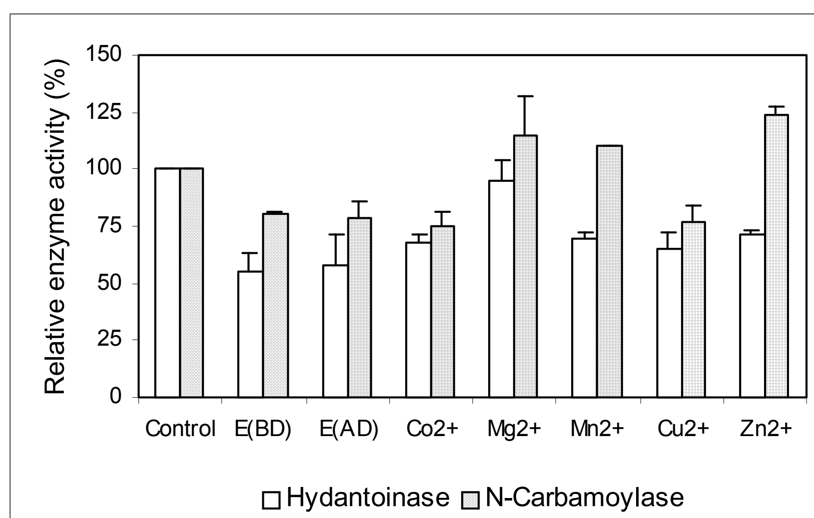


Figure 3.8 Effect of removal and re-addition of metal ions on the hydantoinase and *N*-carbamoylase activities in the enzyme extract. Enzyme activities in treated extracts are expressed as percentages of the activities in the untreated extract (control). E = EDTA; BD = before dialysis; AD = after dialysis.

3.3.9 Effect of ATP on the hydantoinase and *N*-carbamoylase activities in the enzyme extract

The enzyme product yields were measured in the presence of ATP in order to assess the ATP-dependence of the enzymes. The restoration of the hydantoinase and *N*-carbamoylase activities in the presence of Mg^{2+} after EDTA treatment indicated a possible requirement of the enzymes for Mg^{2+} for the utilisation of ATP, thus indicating possible ATP-dependence. ATP-dependent hydantoinases have been reported by Ishikawa *et al.* (1994) and Ogawa *et al.* (1995a). Alternatively, Mg^{2+} ions may have been directly involved in the functioning of the enzymes as the activities of a number of ATP-independent hydantoinases and *N*-carbamoylases have been enhanced by the presence of Mg^{2+} ions (Buson *et al.*, 1996; Sharma and Vohra, 1997; Soong *et al.*, 1999). The effect of ATP on the hydantoinase and *N*-carbamoylase activities in the enzyme extract is shown in Figure 3.9.

The *N*-carbamoylase activity was increased significantly to almost double its original activity in the presence of 0.1mM ATP, while higher concentrations of ATP only slightly enhanced the enzyme activity relative to the control without ATP. The hydantoinase activity was also significantly affected by the presence of ATP as the activity of this enzyme doubled in the presence of 0.1mM ATP and increased further with increasing concentrations of ATP. The elevated levels of hydantoinase and *N*-carbamoylase in the presence of ATP indicated that the enzymes may be dependent on ATP. As noted previously in the metal dependence study in section 3.3.8, the effects of ATP on the enzymes may have been masked by other components in the enzyme extract. The ATP dependence of the enzymes would therefore need to be confirmed by assaying the activity of the purified enzymes in the presence of ATP. However, the results obtained showed that the enzyme activities in the extract were enhanced by the addition of 0.1mM ATP.

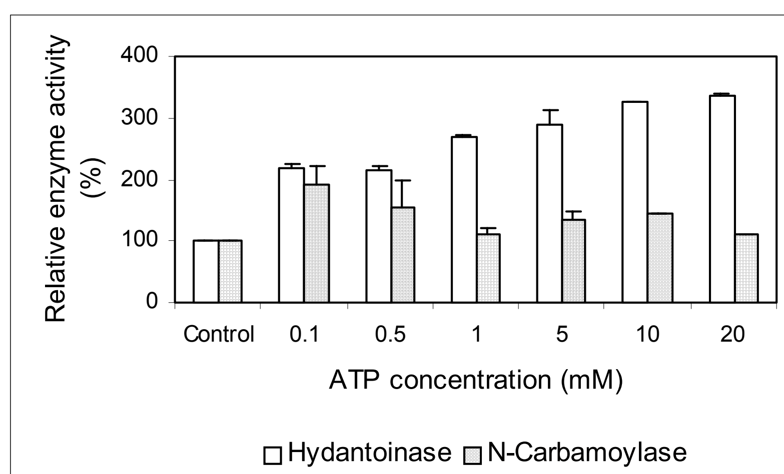


Figure 3.9 Effect of ATP on the hydantoinase and *N*-carbamoylase activities in the enzyme extract. Enzyme activities in the treated extracts are expressed as percentages of the enzyme activities in the untreated extract (control).

3.3.10 Effects of DTT and PMSF on the hydantoinase and *N*-carbamoylase activities in the enzyme extract

Most procedures reported in the literature for the purification of hydantoinase and *N*-carbamoylase enzymes are carried out in the presence of a reducing agent. *N*-carbamoylases, in particular, have been reported to be susceptible to inactivation in the absence of a reducing agent. This inactivation was shown to be due to oxidation of cysteine residues in the enzyme structure in the case of the enzyme from *Agrobacterium radiobacter* (Grifantini *et al.*, 1996). Various reducing agents have been found to stabilise the enzyme activity, including DTT, sodium thioglycollate, reduced glutathione and L-cysteine (Louwrier and Knowles, 1997). DTT has most commonly been used as a stabilizing agent for the purification of both hydantoinase (Ogawa *et al.*, 1995a; Lee and Kim, 1998; Soong *et al.*, 1999) and *N*-carbamoylase (Ogawa *et al.*, 1994b; Kim and Kim, 1995; Nanba *et al.*, 1998a) enzymes. The effect of DTT on the *A. tumefaciens* RU-ORPN1 enzyme activities in the enzyme extract was therefore evaluated by measuring the hydantoinase and *N*-carbamoylase product yields in the presence of various concentrations of DTT (Figure 3.10). A DTT concentration of 0.5mM enhanced the hydantoinase activity in the enzyme extract by 60%. Higher concentrations of DTT had no further effect on the enzyme. DTT concentrations of up to 1mM had no effect on the *N*-carbamoylase activity but concentrations of 5mM and higher appeared to inhibit the enzyme. The increased hydantoinase activity in the presence of DTT indicated that the enzyme without the presence of the reducing agent may have been susceptible to oxidation during the enzyme reaction. The lack of an effect of the DTT on the *N*-carbamoylase enzyme suggested that the enzyme in the absence of the reducing agent was not susceptible to significant oxidation during the enzyme reaction.

A number of protocols reported for the purification of hydantoinase enzymes have included the use of the serine protease inhibitor, PMSF, in order to prevent proteolytic activity in enzyme samples (Runser and Meyer, 1993; Lee *et al.*, 1994; Lee and Kim, 1998). The effect of this protease inhibitor on the *A. tumefaciens* RU-ORPN1 enzyme activities in the enzyme extract was evaluated (Figure 3.11). A PMSF concentration of 1mM increased the hydantoinase activity by 80% and the *N*-carbamoylase activity by 10%. Higher concentrations of PMSF had no further effect on the hydantoinase activity, while the *N*-carbamoylase activity was inhibited at concentrations of 5 and 10mM. The increased hydantoinase and *N*-carbamoylase activities in the presence of PMSF indicated that the enzymes may have been susceptible to some proteolytic attack during the enzyme reaction in the absence of the inhibitor.

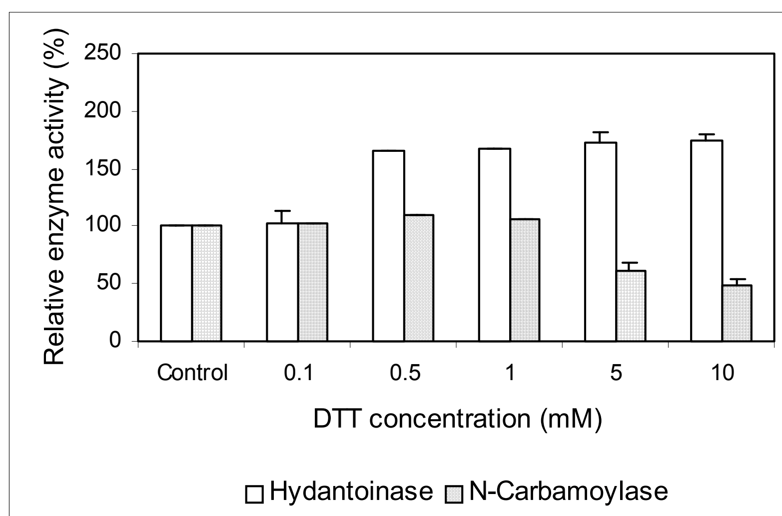


Figure 3.10 Effect of DTT on the hydantoinase and *N*-carbamoylase activities in the enzyme extract. Enzyme activities in the treated extracts are expressed as percentages of the enzyme activities in the untreated extract (control).

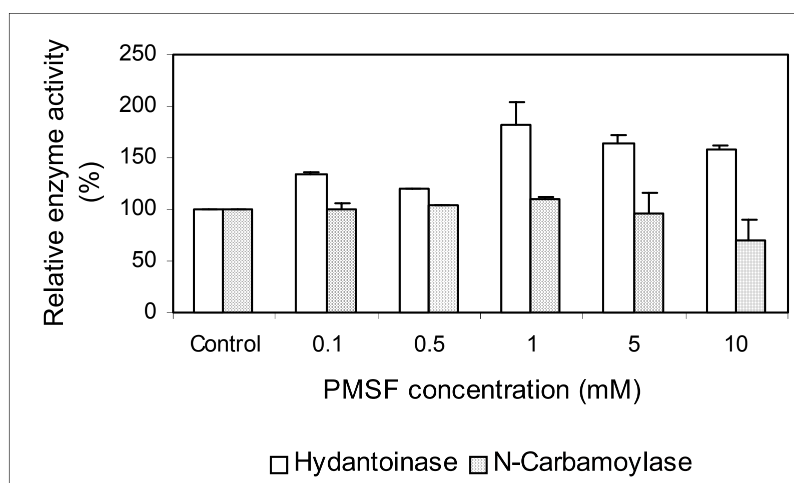


Figure 3.11 Effect of PMSF on the hydantoinase and *N*-carbamoylase activities in the enzyme extract. Enzyme activities in the treated extracts are expressed as percentages of the activities in the untreated extract (control).

3.3.11 Comparison of hydantoin and NCG, and D,L-5-HPH and NC-HPG, as substrates for the hydantoinase and *N*-carbamoylase in the enzyme extract

The substrate specificities of the hydantoinase and *N*-carbamoylase were investigated using hydantoin and D,L-5-HPH as substrates for the hydantoinase, and NCG and NC-HPG as substrates for the *N*-carbamoylase. D-HPG is of industrial interest for use in the production of antibiotics and therefore the conversion efficiency of *A. tumefaciens* RU-ORPN1 for the production of D-HPG from D,L-5-HPH and NC-HPG needed to be evaluated. The enzyme product yields obtained with the various substrates are shown in Table 3.1. The hydantoinase product yield decreased considerably from 2.4: mol/mL when hydantoin was used as substrate, to 0.23: mol/mL when D,L-5-HPH was used as substrate. The *N*-carbamoylase product yield decreased from 3.1: mol/mL when NCG was used as substrate, to 1.4: mol/mL when NC-HPG was used as substrate. The specificities of bacterial hydantoinases for D,L-5-HPH relative to hydantoin are generally low (Table 3.2), with the exception of the hydantoinase from *Agrobacterium tumefaciens* sp. IP-I 671, which had a reported specificity of 148% for D,L-5-HPH relative to hydantoin. *N*-carbamoylase specificities for NC-HPG relative to NCG are generally not reported in the literature, but a number of microbial D-*N*-carbamoylases having relatively high substrate specificities for NC-HPG in comparison to other *N*-carbamyl intermediates have been reported (Olivieri *et al.*, 1981; Ogawa *et al.*, 1994b; Louwrier and Knowles, 1997; Sareen *et al.*, 2001). Interestingly, the *N*-carbamoylases from *Comamonas* sp. (Ogawa *et al.*, 1993) and *Blastobacter* sp. (Ogawa *et al.*, 1994b) were not able to utilise NCG as a substrate at all, while the *N*-carbamoylases from *Agrobacterium* sp. KNK712 (Nanba *et al.*, 1998a) and *Agrobacterium tumefaciens* AM 10 (Sareen *et al.*, 2001) had NCG specificities of only 12 and 1%, respectively, relative to NC-HPG.

The substrate specificity of the *A. tumefaciens* RU-ORPN1 hydantoinase compared favourably with most of the specificities of the other bacterial hydantoinases reported in the literature (Table 3.2). Since the *A. tumefaciens* RU-ORPN1 *N*-carbamoylase NC-HPG specificity was not compared with any *N*-carbamyl intermediates other than NCG, it was difficult to assess the specificity of this enzyme for NC-HPG relative to the other reported *N*-carbamoylases. However, the *A. tumefaciens* RU-ORPN1 enzyme was able to utilise NC-HPG as a substrate indicating that it could be applied in a process for the production of D-HPG.

Table 3.1 Substrate specificities of the hydantoin-hydrolysing enzymes in *A. tumefaciens* RU-ORPN1.

Substrates (12.5mM)	Hydantoinase product yield (: mol/mL)*	*N*-Carbamoylase product yield (: mol/mL)*
Hydantoin/NCG	2.4 ± 0.11	3.1 ± 0.068
D,L-5-HPH/NC-HPG	0.23 ± 0.027	1.4 ± 0.097

* Errors represent SEM (n=3)

Table 3.2 Activity of some bacterial hydantoinases using D,L-5-HPH as a substrate relative to the activities obtained with hydantoin as a substrate.

Strain	Relative activity (%)
Agrobacterium tumefaciens RU-ORPN1	29
Arthrobacter crystallopoietes DSM 20117 (Siemann *et al.*, 1999)	9
Agrobacterium sp. IP-I 671 (Runser *et al.*, 1990)	148
Bacillus sp. AR9 (Sharma and Vohra, 1997)	19
Bacillus stearothermophilus SD-1 (Lee *et al.*, 1995)	17
Pseudomonas sp. NCIM5109 (Sudge *et al.*, 1998)	38

3.3.12 Stability of the hydantoin-hydrolysing activity in the enzyme extract at 25^{0}C and at 40^{0}C over 24 hours

The hydantoinase and *N*-carbamoylase reaction product yields were measured in the enzyme extract following incubation at 25^{0}C and 40^{0}C to evaluate the stability of the enzymes at these temperatures. The stability of the enzymes at 40^{0}C was of particular interest, since this was the temperature at which the enzyme reactions were carried out. The residual enzyme activities measured for each incubation temperature over a period of 24 h are shown in Figure 3.12. The hydantoinase activity gradually decreased to 37% of its original activity during the 24 h incubation at 25^{0}C (Figure 3.12A) but the enzyme activity increased significantly during the 40^{0}C incubation to over 4 times its original activity (Figure 3.12B). Increased hydantoinase activity was also observed after storage of frozen cells at -20^{0}C (section 3.3.2). As previously discussed, the increased hydantoinase activity could have been due to the inactivation of an inhibitor of the enzyme such as a proteolytic enzyme, for example. However, since the enzyme lost activity at 25^{0}C, the inhibitor may have been active at this temperature. The presence of an inhibitor of the hydantoinase enzyme in the enzyme extract is consistent with the results obtained in section 3.3.10 which

showed that the hydantoinase activity in the extract could be increased by the addition of PMSF, a serine protease inhibitor.

The *N*-carbamoylase activity decreased during the incubation at both temperatures but was more stable at 25^{0}C (Figure 3.12A) than at 40^{0}C (Figure 3.12B). The enzyme decreased to 37% and 4% of its original activity after 24 hours incubation at these temperatures, respectively. The loss of the enzyme activity during the incubation at these temperature suggested that the enzyme was susceptible to oxidation, a common characteristic of these enzymes (Grifantini *et al.*, 1996). The oxidation would have been more rapid at 40^{0}C due to the elevated temperature resulting in the greater loss of activity in this sample after the same length of time. Very few reports on the thermostability of hydantoinases and *N*-carbamoylases at these temperatures have been made in the literature. However, the hydantoinase from a *Bacillus* sp. was stable for 8 hours at 40^{0}C (Luksa *et al.*, 1997) and the *N*-carbamoylase from *Agrobacterium radiobacter* remained stable at 40^{0}C for 24 hours (Olivieri *et al.*, 1979). Most *N*-carbamoylase enzymes reported in the literature have been reported to be stable at temperatures between 40 and 55^{0}C. However, in most cases, the thermostabilities were only tested over 10 to 30 minutes at these temperatures (Nanba *et al.*, 1998a; Kim and Kim, 1995; Ogawa *et al.*, 1994b). Likewise, most hydantoinases reported in the literature have been reported to be stable at temperatures between 50 and 70^{0}C but in most cases the thermostabilities were only tested over 10 to 30 minutes (Möller *et al.*, 1988; Lee *et al.*, 1994; Kim and Kim, 1995). A *N*-carbamoylase from an *Agrobacterium* sp., however, was stable for 4 hours at 50^{0}C (Louwrier and Knowles, 1997) and a hydantoinase from *Arthrobacter* sp. had a half-life of 6 hours at 50^{0}C (Siemann *et al.*, 1999). Recently, a thermostable L-hydantoinase from a hyperthermophilic archeon was described which had a half-life of 100 minutes at 90^{0}C (Chung *et al.*, 2002).

The results of this investigation indicated that the enzymes from *A. tumefaciens* RU-ORPN1 were less thermostable than most of the enzymes reported in the literature. Thus, the stabilisation of the enzyme activities would be essential for the successful development of a commercially viable hydantoin-hydrolysing biocatalyst. Various stabilisation strategies were therefore later assessed and are described in Chapter 5.

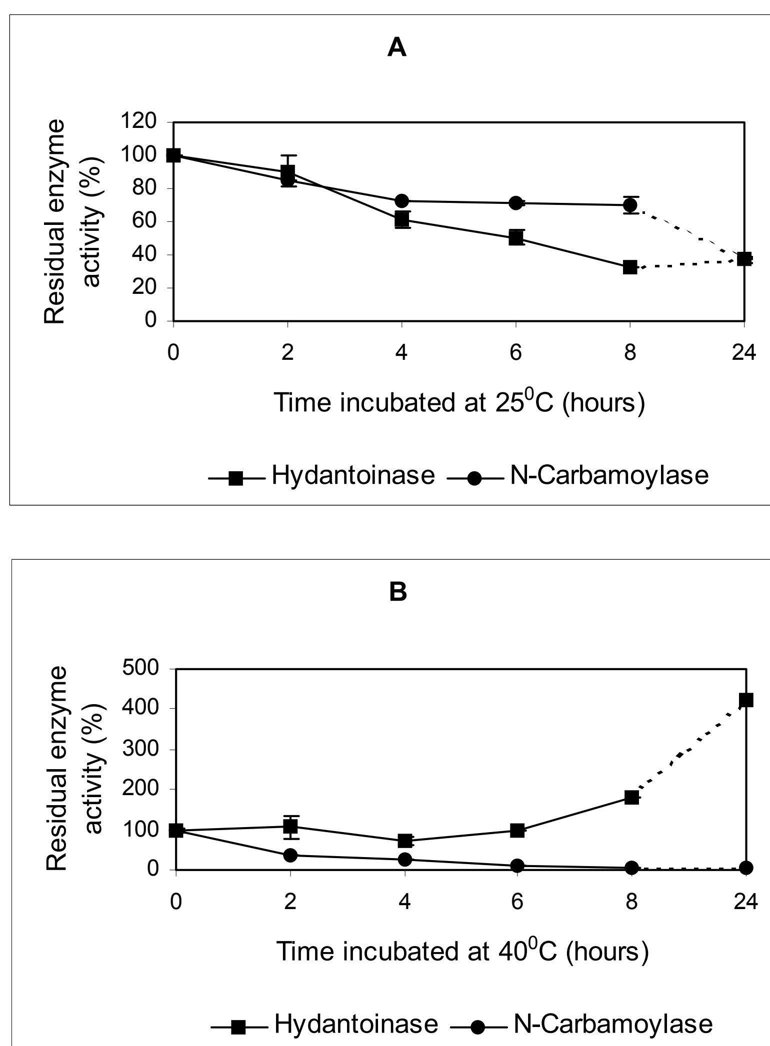


Figure 3.12 Stability of the hydantoinase and *N*-carbamoylase enzymes in the extract during incubation at 25^0C (**A**) and at 40^0C (**B**).

3.3.13 Effect of freeze-drying on the hydantoin-hydrolysing activity in the enzyme extract

The effect of freeze-drying on the hydantoinase and *N*-carbamoylase activities in the enzyme extract was investigated in order to evaluate freeze-drying as a method for the storage of the enzyme extract. The enzyme reaction product yields measured before and after freeze-drying of the enzyme extract are shown in Figure 3.13 and the residual enzyme activities in the freeze-dried extract after storage at -20^{0}C and at 25^{0}C are shown in Figures 3.14 and 3.15, respectively. The hydantoinase and *N*-carbamoylase enzymes retained 73% and 90% activity, respectively, after freeze-drying (Figure 3.13). The hydantoinase retained 100% of its original activity in the freeze-dried extract stored at -20^{0}C after 4 weeks, while the *N*-carbamoylase decreased to 48% of its original activity after 4 weeks at this temperature (Figure 3.14). The hydantoinase activity in the freeze-dried extract stored at 25^{0}C remained stable for the first 2 weeks of storage and then decreased to 70% of its original activity after 4 weeks. The *N*-carbamoylase in this extract remained stable for 7 days after which it decreased to 12% of its original activity (Figure 3.15). Freeze-drying was therefore an effective method for the storage of the enzyme extract. Using this method, the enzyme extract could be stored for 2 weeks at -20^{0}C with over 70% activity retention for both enzymes and the enzymes could be stored at room temperature for 1 week without any loss of activity. The effect of freeze-drying on hydantoinase or *N*-carbamoylase enzymes has not been reported in the literature.

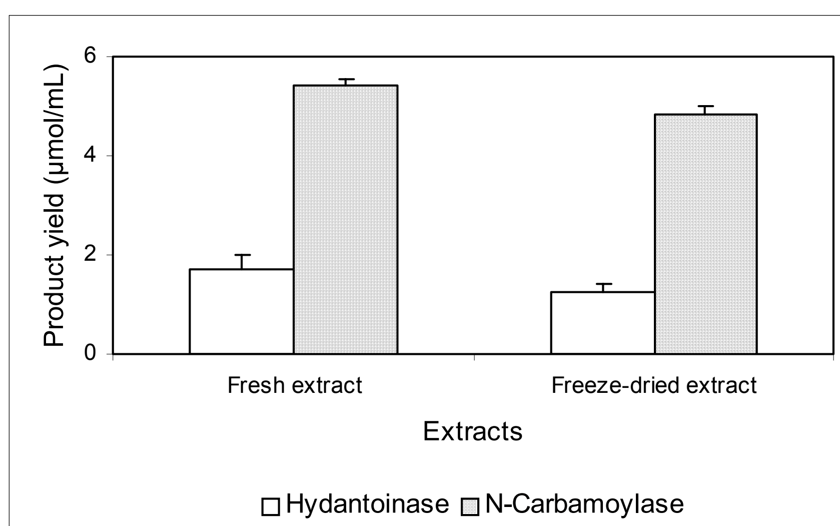


Figure 3.13 Hydantoinase and *N*-carbamoylase activities measured before and after freeze-drying the enzyme extract.

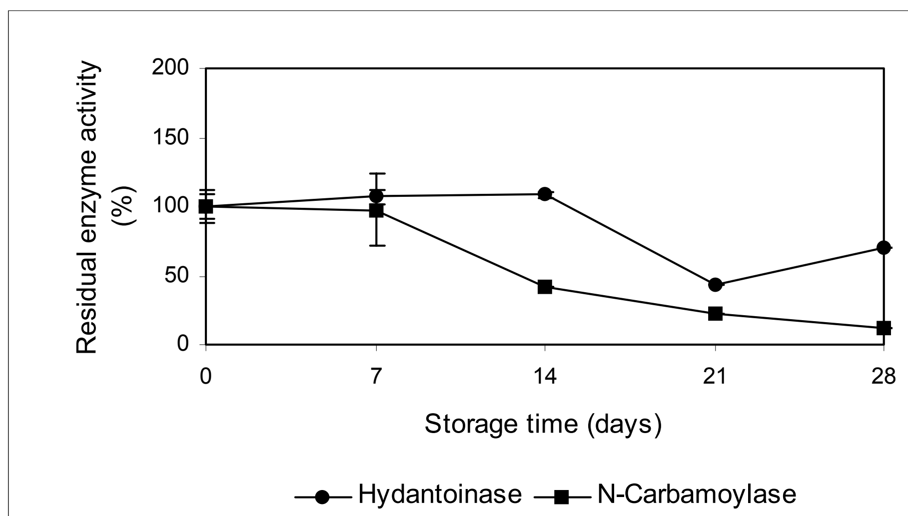


Figure 3.14 Storage stability of the hydantoin-hydrolysing enzyme activity in the freeze-dried enzyme extract at -20^{0}C.

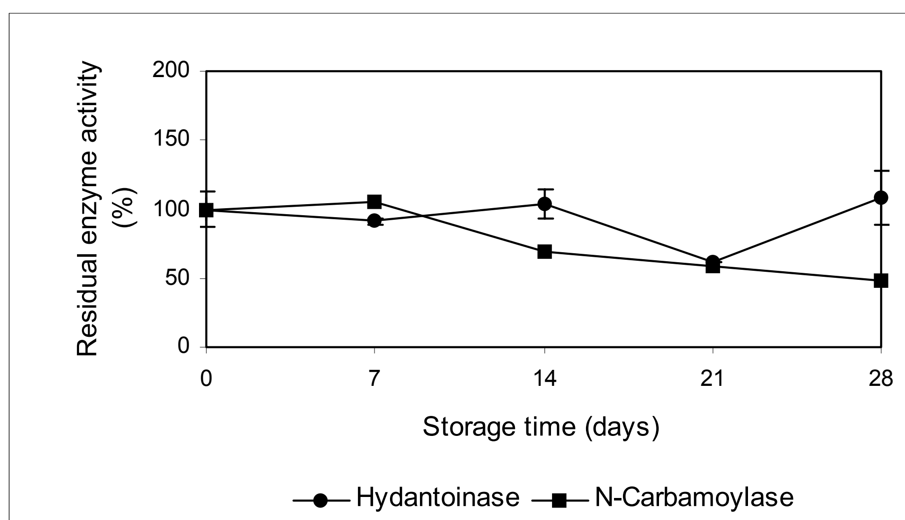


Figure 3.15 Storage stability of the hydantoin-hydrolysing enzyme activity in the freeze-dried enzyme extract at 25^{0}C.

3.4 CONCLUSIONS

A number of conclusions were reached from the results of this work. Various conditions for the production of the enzyme extract were evaluated. Interestingly, frozen cells were found to produce higher *N*-carbamoylase product yields in comparison to freshly harvested cells in resting cell assays, and cells could be frozen for 3 weeks without any loss of hydantoinase or *N*-carbamoylase activities. The increased *N*-carbamoylase activity was thought to be due to the partial breakdown of the cell wall due to the freeze-thaw process increasing the mass transfer of substrates into the cells.

Enzyme extracts produced from frozen cells also yielded higher enzyme product yields in comparison to extracts produced from fresh cells. Sonication of frozen cells for 5 minutes produced the enzyme extract with highest hydantoinase and *N*-carbamoylase activities in comparison to those produced by french-pressing and by the two techniques combined. This method was therefore chosen as the standard method for the production of the enzyme extract.

Maximum hydantoinase and *N*-carbamoylase product yields appeared to be reached after 6 hours incubation in the presence of the substrates. Conversion of the substrates, however, was not complete after this length of time, indicating that the enzymes may have been susceptible to oxidation during the enzyme reaction, resulting in activity loss. The susceptibility of the *N*-carbamoylase enzyme, in particular, to oxidising conditions is well known and often, strictly anaerobic conditions are required for the complete conversion of substrates to the corresponding amino acids (Grifantini *et al.*, 1996; Chao *et al.*, 1999b; Nanba *et al.*, 1999). This would need to be considered with the application of these enzymes in a process for the production of D-HPG.

The hydantoinase and *N*-carbamoylase in the enzyme extract retained 74% and 83% activity, respectively, after 7 days storage at 4^0C. The *N*-carbamoylase enzyme was therefore found to be more stable than other *N*-carbamoylases reported in the literature at this temperature. The hydantoinase activity in the extract decreased after freezing, indicating a possible sensitivity of this enzyme to the freeze-thaw process. The enzyme extract was therefore freshly prepared for all subsequent experiments.

The apparent pH optima for the hydantoinase and *N*-carbamoylase enzymes in the enzyme extract were found to be similar to those reported for other hydantoinases and *N*-carbamoylases in the literature. The

apparent optimal pH for the hydantoinase was pH 10, while the apparent pH optimum for the *N*-carbamoylase was pH 9. The apparent temperature optima for the enzymes were found to be amongst the lower range of temperature optima reported in the literature. The apparent temperature optima for the hydantoinase and *N*-carbamoylase in the extract were 30^0C and 40^0C, respectively.

Both the hydantoinase and *N*-carbamoylase activities were decreased by the removal of metal ions from the extract as a result of EDTA treatment. The hydantoinase activity was restored by the addition of Mg^{2+} ions, while the *N*-carbamoylase activity was restored in the presence of Mg^{2+}, Mn^{2+} or Zn^{2+} ions. The enhanced enzyme activities in the presence of Mg^{2+} suggested that the enzymes may have been ATP-dependent, since Mg^{2+} is required for ATP utilisation. The enzymes were also activated in the presence of ATP. This study was done using the enzyme extract which consisted of the unclarified cell homogenate. The effects of the various additives therefore may have been masked by components in the extract and the ATP-dependence of the enzymes, therefore, would need to be confirmed by examining the effects of the additives on the purified enzymes.

The hydantoinase activity in the enzyme extract was enhanced by 0.5mM DTT and by 1mM PMSF. The *N*-carbamoylase was slightly enhanced by the presence of 1mM PMSF while DTT had no positive effect on the activity of this enzyme. The enhanced enzyme activities in the presence of PMSF indicated that the enzymes may have been exposed to some proteolytic activity in the enzyme extract. The lack of an effect of the DTT on the *N*-carbamoylase enzyme was surprising since these enzymes are usually stabilised in the presence of reducing agents. However, interference from other components in the enzyme extract may have shielded the effect of the DTT.

The enzymes were able to utilise D,L-5-HPH and NC-HPG as substrates for the production of D-HPG. This was important, since the biocatalyst could be developed for the production of D-HPG. The hydantoinase activity increased significantly during incubation at 40^0C, while the *N*-carbamoylase had a half-life of approximately 5 hours at this temperature. The hydantoinase and *N*-carbamoylase activities gradually decreased at 25^0C over 24 hours. The enzymes were therefore sensitive to thermal inactivation and improved thermostability would be necessary for the development of a successful biocatalyst utilising these enzymes.

Finally, freeze-drying was found to be an efficient method for the storage of the enzyme extract. The enzymes were significantly stabilised by the freeze-drying process and could be stored for a week at room temperature in this form without any loss of activity, while the non-freeze-dried enzymes were almost completely inactivated after 24-hours storage at this temperature.

CHAPTER 4

ENZYME SOLUBILISATION STRATEGIES

4.1 INTRODUCTION

One of the objectives set at the outset of this project was to purify the hydantoinase and *N*-carbamoylase enzymes from *A. tumefaciens* RU-ORPN1. Purified enzymes are required for detailed biochemical and

kinetic analyses which allow for a deeper understanding of the mechanism of enzyme interaction. One of the prerequisites for purifying an enzyme is a source of large quantities of the enzyme in a soluble form and many procedures reported for the purification of hydantoinases and *N*-carbamoylases in the literature involve the purification of these enzymes from genetically modified organisms over-expressing large quantities of the enzymes in a soluble form (Grifantini *et al.*, 1996; Ishikawa *et al.*, 1996; Nanba *et al.*, 1998a and Pietzsch *et al.*, 2000). However, hydantoinases have been purified from a number of native species including *Agrobacterium* sp. (Runser and Meyer, 1993), *Arthrobacter* sp. (May *et al.*, 1998d; Siemann *et al.*, 1999), *Bacillus* sp. (Lee *et al.*, 1995; Luksa *et al.*, 1997), *Blastobacter* sp. (Soong *et al.*, 1999) and *Pseudomonas* sp. (Takahashi *et al.*, 1978). Similarly, *N*-carbamoylases have been purified from a number of native species including *Comamonas* sp. (Ogawa *et al.*, 1993), *Blastobacter* sp. (Ogawa *et al.*, 1994b), *Agrobacterium* sp. (Kim and Kim, 1995; Sareen *et al.*, 2001) and *Pseudomonas* sp. (Ikenaka *et al.*, 1998b). In each case, the enzyme was present in a soluble form and was purified from cell-free extracts following cell disruption. However, following disruption of *A. tumefaciens* RU-ORPN1 cells and clarification of the lysate by centrifugation, it was found that the majority of the hydantoinase and *N*-carbamoylase activities were embedded in the cell pellet, either due to covalent bonding, or strong binding, indicating that the enzymes were insoluble or membrane-bound.

Various strategies were adopted in an attempt to solubilise the *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase in an active form for further study and in order to elucidate the nature of the enzyme insolubility. Each method was evaluated by measuring the enzyme activities in the pellet and supernatant of the treated enzyme extracts and comparing the percentage of the total enzyme activity present in the supernatant after each treatment, with the percentage of the total enzyme activity present in the supernatant of the untreated enzyme extract.
Initially, the effects of different cell disruption methods on the solubilities of the enzymes were investigated. Mechanical disruption methods such as sonication, bead-beating and french-pressing were compared. These vigorous treatments were then compared with a less robust method of cell lysis using lysozyme treatment. The effects of minimising proteolytic activity in the enzyme extract and the addition of a reducing agent on the enzyme activity levels in the supernatant of the enzyme extract were investigated.

Various techniques commonly used for the solubilisation of membrane-associated proteins were then investigated. The association of proteins with membranes can be either peripheral, anchored or integral. Peripheral membrane proteins are associated with the membrane bilayer via a combination of electrostatic and hydrophobic non-covalent interactions and can normally be released from the membrane by mild treatments such as changing the pH or ionic strength (de Lima Santos and Ciancaglini, 2000). The effects of ionic strength and pH on the solubilities of the hydantoinase and *N*-carbamoylase were therefore determined.

Anchored membrane proteins have a hydrophobic, covalently bound, lipid or alkyl chain by which they are attached to the membrane. These proteins can be solubilised by treatment with detergents or with certain hydrolytic enzymes (de Lima Santos and Ciancaglini, 2000). Thus, chymotrypsin and phospholipase were used here for the potential cleavage of membrane-bound polypeptides or alkyl chains from the enzyme protein structures, with the aim of releasing the enzymes into the supernatant in an active, soluble form.

Integral membrane proteins can only be released from membranes by treatment with detergents or chaotropic agents that disrupt the membrane structure, because they have hydrophobic peptide chains that partially or completely cross the lipid bilayer (de Lima Santos and Ciancaglini, 2000). Thus, the enzyme extract was treated with non-ionic detergents in order to solubilise the cell membranes and to evaluate the effect of this on the solubility of the enzymes.

Finally, hydropathy plots and transmembrane prediction plots of the predicted amino acid sequences derived from two *N*-carbamoylase genes (*NcaR1* and *NcaR2*), identified in the *A. tumefaciens* RU-OR genome by Hartley, (2001), were analysed to identify any hydrophobic regions of the protein which may be membrane-associated. The predicted amino acid sequences derived from the wild-type *A. tumefaciens* RU-OR *N*-carbamoylase genes were analysed on the assumption that these sequences would be the same for the mutant strain *A. tumefaciens* RU-ORPN1. This latter strain was selected on the basis of a change in the regulation of the enzyme expression, and the mutation in this strain is therefore assumed to be in the regulatory DNA sequences of the genome and not within the *NcaR1* or *NcaR2* gene sequences.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Lysozyme was purchased from Boeringer Mannheim, Triton X-100 was purchased from BDH Chemicals Ltd. and Tween-20 was purchased from Merck Laboratory Supplies (PTY) Ltd. Chymotrypsin and phospholipase were purchased from Sigma Chemical Co. All reagents were analytical grade.

4.2.2 Methods

4.2.2.1 Investigation of the enzyme activities in the pellet and the supernatant of the enzyme extract Enzyme extract was produced by the disruption of frozen cells (10mL, 40mg/mL) by 5 minutes sonication as described in Chapter 3, section 3.2.2.4. Cell lysis was investigated by observing a sample of the cells under a light microscope before and after sonication. The cells were centrifuged at 13 000g for 3 minutes before and after 1, 3 and 5 minutes sonication. Cell lysis was further investigated by subjecting the resulting supernatants to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The resulting pellet was resuspended in the original volume of potassium phosphate buffer (0.1M, pH 9) and the pellet (1mL) and supernatant (1mL) were assayed for hydantoinase and *N*-carbamoylase activities as described for the enzyme extract in Chapter 3, section 3.2.2.6. The percentages of the enzyme activities present in the supernatant were calculated relative to the total enzyme activities in the enzyme extract. The protein concentration in the supernatant before and after 5 minutes sonication was determined.

4.2.2.2 Effect of bead-beating on the enzyme activities in the supernatant of the enzyme extract

Whole cells (7mL, 40mg/mL) resuspended in potassium phosphate buffer (0.1M, pH 9) were disrupted with glass beads of 0.25mm diameter in a glass bead homogenizer (BioSpec Products, Inc., Oklahoma, USA) for 0, 5, 10, 15, 20, 25 and 30 minutes. The samples were centrifuged at 13 000g for 3 minutes. The hydantoinase and *N*-carbamoylase activities in the resulting pellets and supernatants were assayed as described in section 4.2.2.1. The protein concentrations were assayed in the supernatants. The percentages of the enzyme activities present in the supernatants were calculated relative to the total enzyme activities in the enzyme extract.

4.2.2.3 Effect of french-pressing on the enzyme activities in the supernatant of the enzyme extract

Whole cells (21mL, 40mg/mL) resuspended in potassium phosphate buffer (0.1M, pH 9) were disrupted by three passes through a french pressure cell (Yeda-Press, (LINCA Lamon Instrumentations Co., Tel Aviv, Israel) at 15kPa and 4^0C. Samples (7mL) were collected after each pass and centrifuged at 13 000g for 3 minutes. The hydantoinase and *N*-carbamoylase activities in the resulting pellets and supernatants were assayed as described in section 4.2.2.1. The protein concentrations were assayed in the supernatants. The percentages of the enzyme activities present in the supernatants were calculated relative to the total enzyme activities in the enzyme extract.

4.2.2.4 Effect of increasing exposure to sonication on the enzyme activities in the supernatant of the enzyme extract

Whole cells (7mL, 40mg/mL) resuspended in potassium phosphate buffer (0.1M, pH 9) were disrupted by 0, 5, 10, 15, 20, 25 and 30 minutes sonication using 10 second blasts and 10 second rests at 4^0C using a Vibria cell sonicator (Sonics and Materials Inc., Danbury, USA). The samples were centrifuged for 3 minutes at 13 000g. The hydantoinase and *N*-carbamoylase activities in the resulting pellets and supernatants were assayed as described in section 4.2.2.1. The protein concentrations were assayed in the supernatants. The percentages of the enzyme activities present in the supernatants were calculated relative to the total enzyme activities in the enzyme extract.

4.2.2.5 Effect of lysozyme disruption of *A. tumefaciens* RU-ORPN1 cells on the soluble enzyme activity levels

Whole cells (20mg/mL) resuspended in 10mL potassium phosphate buffer (0.1M, pH 9) containing 1mg/mL lysozyme were incubated for 30 minutes at 37^0C. Following incubation, the cells were centrifuged for 20 minutes at 13 000g at 25^0C. The resulting supernatant (periplasmic fraction) was kept on ice for enzyme and protein assays. The pellet, consisting of protoplasts, was resuspended in 10mL of the same ice cold buffer in order to lyse the protoplasts. The lysate was centrifuged at 13 000g for 20 minutes. The resulting supernatant (cytosolic fraction) and pellet (membrane fraction) were kept on ice for enzyme and protein assays. The various fractions (1mL) were assayed for hydantoinase and *N*-carbamoylase activities (section 4.2.2.1) and protein concentration.

4.2.2.6 The effects of DTT, PMSF and EDTA on the enzyme activities in the supernatant of the enzyme extract

Enzyme extracts were prepared as described in section 4.2.2.1 in the presence of 0.5mM DTT, 1mM PMSF and 1mM EDTA and centrifuged for 3 minutes at 13 000g. The hydantoinase and *N*-carbamoylase activities in the resulting pellets and supernatants were assayed as described in section 4.2.2.1. The protein concentrations were assayed in the supernatants. The percentages of the enzyme activities present in the supernatants were calculated relative to the total enzyme activities in the enzyme extract.

4.2.2.7 Effect of pH on the enzyme activities in the supernatant of the enzyme extract

Enzyme extracts (7mL) were prepared as described in section 4.2.2.1, except potassium phosphate buffer (0.1M) at pH 4 , pH 6, pH 8 and pH 10 was used. The enzyme extracts were then centrifuged for 3 minutes at 13 000g. The hydantoinase and *N*-carbamoylase activities in the resulting pellets and supernatants were assayed as described in section 4.2.2.1. The protein concentrations were assayed in the supernatants. The percentages of the enzyme activities present in the supernatants were calculated relative to the total enzyme activities in the enzyme extract.

4.2.2.8 Effect of ionic strength on the enzyme activities in the supernatant of the enzyme extract

Enzyme extracts (7mL) were prepared as described in section 4.2.2.1, except potassium phosphate buffer (pH 9) at ionic strengths of 0.01M, 0.1M and 1M was used. An extract was also prepared using deionised water, as a control. The extracts were then centrifuged at 13 000g for 3 minutes. The hydantoinase and *N*-carbamoylase activities in the resulting pellets and supernatants were assayed as described in section 4.2.2.1. The protein concentrations were assayed in the supernatants. The percentages of the enzyme activities present in the supernatants were calculated relative to the total enzyme activities in the enzyme extract.

4.2.2.9 Effect of hydrolysing enzymes on the enzyme activities in the supernatant of the enzyme extract

Enzyme extract (40mL) prepared as described in section 4.2.2.1 was incubated in the presence of chymotrypsin (0.5μg/mL) for 24 h at 25^{0}C in potassium phosphate buffer (0.1M, pH 7.8). Samples (8mL) were collected after 0, 3, 6, 12 and 24 h incubation and centrifuged at 13 000g for 3 minutes. The enzyme extract (40mL) was also incubated in the presence of phospholipase (1μg/mL) for 24 h at 37^{0}C in potassium phosphate buffer (0.1M, pH 8). Samples were collected after 0, 3, 6, 12 and 24 h incubation and centrifuged at 13 000g for 3 minutes. The hydantoinase and *N*-carbamoylase activities in the resulting pellets and supernatants were assayed as described in section 4.2.2.1. The percentages of the enzyme activities present in the supernatants were calculated relative to the total enzyme activities in the enzyme extract.

4.2.2.10 Effect of non-ionic detergents on the enzyme activities in the supernatant of the enzyme extract

Enzyme extracts (7mL) were prepared as described in section 4.2.2.1 in the presence of 0, 1, 2 and 3% Triton X-100 and in the presence of 0, 1, 2 and 3% Tween-20 and the various enzyme extracts were centrifuged at 13 000g for 3 minutes. The hydantoinase and *N*-carbamoylase activities in the resulting pellets and supernatants were assayed as described in section 4.2.2.1. The protein concentrations were assayed in the supernatants. The percentages of the enzyme activities present in the supernatants were calculated relative to the total enzyme activities in the enzyme extract.

4.2.2.11 Hydropathy and transmembrane prediction plots of *A. tumefaciens* RU-OR *N*-carbamoylase predicted amino acid sequences

Two genes coding for the *N*-carbamoylase protein in *A. tumefaciens* RU-OR were identified, cloned and sequenced by Hartley, (2001). From these DNA sequences, the predicted amino acid sequences for the two genes, *NcaR1* and *NcaR2*, were generated (Hartley, 2001) and are shown in Appendix E. Hydropathy plots of the predicted amino acid sequences were generated according to the method of Kyte and Doolittle (1982). Transmembrane prediction plots were generated according to the method of Engelman, Steitz and Goldman (Engelman *et al.*, 1986).

4.2.2.12 Protein determinations and calculation of specific activity

The concentration of soluble protein in the supernatants of the various enzyme extracts were determined using the Bradford method (Bradford, 1976). Standard protein solutions were prepared using bovine serum

albumin in 100: L volumes. Bradford's reagent (1mL) was added to each standard, and to the supernatant samples (100: L), and the absorbance of each was read at 595nm after 2 minutes. The protein concentration in the samples was then calculated by interpolation from the protein standard curve (Appendix D). Protein determinations were conducted in duplicate and mean values are reported. All spectrophotometric measurements were made using a Shimadzu UV-160A spectrophotometer.

Specific hydantoinase and *N*-carbamoylase activities were calculated and are expressed as Units (enzyme activity)/mg protein where Units are expressed as : mol product produced per minute over 4 hours (linear portion of enzyme reaction, section 3.3.4, Chapter 3).

4.3 RESULTS AND DISCUSSION

4.3.1 Investigation of the enzyme activities in the pellet and supernatant of the enzyme extract

The hydantoinase and *N*-carbamoylase activities in the pellet and supernatant of the enzyme extract, which comprised the complete cell lysate, were assayed in order to evaluate the solubilities of the enzymes. The solubility of the enzymes was assessed by determining the amounts of enzyme activity present in the supernatant as a percentage of the total activities present in the enzyme extract (Table 4.1). The hydantoinase activity measured in the supernatant ranged from 5% to 28% of the total hydantoinase activity in the enzyme extract, over 6 experiments. The *N*-carbamoylase present in the supernatant ranged from 3% to 17% of the total *N*-carbamoylase activity in the enzyme extract, over 6 experiments. The low amounts of the enzymes present in the supernatant from the enzyme extract indicated that the enzymes were either present in the homogenate in an aggregated (but active) form, or were membrane-associated, causing the majority of the enzyme activity to remain in the cell debris. Incomplete cell lysis could also have resulted in the pelleting of unlysed cells, resulting in enzyme activity in the pellet.

Photographs of *A. tumefaciens* RU-ORPN1 cells taken under a light microscope at 100× magnification before and after sonication are shown in Figure 4.1. Although the low resolution of light microscopy is too low to prove cell disintegration, the general morphologies and densities of the cells appeared to change after sonication suggesting that the cells were successfully lysed. Cell lysis was further investigated by subjecting the supernatant of the cell suspension to SDS-PAGE before and after one, three and five minutes sonication. The protein bands resulting from the release of proteins from the lysed cells are clearly

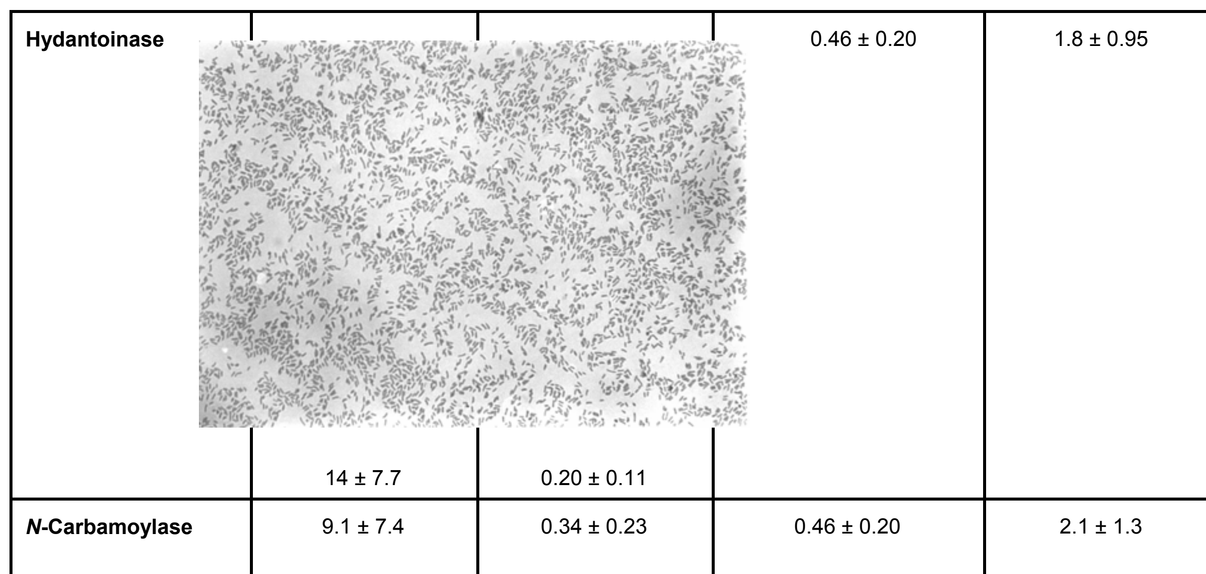
visible in Figure 4.2. Furthermore, the protein yield measured in the supernatant of the enzyme extract after 5 minutes sonication, relative to the concentration of the cell suspension before sonication (Table 4.1), was found to be similar to that reported by Sareen *et al.* (2001) for an *Agrobacterium* strain. These authors reported a protein yield of 6% (w/w) relative to the concentration of the cell suspension after disruption of the cells by sonication. This was in the same order of magnitude as the 1% (w/w) protein yield reported in this study. However, the average specific activity measured for the *A. tumefaciens* RU-ORPN1 hydantoinase was amongst the lowest reported in the literature in supernatants produced after sonication in other strains, which range from 0.105 nmol *N*-carbamyl-$-alanine/min/mg protein (Xu and West, 1994) to 0.18 : mol ureidic acid/min/mg protein (Luksa *et al.*, 1997). The *A. tumefaciens* RU-ORPN1 *N*-carbamoylase specific activity (Table 4.1) was considerably lower than the specific activities reported in supernatants after sonication of other strains. Specific activities for the *N*-carbamoylases from an *Agrobacterium* sp. (Sareen *et al.*, 2001) and from a *Comamonas* sp. (Ogawa *et al.*, 1993) were 0.033 : mol D-phenylglycine/min/mg protein and 2.9 : mol D-HPG/min/mg protein, respectively, after sonication. These specific activities were 10 and 1000 times higher than the specific activity reported for this enzyme in this study.

Since the cells appeared to be successfully lysed and the majority of the enzyme activities remained in the cell pellet after sonication, the enzymes were assumed to be insoluble or membrane bound. The low specific activities measured in the supernatant after sonication in comparison to those reported in the literature further substantiated this assumption. This novel property of these enzymes has been reported for the wild-type strain *A. tumefaciens* RU-OR (Burton *et al.*, 1998). No other reports of insoluble hydantoinases or *N*-carbamoylases of this nature have been made in the literature for wild-type strains, although other researchers have experienced some difficulty in solubilising these enzymes (O. May and C. Syldatk, *pers. comm.*). The formation of inclusion bodies (Pietzch *et al.*, 2000) or insoluble protein aggregates (Chao *et al.*, 2000a) on expressing these enzymes in *E.coli* has been reported.

Table 4.1 Average hydantoinase and *N*-carbamoylase activities and protein concentration in the supernatant of the enzyme extract.

Enzyme	Percentage of total activity in supernatant*	Product yield (: mol/mL)*	Protein concentration (mg/mL)*	Specific activity (10^{-3} Units#/mg protein)*

Hydantoinase	14 ± 7.7	0.20 ± 0.11	0.46 ± 0.20	1.8 ± 0.95
***N*-Carbamoylase**	9.1 ± 7.4	0.34 ± 0.23	0.46 ± 0.20	2.1 ± 1.3



* Errors represent SEM (n=18)

Units are expressed as : mol product produced per minute over 4 hours

A

B

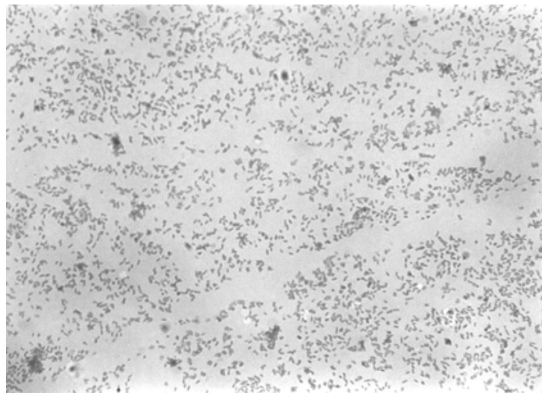


Figure 4.1 Photographs of *A. tumefaciens* RU-ORPN1 cells taken under a light microscope before (**A**) and after (**B**) sonication (100× magnification).

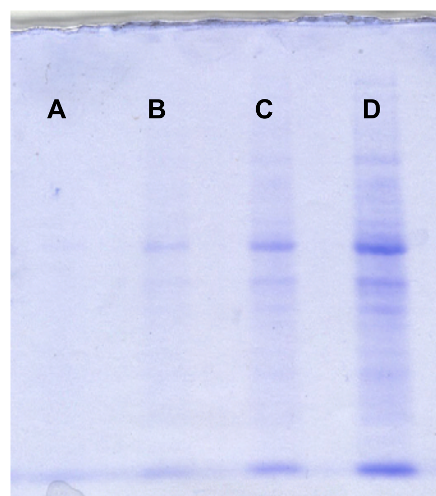


Figure 4.2 SDS-PAGE of *A. tumefaciens* RU-ORPN1 supernatants produced after 0 (**A**), 1(**B**), 3 (**C**) and 5 (**D**) minutes sonication of frozen cells.

4.3.2 Effect of bead-beating on the enzyme activities in the supernatant of the enzyme extract

Hydantoinases from *Pseudomonas* sp. (Takahashi *et al.*, 1978), *Arthrobacter* sp. (May *et al.*, 1998d; Siemann *et al.*, 1999) and *Blastobacter* sp. (Soong *et al.*, 1999) cells have been successfully released in soluble form by bead-beating. Bead-beating has also been successfully used for the disruption and release of soluble *N*-

carbamoylase enzymes from *Blastobacter* sp. (Ogawa *et al.*, 1994b). Bead-beating was therefore evaluated as a means of disrupting the *A. tumefaciens* RU-ORPN1 cells by determining the hydantoinase and *N*-carbamoylase activities released into the supernatant, expressed as percentages of the total enzyme activities in the enzyme extract, with increasing bead-beating time (Figure 4.3). The effect of increasing the bead-beating time on the protein concentrations and specific activities in the supernatants was also investigated (Table 4.2). Bead-beating resulted in a significant increase in protein concentration over the first 10 minutes, after which the protein concentration remained fairly constant suggesting that cell lysis was complete. The hydantoinase activity in the supernatant reached a maximum of 8.7% of the total hydantoinase activity in the enzyme extract, after 5 minutes bead-beating while the *N*-carbamoylase reached a maximum in the supernatant of 3.5% of the total *N*-carbamoylase activity in the enzyme extract, after 10 minutes (Figure 4.3). Only very small quantities of active, solubilised hydantoinase and *N*-carbamoylase were therefore released into the supernatant by bead-beating. Further bead-beating resulted in the gradual loss of both enzyme activities, probably due to denaturation of the enzymes.

The protein yielded (0.322mg/mL) after 30 minutes bead-beating relative to the cell concentration (40mg/mL) was 0.8% (w/w)(Table 4.2). This protein yield was within the range of protein yields relative to cell concentrations reported in the literature which range from 0.74% (w/w)(Soong *et al.*, 1999) to 7% (w/w)(Ogawa *et al.*, 1994b) after disruption of cells by bead-beating. The hydantoinase specific activities in supernatants reported in the literature after disruption of cells by bead-beating range from 0.064 : mol *N*-carbamyl-$-alanine/min/mg (Soong *et al.*, 1999) to 5.2 : mol phenylglycine/min/mg (Siemann *et al.*, 1999). The highest *A. tumefaciens* RU-ORPN1 hydantoinase specific activity (0.007 : mol/min/mg) was therefore considerably lower than those reported in the literature (Table 4.2). The highest *A. tumefaciens* RU-ORPN1 *N*-carbamoylase specific activity was 0.002 : mol/min/mg which was similar to the *N*-carbamoylase specific activity reported for a *Blastobacter* sp. of 0.001 : mol D-HPG/min/mg (Ogawa *et al.*, 1994b) after bead-beating. However, these *N*-carbamoylase specific activities were low in comparison to the specific activities reported in the literature for these enzymes in other strains (Sareen *et al.*, 2001; Ogawa *et al.*, 1993).

Disruption of *A. tumefaciens* RU-ORPN1 cells by bead-beating resulted in low levels of hydantoinase and *N*-carbamoylase activity in the supernatant of the resulting enzyme extract and the specific activities of the enzymes in the supernatant were low in comparison to those reported for these enzymes in the literature

in enzyme extracts produced by this method. Bead-beating was therefore not suitable for the release of high levels of active, soluble enzymes into the supernatant of the enzyme extract.

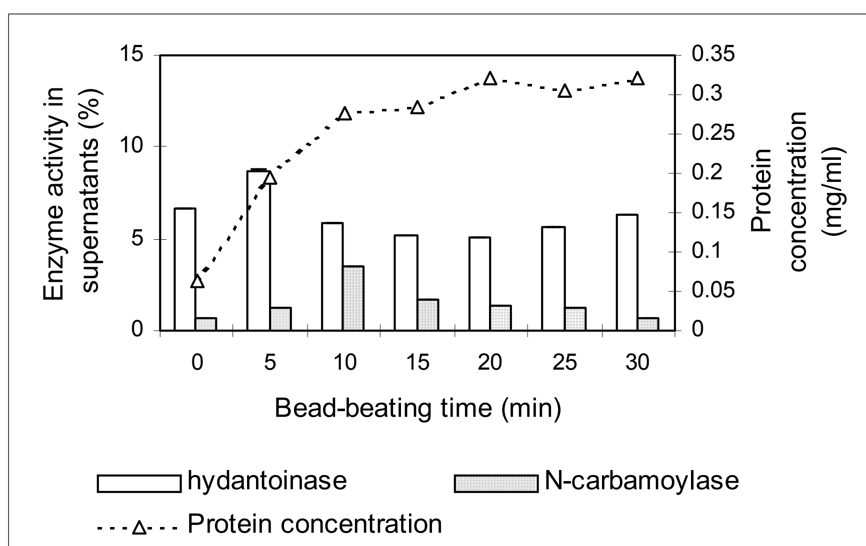


Figure 4.3 Hydantoinase and *N*-carbamoylase activities in the supernatants after increasing exposure to bead-beating, expressed as percentages of the total hydantoinase and *N*-carbamoylase activities in the enzyme extract.

Table 4.2 Hydantoinase and *N*-carbamoylase specific activities in the supernatant after increasing bead-beating time.

Time (min)	HYD product yield (: mol/mL)	NCAAH product yield (: mol/mL)	Protein concentration (mg/mL)	HYD specific activity (10^{-3} Units/mg protein)*	NCAAH specific activity (10^{-3} Units/mg protein)*
0	0.086	0.025	0.062	5.8	1.7
5	0.114	0.045	0.195	7.1	0.96
10	0.076	0.126	0.276	1.1	1.9
15	0.068	0.061	0.285	1.0	0.89
20	0.066	0.051	0.322	0.85	0.66
25	0.073	0.045	0.304	1.0	0.62
30	0.082	0.025	0.322	1.1	0.33

***** Units are expressed as : mol product produced per minute over 4 hours
HYD = hydantoinase
NCAAH = *N*-carbamoylase

4.3.3 Effect of french-pressing on the enzyme activities in the supernatant of the enzyme extract

Hydantoinases have been successfully released from *Agrobacterium* sp. (Runser and Ohleyer, 1990; Durham and Weber, 1995) and *Bacillus* sp. (Lee *et al.*, 1995) by french-pressing. Similarly, french-pressing has been used for the successful disruption and release of soluble *N*-carbamoylase enzymes from *Agrobacterium* sp. (Kim and Kim, 1994; Louwrier and Knowles, 1996). The effect of french-pressing on the release of active, soluble enzymes from *A. tumefaciens* RU-ORPN1 cells was therefore evaluated. The hydantoinase and *N*-carbamoylase activities in the supernatant, expressed as percentages of the total enzyme activities in the enzyme extract, measured after repeated passes through the french-press are shown in Figure 4.4. The protein concentrations and specific activities in the supernatants were also calculated (Table 4.3).

The protein concentration in the supernatant increased after the first pass through the french-press indicating that cell lysis had occurred. The protein concentration increased only slightly after repeated passes through the press, indicating lysis of a small percentage of unlysed cells still remaining in the extract. The highest hydantoinase specific activity in the supernatant was 0.007 : mol/min/mg (Table 4.3) which was considerably lower than the specific activities of 2.6 : mol NCG/min/mg reported for the hydantoinase from *Bacillus stearothermophilus* SD-1 (Lee *et al.*, 1995) and 1.5 : mol *N*-carbamyl D-alanine/min/mg for the hydantoinase from *Agrobacterium tumefaciens* 47C (Durham and Weber, 1995), in supernatants after french-pressing.

The hydantoinase activity in the supernatant reached a maximum of only 2% of the total hydantoinase activity in the enzyme extract, after the first pass through the press, and then gradually lost activity with repeated passes through the press, probably due to denaturation of the enzyme (Figure 4.4). *N*-carbamoylase activity could not be detected in the supernatant at all after french-pressing of the *A. tumefaciens* RU-ORPN1 cells, possibly due to denaturation of any soluble enzyme released under the high pressure conditions. French-pressing therefore resulted in very low yields of enzyme activity in the supernatant and the specific activities of the enzymes were also low in comparison to those reported in the literature.

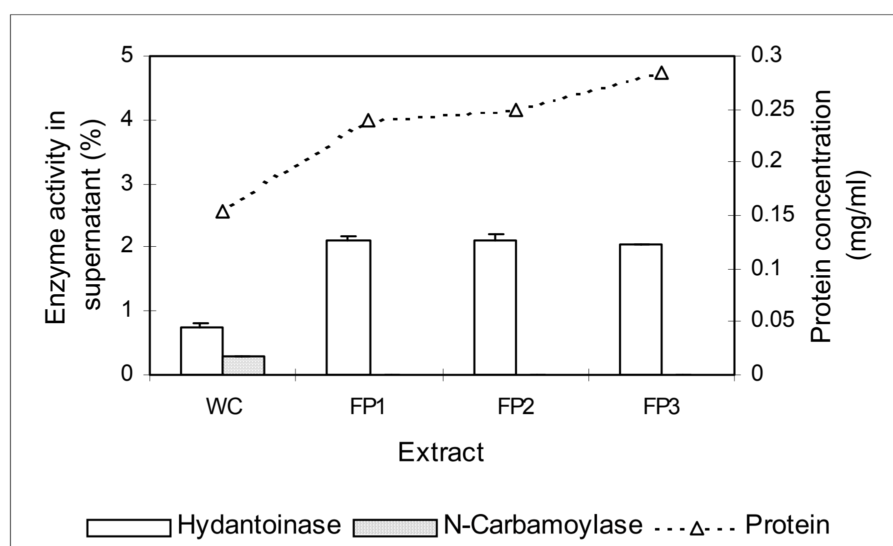


Figure 4.4 Hydantoinase and *N*-carbamoylase activities in the supernatant after repeated french-pressing, expressed as percentages of the total hydantoinase and *N*-carbamoylase activities in the enzyme extract. WC = whole cells; FP1 = french-pressed once; FP2 = french-pressed twice; FP3 = french-pressed three times.

Table 4.3 Hydantoinase and *N*-carbamoylase specific activities in the supernatant after repeated french-pressing.

Number of passes through french-press	HYD product yield (: mol/mL)	NCAAH product yield (: mol/mL)	Protein concentration (mg/mL)	HYD specific activity (10^{-3} Units/mg protein)*	NCAAH specific activity (10^{-3} Units/mg protein)*
0	0.142	0.017	0.154	3.8	0.4
1	0.386	ND	0.240	6.7	-
2	0.386	ND	0.250	6.4	-
3	0.378	ND	0.285	5.5	-

* Units are expressed as : mol product produced per minute over 4 hours
HYD = hydantoinase
NCAAH = *N*-carbamoylase
ND = not detected

4.3.4 Effect of increasing exposure to sonication on the enzyme activities in the supernatant of the enzyme extract Hydantoinase enzymes have been successfully released from *Arthrobacter* sp. (Möller *et al.*, 1988), *Pseudomonas* sp. (Morin *et al.*, 1986; Xu and West, 1994; Ogawa *et al.*, 1995a) and *Bacillus* sp. (Sharma and Vohra, 1997; Luksa *et al.*, 1997) in soluble form by sonication. Similarly, sonication has been used for the successful release of soluble *N*-carbamoylase enzymes from *Comamonas* sp. (Ogawa *et al.*, 1993), *Pseudomonas* sp. (Ikenaka *et al.*, 1998b) and *Agrobacterium* sp. (Sareen *et al.*, 2001). The levels of soluble hydantoinase and *N*-carbamoylase in the supernatant after 5 minutes sonication of *A. tumefaciens* RU-ORPN1 cells were found to be low in comparison to those reported in the literature for these enzymes after cell disruption by sonication (section 4.3.1). The effect of increasing the sonication time on the levels of soluble enzyme was therefore evaluated.

The hydantoinase specific activity in the supernatant increased from 0.73×10^{-3} μmol/min/mg after 5 minutes to 1.2×10^{-3} μmol/min/mg after 15 minutes, while the *N*-carbamoylase specific activity in the supernatant increased from 0.9×10^{-3} μmol/min/mg after 5 minutes to 2×10^{-3} μmol/min/mg after 10 minutes (Table 4.4). Increasing the sonication time therefore slightly increased the specific activities of the enzymes in the supernatant but the specific activities remained low in comparison to those reported in the literature (section 4.3.1) for these enzymes after sonication.

The hydantoinase and *N*-carbamoylase activities in the supernatants, expressed as percentages of the total enzyme activities in the enzyme extract, measured after increasing exposure to sonication, and

protein concentrations, are shown in Figure 4.5. The hydantoinase activity reached a maximum in the supernatant of 8% of the total hydantoinase activity after 15 minutes sonication, and the *N*-carbamoylase activity reached a maximum in the supernatant of 7.7% of the total *N*-carbamoylase activity after 25 minutes sonication. Although increasing the sonication time slightly improved the levels of soluble hydantoinase and *N*-carbamoylase detected in the supernatant, the majority of the enzyme activities remained in the cell pellet. Sonication produced the highest levels of soluble hydantoinase and *N*-carbamoylase in the supernatant of the three mechanical disruption techniques tested and this method was used in all subsequent experiments for cell lysis.

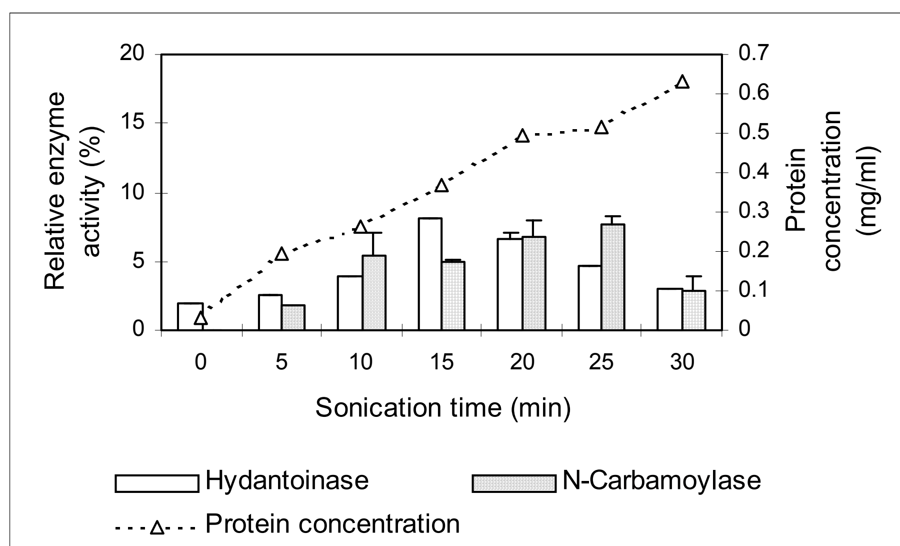


Figure 4.5 Hydantoinase and *N*-carbamoylase activities in the supernatant after increasing sonication times, expressed as percentages of the total hydantoinase and *N*-carbamoylase activities in the enzyme extract.

Table 4.4 Hydantoinase and *N*-carbamoylase specific activities in the supernatant after increasing sonication time.

Time (min)	HYD product yield (: mol/mL)	NCAAH product yield (: mol/mL)	Protein concentration (mg/mL)	HYD specific activity (10^{-3} Units/mg protein)*	NCAAH specific activity (10^{-3} Units/mg protein)*
0	0.025	ND	0.034	3.1	-
5	0.034	0.042	0.195	0.73	0.90
10	0.052	0.126	0.264	0.82	2.0
15	0.106	0.116	0.370	1.2	1.3
20	0.086	0.159	0.493	0.73	1.3
25	0.061	0.179	0.518	0.49	1.4
30	0.049	0.067	0.629	0.33	0.44

***** Units are expressed as : mol product produced per minute over 4 hours
HYD = hydantoinase
NCAAH = *N*-carbamoylase
ND = not detected

4.3.5 Effect of lysozyme disruption on the enzyme solubilities in the enzyme extract

Lysozyme treatment of *A. tumefaciens* RU-ORPN1 cells was evaluated as an alternative method for the disruption and release of the hydantoinase and *N*-carbamoylase enzymes from the cells. The various cell fractions produced after cell lysis using lysozyme were assayed for hydantoinase and *N*-carbamoylase activities (Figure 4.6). Of the three fractions collected after the lysozyme disruption, the membrane fraction contained 64% and 97% of the total hydantoinase and *N*-carbamoylase activities, respectively. The periplasmic fraction contained 22% of the hydantoinase activity and 2.5% of the *N*-carbamoylase activity and the cytoplasmic fraction contained 14% of the hydantoinase and 0.5% of the *N*-carbamoylase activities. Thus, the major proportion of the hydantoinase and *N*-carbamoylase activities was present in the cell membrane fraction, again confirming their association with the insoluble cell components. Lysozyme disruption was therefore ineffective in terms of releasing the enzymes in an active, soluble form into the supernatant.

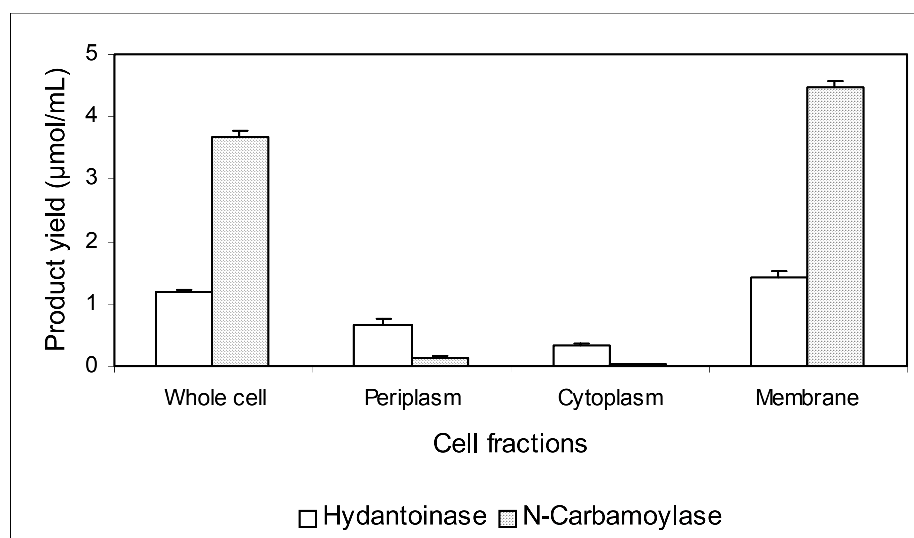


Figure 4.6
Hydantoinase and *N*-carbamoylase activities present in the cell fractions after lysozyme disruption of *A. tumefaciens* RU-ORPN1 cells.

4.3.6 Effects of DTT, PMSF and EDTA on the enzyme activities in the supernatant of the enzyme extract

The effect of minimising proteolytic activity in the enzyme extract in order to optimise the levels of the active, soluble enzymes was investigated by producing the extract in the presence of various protease inhibitors. EDTA, a commonly used metal chelator (Scopes, 1987), was used for the inhibition of metalloproteases, and PMSF was added to the enzyme extract for the inhibition of serine proteases. PMSF is commonly used in protocols for the purification of the hydantoinase enzyme (Runser and Meyer, 1993; Lee and Kim, 1998; Abendroth *et al.*, 2000a). The enzyme extract was also produced in the presence of a reducing agent, DTT, which has been used extensively in the literature dealing with the purification of these enzymes to prevent oxidation and denaturing of the *N*-carbamoylase, in particular (Ogawa *et al.*, 1993; Louwrier and Knowles, 1996; Nanba *et al.*, 1998a; Soong *et al.*, 1999; Sareen *et al.*, 2000; Kim and Kim, 1995).

The enzyme extract was prepared in the presence of 0.5mM DTT, 1mM PMSF and 1mM EDTA. These concentrations of DTT and PMSF were previously found to be optimal for enzyme activity in the enzyme extract (Chapter 3, section 3.3.10). The percentages of the total hydantoinase and *N*-carbamoylase activities present in the supernatants were calculated and the effect of the additives on the protein

concentration in the supernatants was evaluated (Figure 4.7). The addition of DTT and EDTA resulted in decreased levels of soluble enzyme activities detected in the supernatants in comparison to the control having no additives. The hydantoinase activity in the supernatant decreased from 8.2% of the total hydantoinase activity in the control, to 4.9% and 4.3% of the total activity in the supernatants containing DTT and EDTA, respectively. The *N*-carbamoylase activity in the supernatant decreased from 17% of the total *N*-carbamoylase activity in the control, to 0.5% and 11% of the total activity in the supernatants containing DTT and EDTA, respectively. In the enzyme extract treated with PMSF, the hydantoinase activity in the supernatant increased slightly, from 8.2% of the total activity in the control, to 8.4% of the total activity, in the supernatant containing PMSF. The *N*-carbamoylase activity in the supernatant containing PMSF decreased from 17% of the total *N*-carbamoylase activity in the control, to 8.3% of the total activity.

The protein concentrations in the supernatants were unaffected by the presence of the additives. The specific activity of the hydantoinase enzyme in the supernatant increased in the presence of PMSF (Table 4.5). In every other case, the specific activities of the enzymes in the supernatants decreased in the presence of the additives (Table 4.5). Therefore, in most cases the additives did not promote the release of active, soluble enzymes into the supernatant. In fact, with additives present, a loss of soluble enzyme activity was observed in the supernatants since the specific activities decreased. The lack of effect of the DTT indicated that the enzymes were probably only susceptible to very low levels of oxidation during the enzyme assay time. The effect of the reducing agent would probably be better evaluated over an extended period where the enzymes would be exposed to oxidising conditions for longer. The lack of effect of the protease inhibitors suggests that the enzymes may not have been exposed to proteolytic attack; alternatively, the lack of effect could have been due to the alkaline pH of the enzyme extract which was prepared in potassium phosphate buffer, pH 9, since proteases generally have little activity at pH 9-10 (Scopes, 1987).

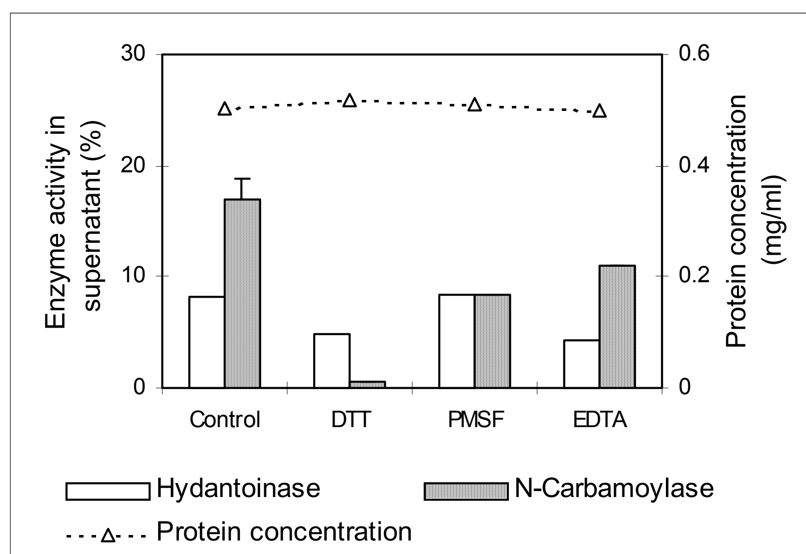


Figure 4.7 Hydantoinase and *N*-carbamoylase activities present in the supernatants, expressed as percentages of the total activities in the enzyme extracts, after treatment of the enzyme extract with DTT, PMSF and EDTA.

Table 4.5 Hydantoinase and *N*-carbamoylase specific activities in the supernatant after production of the enzyme extract in the presence of DTT, PMSF and EDTA.

Additive	HYD product yield (: mol/mL)	NCAAH product yield (: mol/mL)	Protein concentration (mg/mL)	HYD specific activity (10^{-3} Units/mg protein)*	NCAAH specific activity (10^{-3} Units/mg protein)*
None	0.045	0.429	0.503	0.37	3.5
DTT	0.045	0.013	0.517	0.36	0.13
PMSF	0.136	0.329	0.509	1.1	2.7
EDTA	0.013	0.165	0.500	0.13	1.4

* Units are expressed as : mol product produced per minute over 4 hours

HYD = hydantoinase

NCAAH = *N*-carbamoylase

4.3.7 Effect of pH on the enzyme activities in the supernatant of the enzyme extract

The *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase were assumed to be membrane-bound or insoluble as a result of the previous experiments, since after each treatment, the enzymes remained associated with the particulate parts of the cell after cell disruption. The solubility of a protein molecule in an aqueous environment is determined by the relative proportions and distribution of charged hydrophilic and hydrophobic groups on its surface (Harris and Angal, 1994). Peripheral membrane proteins are associated with membranes via these electrostatic and hydrophobic interactions. Therefore, by altering the charges on the surfaces of proteins, the electrostatic or hydrophobic interactions can be disrupted, releasing the proteins from the membranes. At the pH equal to the pI of a protein, the net charge on the surface of the protein is neutral and the electrostatic interactions with other molecules are minimised, often resulting in the aggregation and precipitation of the protein molecules. The protein is, therefore, in its least soluble form at its pI. Conversely, at pHs above or below the pI of a protein, the charges on the surface will be predominantly negative or positive, respectively, and the electrostatic forces of attraction and repulsion between the protein and other molecules will be maximised. The effects of pH and ionic strength on the solubilities of the *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase were therefore determined by comparing the percentages of the total hydantoinase and *N*-carbamoylase activities present in the supernatant at various pH and ionic strength of the enzyme extract. This study describes the effect of pH on the enzyme solubilities.

The pI values of the *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase were unknown and therefore enzyme extracts were prepared in potassium phosphate buffer in a pH range from 4 to 10 in order to assess the solubilities of the enzymes in a range of pH where the enzymes would most likely have different charges on their surfaces, and therefore different solubilities. The protein concentration and hydantoinase and *N*-carbamoylase activities measured in the supernatant of each enzyme extract, expressed as percentages of the total hydantoinase and *N*-carbamoylase activities in the enzyme extracts, are shown in Figure 4.8. The specific activities of the enzymes in the supernatants are listed in Table 4.6. The pH of the enzyme extracts had some effect on the enzyme solubilities with the hydantoinase activity in the supernatants varying from 23% of the total hydantoinase activity at pH 10 to 40% of the total hydantoinase activity at pH 4. The *N*-carbamoylase activity in the supernatants varied from 0% of the total *N*-carbamoylase activity at pH 4 and 10 to 12% of the total activity at pH 8. The pH values at which the enzymes were most soluble were therefore pH 4 and pH 8 for the hydantoinase and *N*-carbamoylase,

respectively. The hydantoinase and *N*-carbamoylase had optimal activity at pH 9 (see Chapter 3, section 3.3.6) and thus the pH at which the hydantoinase was most soluble did not correlate with the pH for optimal activity of this enzyme. The *N*-carbamoylase was most soluble at pH 8 which was close to its optimal pH for activity.

The protein concentration in the supernatant was highest at pH 6 and was significantly reduced at pH 4 probably due to the denaturation and precipitation of proteins out of the supernatant. The specific activity of the hydantoinase was highest at pH 4. This pH was therefore found to favourable for the partial solubilisation of the enzyme as well as for partial isolation of the enzyme. The specific activity of the *N*-carbamoylase was highest at pH 8. However, the specific activities of the enzymes remained considerably lower than the specific activities reported in the literature for these enzymes after cell disruption (Ogawa *et al.*, 1993; Luksa *et al.*, 1997; Sareen *et al.*, 2001).

These data indicate that the hydantoinase may be a peripheral membrane protein since pH alteration partially improved the solubility of the enzyme. The *N*-carbamoylase, on the other hand, appeared to be bound more tightly to the membranes than by electrostatic or hydrophobic interactions and therefore was unlikely to be a peripheral membrane protein.

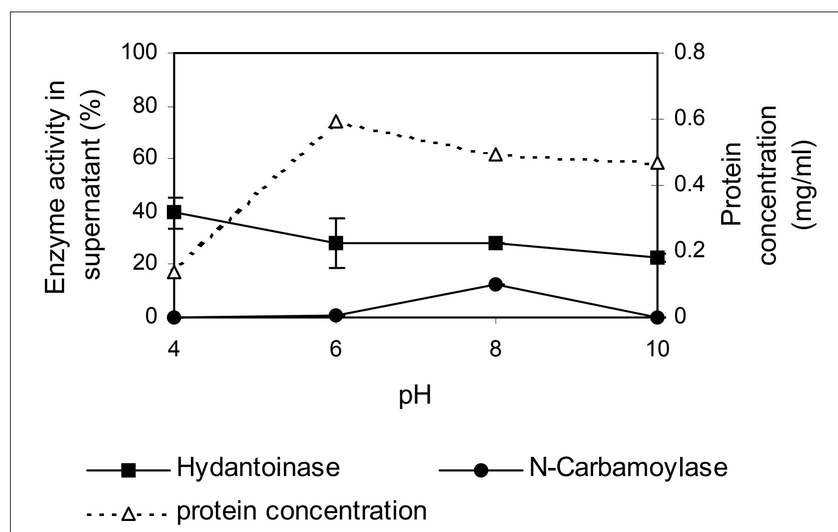


Figure 4.8 Hydantoinase and *N*-carbamoylase activities present in the supernatant, expressed as percentages of the total hydantoinase and *N*-carbamoylase activities in the enzyme extracts, after the production of the enzyme extracts at different pH.

Table 4.6 Hydantoinase and *N*-carbamoylase specific activities in the supernatant after production of the enzyme extract at various pH.

pH	HYD product yield (: mol/mL)	NCAAH product yield (: mol/mL)	Protein concentration (mg/mL)	HYD specific activity (10^{-3} Units/mg protein)*	NCAAH specific activity (10^{-3} Units/mg protein)*
4	0.338	ND	0.137	10.3	-
6	0.294	0.024	0.594	2.1	0.17
8	0.353	0.256	0.493	3.0	2.2
10	0.215	ND	0.468	1.9	**-**

* Units are expressed as : mol product produced per minute over 4 hours

HYD = hydantoinase

NCAAH = *N*-carbamoylase

ND = not detected

4.3.8 The effect of ionic strength on the enzyme activities in the supernatant of the enzyme extract

Proteins are commonly precipitated from aqueous solutions by increasing the ionic strength of the solution. The precipitation is dependent on the hydrophobic nature of the surface of the protein. Water interacts with the hydrophobic regions on the surfaces of proteins but as salt ions are added to the system, the water solvates the salt ions, exposing the hydrophobic regions. Interactions between these hydrophobic regions then cause proteins to aggregate and precipitate (Harris and Angal, 1994). Changes in ionic strength, therefore, alter the polar nature of proteins, thereby changing their solubility.

The effect of changing the ionic strength of the potassium phosphate buffer used for the preparation of the enzyme extract on the solubility of the *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase was investigated. The enzyme extract was prepared using distilled water as a control, and using 0.01M, 0.1M and 1M potassium phosphate buffer and the percentage of the enzyme activities present in the supernatant, and protein concentrations, are shown in Figure 4.9. The specific enzyme activities in the supernatants are listed in Table 4.7. The hydantoinase activity in the supernatants varied from 5% of the total hydantoinase activity in 0.1M buffer to 24% of the total hydantoinase activity in 1M buffer. The *N*-carbamoylase activity in the supernatants varied from 1.6% of the total *N*-carbamoylase activity in distilled water to 17% of the total *N*-carbamoylase activity in 0.1M potassium phosphate buffer.

Ionic strength did not have a significant effect on the protein concentration in the supernatant. The hydantoinase specific activity was highest in the supernatant prepared with 1M buffer while the *N*-carbamoylase specific activity in the supernatant was highest in the presence of 0.1M buffer. However, the specific activities were similar to those described in the previous experiments. These results showed that the solubilities of the enzymes were not significantly increased by simply altering their surface hydrophobicities and showed that they were not likely to be peripheral membrane proteins.

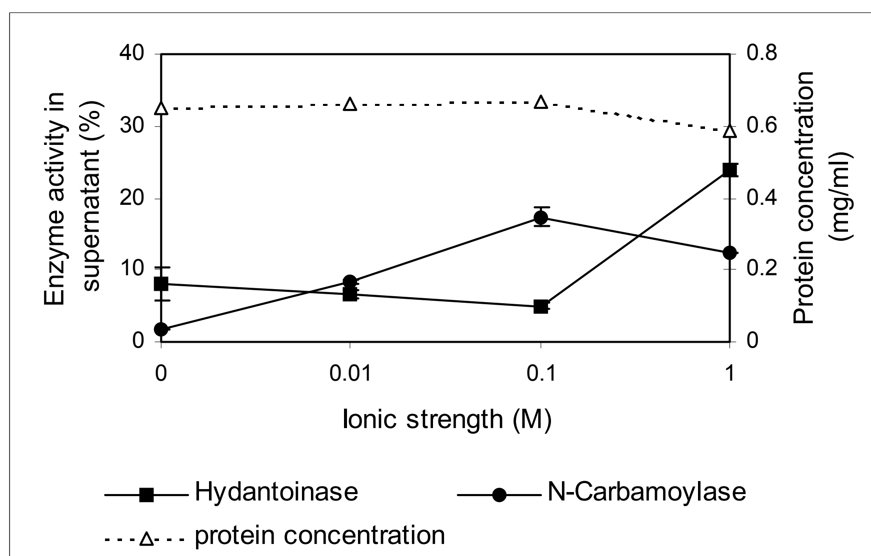


Figure 4.9
Hydantoinase and *N*-carbamoylase activities present in the supernatants, expressed as percentages of the total hydantoinase and *N*-carbamoylase activities in the enzyme extracts, after the production of the enzyme extract at varying buffer ionic strength.

Table 4.7 Hydantoinase and *N*-carbamoylase specific activities in the supernatant after production of the enzyme extract at various buffer ionic strength.

Ionic strength	HYD product yield (: mol/mL)	NCAAH product yield (: mol/mL)	Protein concentration (mg/mL)	HYD specific activity (10^{-3} Units/mg protein)*	NCAAH specific activity (10^{-3} Units/mg protein)*
Control	0.113	0.038	0.653	0.72	0.24
0.01M	0.148	0.221	0.662	0.93	1.4
0.1M	0.132	0.492	0.667	0.82	3.1
1M	0.292	0.164	0.588	2.1	1.2

* Units are expressed as : mol product produced per minute over 4 hours
HYD = hydantoinase
NCAAH = *N*-carbamoylase

4.3.9 Effect of hydrolysing enzymes on the enzyme activities in the supernatant of the enzyme extract

The results of the previous experiments indicated that the *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase were not peripheral membrane proteins. The enzyme extract was therefore subjected to mild proteolysis by incubation in the presence of low concentrations of chymotrypsin to investigate whether the enzymes were anchored membrane proteins. Mild proteolysis has been shown to cleave certain membrane-anchored proteins from membranes by the cleavage of specific peptide bonds within the protein structure, resulting in the release of proteins from the membranes without compromising activity in the case of enzymes (Scopes, 1987). The proteins may also be released by the mild disruption of the membrane-phospholipid bilayer by treatment with phospholipase enzymes. The enzyme extract was subjected to this form of hydrolysis by incubation in the presence of low concentrations of phospholipase.

Incubation of the enzyme extract in the presence of chymotrypsin resulted in a gradual increase in the percentage of the total hydantoinase activity measured in the supernatant, from 9% to 11% of the total activity after 12 hours (Figure 4.10). The percentage of the total *N*-carbamoylase activity measured in the supernatant over the 24-hour incubation with chymotrypsin also gradually increased, from 3% to 8% of the total activity after 24 hours (Figure 4.10). Incubation of the enzyme extract in the presence of phospholipase resulted in an increase in the percentage of the total hydantoinase activity measured in the supernatant of the enzyme extract from 14% to 29% of the total activity after 6 hours (Figure 4.11). The percentage of the total *N*-carbamoylase activity measured in the supernatant of the enzyme extract was highest after 3 hours incubation with phospholipase, with 16% of the activity being present (Figure 4.11).

The hydantoinase and *N*-carbamoylase specific activities in the supernatants after treatment with chymotrypsin and phospholipase are shown in Tables 4.8 and 4.9, respectively. Despite the increases in the percentages of the hydantoinase and *N*-carbamoylase activities present in the supernatant after incubation with chymotrypsin, the specific activity of the hydantoinase gradually decreased in the presence of the hydrolytic enzyme, indicating that the chymotrypsin may have had a denaturing effect on the enzyme. Similarly, the *N*-carbamoylase specific activity was highest in the supernatant after 3 hours incubation with chymotrypsin and then decreased with further incubation, indicating a possible denaturing effect on the enzyme. The specific activity of the hydantoinase enzyme was highest in the supernatant after

6 hours incubation with phospholipase, and then decreased. The *N*-carbamoylase specific activity in the supernatant was highest before treatment with phospholipase and decreased after incubation of the enzyme extract in the presence of the enzyme. The highest specific activities measured in the supernatants for the hydantoinase and *N*-carbamoylase in the presence of the hydrolytic enzymes remained low relative to those reported in the literature (sections 4.3.2 to 4.3.4) for these enzymes after cell lysis.

The very small increases in the percentages of the total hydantoinase and *N*-carbamoylase activities measured in the supernatants in the presence of the chymotrypsin and phospholipase indicated that the hydantoinase and *N*-carbamoylase were either, not anchored to membranes via alkyl, lipid or peptide chains or, if the enzymes were membrane-anchored proteins, chymotrypsin and phospholipase were ineffective for the cleavage of the enzymes from the membranes.

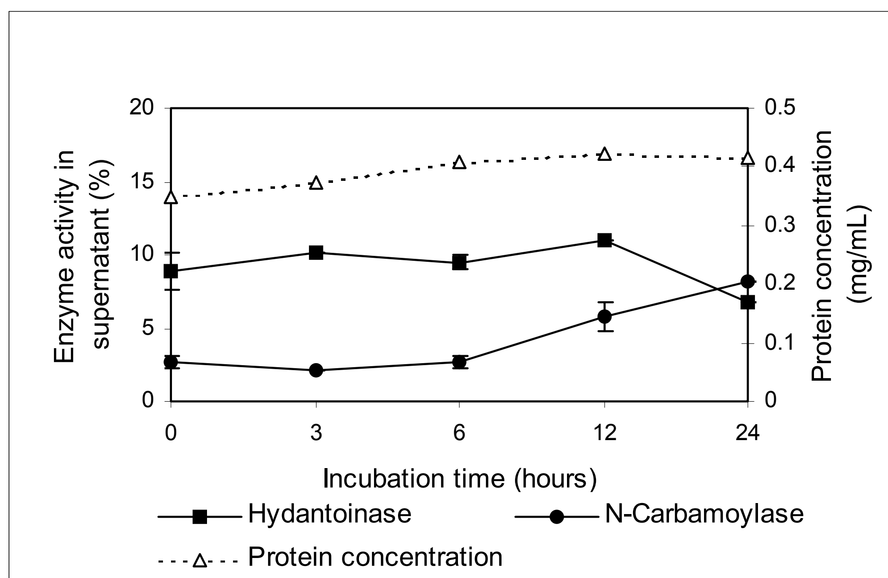


Figure 4.10

The effect of incubation in the presence of chymotrypsin on the hydantoinase and *N*-carbamoylase activities in the supernatant of the enzyme extract, expressed as percentages of the total enzyme activities in the enzyme extract.

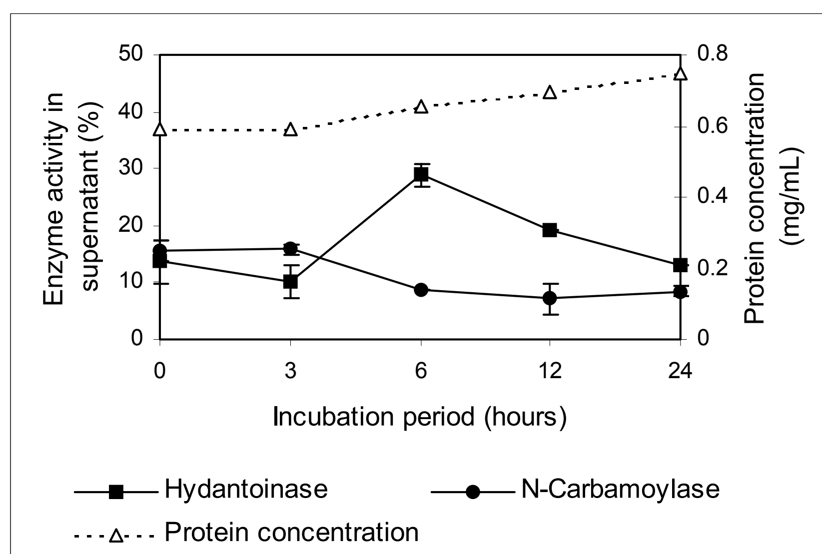


Figure 4.11

The effect of incubation in the presence of phospholipase on the hydantoinase and *N*-carbamoylase activities in the supernatant of the enzyme extract, expressed as percentages of the total enzyme activities in the enzyme extract.

Table 4.8 Hydantoinase and *N*-carbamoylase specific activities in the supernatant after incubation of the enzyme extract in the presence of chymotrypsin.

Incubation time (h)	HYD product yield (: mol/mL)	NCAAH product yield (: mol/mL)	Protein concentration (mg/mL)	HYD specific activity (10^{-3} Units/mg protein)*	NCAAH specific activity (10^{-3} Units/mg protein)*
0	0.177	0.103	0.347	2.1	1.2
3	0.130	0.421	0.375	1.4	4.7
6	0.138	0.147	0.410	1.4	1.5
12	0.079	0.241	0.423	0.78	2.4
24	0.018	0.313	0.417	0.18	3.1

* Units are expressed as : mol product produced per minute over 4 hours
HYD = hydantoinase
NCAAH = *N*-carbamoylase

Table 4.9 Hydantoinase and *N*-carbamoylase specific activities in the supernatant after incubation of the enzyme extract in the presence of phospholipase.

Incubation time (h)	HYD product yield (: mol/mL)	NCAAH product yield (: mol/mL)	Protein concentration (mg/mL)	HYD specific activity (10^{-3} Units/mg protein)*	NCAAH specific activity (10^{-3} Units/mg protein)*
0	0.243	0.674	0.589	1.7	4.8
3	0.246	0.623	0.594	1.7	4.4
6	1.001	0.200	0.654	6.4	1.3
12	0.227	0.087	0.694	1.4	0.52
24	0.219	0.078	0.745	1.2	0.44

* Units are expressed as : mol product produced per minute over 4 hours
HYD = hydantoinase
NCAAH = *N*-carbamoylase

4.3.10 Effects of non-ionic detergents on the enzyme activities in the supernatant of the enzyme extract

Detergents are capable of displacing a protein which is tightly bound by hydrophobic forces within a membrane, firstly, by dissolving the membrane and secondly, by replacing the membrane by aliphatic or aromatic chains which form part of the detergent (Scopes, 1987). Triton X-100, a non-ionic detergent, is widely used for this purpose, since it is mild in its action and therefore, solubilises proteins without

denaturing them. Many anionic detergents, while efficient solubilisers, are extremely denaturing and are, therefore, not useful for the isolation of enzymes (Scopes, 1987).

The hydantoinase and *N*-carbamoylase activities in the supernatant were assayed in the presence of 0, 1, 2 and 3% Triton X-100 and in the presence of the same concentrations of Tween-20 to evaluate the effect of these non-ionic detergents on the solubilities of the enzymes. Non-ionic detergents were used because of their less denaturing effect on proteins (Neugebauer, 1988; Bollag and Edelstein, 1991). The levels of soluble hydantoinase and *N*-carbamoylase in the supernatant of the enzyme extract, increased significantly in the presence of Triton X-100 (Figure 4.12). The optimal levels of Triton X-100 for the solubilisation of the enzymes were 2% and 1% for the hydantoinase and *N*-carbamoylase, respectively. In the presence of these levels of Triton X-100, 57% of the total hydantoinase activity in the enzyme extract and 55% of the total *N*-carbamoylase activity in the enzyme extract were released into the supernatant. The protein concentration in the supernatant increased slightly with increasing Triton X-100 concentration indicating the release of membrane-bound proteins (Figure 4.12). Although the percentage of the total activities present in the supernatant increased significantly in the presence of Triton X-100, almost half the enzyme activities remained in the cell pellet. In a study of the effect of detergents on the hydantoinase activity in crude extracts, Morin *et al.* (1990) found no hydantoinase activity in cell pellets after centrifugation of lysed *Pseudomonas putida* DSM 84 cells following treatment with detergents.

The hydantoinase and *N*-carbamoylase specific activities in the supernatants in the presence of Triton X-100 were highest in the presence of 1% Triton X-100 (Table 4.10). The hydantoinase and *N*-carbamoylase specific activities in this supernatant were 3.5nmol/min/mg and 5.1nmol/min/mg, respectively. These specific activities were expected to be higher considering the 3-fold increase in the percentage of the enzymes present in the supernatant (Figure 4.12), however, the large decrease in the total enzyme activities in the presence of Triton X-100 negated this effect. Although these specific activities were higher than those obtained in previous experiments, they remained low in comparison to those reported in the literature for these enzymes in supernatants after cell lysis (e.g. Ogawa *et al.*, 1993; Luksa *et al.*, 1997). The hydantoinase and *N*-carbamoylase specific activities in the supernatant decreased with increasing Triton X-100 concentration.

Tween-20 was less effective than the Triton X-100 in solubilising the enzymes. The hydantoinase and *N*-carbamoylase activities, expressed as percentages of the total enzyme activities in the enzyme extract, and the protein concentrations, in the supernatants treated with Tween-20 are shown in Figure 4.13. The highest levels of soluble hydantoinase and *N*-carbamoylase were detected in the supernatant of the enzyme extract containing 2% Tween-20. This supernatant contained 28% of the total hydantoinase activity in the enzyme extract and 3% of the total *N*-carbamoylase activity in the enzyme extract. The protein concentration in the supernatant increased with increasing concentrations of Tween-20, indicating the release of membrane proteins by the detergent. The hydantoinase specific activity in the supernatant was highest before treatment with Tween-20 and decreased in the presence of Tween-20 (Table 4.11). The *N*-carbamoylase specific activity in the supernatant was highest in the presence of 2% Tween-20 but remained similar to the specific activities obtained in the previous experiments (Table 4.11).

The solubilising effect of the Triton X-100, in particular, on the hydantoinase and *N*-carbamoylase was a significant result, indicating that the enzymes could be integral or anchored membrane proteins. This property of the enzymes has been reported for the hydantoinase enzymes in hydantoin-hydrolysing *Agrobacterium* sp. and *Pseudomonas* sp. strains researched by the Rhodes Hydantoinase Group (Burton *et al.*, 1998). These authors reported significantly higher enzyme activities in enzyme extracts containing the cell debris after french-pressing of cells than in enzyme extracts excluding the cell debris, and increased levels of enzyme activity were obtained in the presence of a detergent, suggesting that the enzymes were membrane-bound. The majority of hydantoinases and *N*-carbamoylases in the literature have been reported to be present in high levels in supernatants after cell lysis, in a soluble form (e.g. Ogawa *et al.*, 1993; Soong *et al.*, 1999; Siemann *et al.*, 1999).

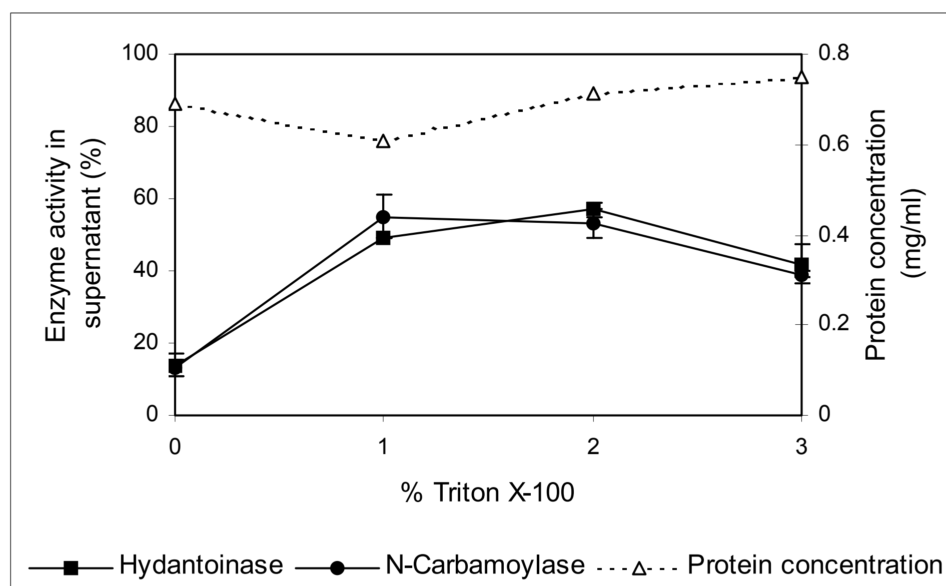


Figure 4.12 Effect of Triton X-100 on the hydantoinase and *N*-carbamoylase activities present in the supernatant of the enzyme extract, expressed as percentages of the total enzyme activities in the enzyme extract.

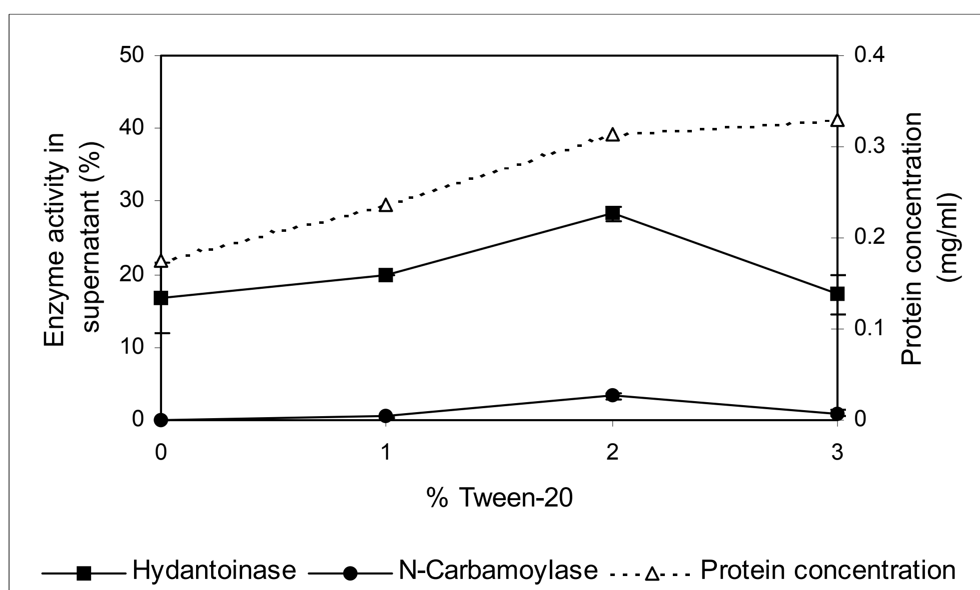


Figure 4.13 Effect of Tween-20 on the hydantoinase and *N*-carbamoylase activities present in the supernatant of the enzyme extract, expressed as percentages of the total enzyme activities in the enzyme extract.

Table 4.10 Hydantoinase and *N*-carbamoylase specific activities in the supernatant after production of the enzyme extract in the presence of Triton X-100.

Triton X-100 concentration (%)	HYD product yield (: mol/mL)	NCAAH product yield (: mol/mL)	Protein concentration (mg/mL)	HYD specific activity (10^{-3} Units/mg protein)*	NCAAH specific activity (10^{-3} Units/mg protein)*
0	0.324	0.460	0.692	2.0	2.8
1	0.511	0.737	0.607	3.5	5.1
2	0.533	0.663	0.713	3.1	3.9
3	0.211	0.359	0.750	1.2	2.0

* Units are expressed as : mol product produced per minute over 4 hours
HYD = hydantoinase
NCAAH = *N*-carbamoylase

Table 4.11 Hydantoinase and *N*-carbamoylase specific activities in the supernatant after production of the enzyme extract in the presence of Tween-20.

Tween-20 concentration (%)	HYD product yield (: mol/mL)	NCAAH product yield (: mol/mL)	Protein concentration (mg/mL)	HYD specific activity (10^{-3} Units/mg protein)*	NCAAH specific activity (10^{-3} Units/mg protein)*
0	0.113	ND	0.174	2.7	-
1	0.136	0.037	0.237	2.4	0.65
2	0.136	0.187	0.314	1.8	2.5
3	0.057	0.044	0.330	0.72	0.56

* Units are expressed as : mol product produced per minute over 4 hours
HYD = hydantoinase
NCAAH = *N*-carbamoylase
ND = not detected

4.3.11 Hydropathy and transmembrane prediction plots of *A. tumefaciens* RUOR *N*-carbamoylase predicted amino acid sequences

Kyte-Doolittle hydropathy plots of the predicted amino acid sequences for the two *N*-carbamoylase genes, *NcaR1* (Figure 4.19A) and *NcaR2* (Figure 4.20A), were generated in order to assess the hydrophobicity of the proteins. The values used for the amino acids are the water-vapour transfer free energies with adjustments for the preferences of the amino acids for internal versus exposed environments (Kyte and Doolittle, 1982). The peaks are considered hydrophobic and these plots are useful for finding transmembrane regions. An amino acid sequence for the *A. tumefaciens* RU-OR hydantoinase was unavailable and therefore the hydrophobicity of this enzyme could not be assessed.

The Goldman, Engelman and Steitz method is usually used to identify transmembrane regions. The values used are the sums of the hydrophobic and hydrophilic components of each amino acid in the sequence. The hydrophobic components are derived from the free energy of transfer of water-oil for the amino acid side chains (Engelman *et al.*, 1986). The hydrophilic component is derived from the free energy for the insertion of charged groups into a membrane bilayer, along with the free energy of the hydrogen bonding with water and the carbonyl groups of the protein background (Engelman *et al.*, 1986). The graph plots hydrophobicity as positive and hydrophilicity as negative and transmembrane regions usually appear as positive peaks. The transmembrane prediction plots of the *NcaR1* and *NcaR2* predicted amino acid sequences are shown in Figure 4.19B and 4.20B, respectively.

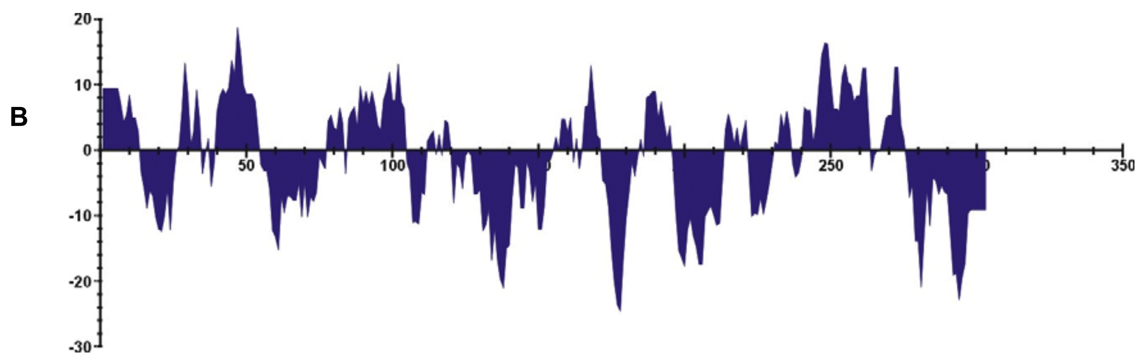
The hydropathy and transmembrane plots for the *NcaR1* predicted amino acid sequence indicated a fairly equal division of hydrophobic and hydrophylic regions in the amino acid sequence (Figure 4.19). A particularly hydrophobic region was identified in the region of amino acids 240 to 265 (Figure 4.19A) which corresponded to a possible transmembrane region in the same area identified by the transmembrane prediction plot (Figure 4.19B). Other, much smaller hydrophobic regions were identified by the plots but none of these were considered large enough to be possible transmembrane regions.

The hydropathy and transmembrane prediction plots of the *NcaR2* predicted amino acid sequence contrasted quite significantly with the *NcaR1* plots (Figure 4.20). The *NcaR2* amino acid sequence had considerably less hydrophobic regions (Figure 4.20A). One hydrophobic region from amino acids 150 to 170 corresponded to a predicted transmembrane region spanning amino acids 155 to 165. No other

transmembrane regions were identified (Figure 4.20B). The data generated from the predicted amino acid sequences of these two genes therefore identified a possible transmembrane region in each sequence. This data, therefore, supported the hypothesis that the enzyme could be membrane-bound.

A

B



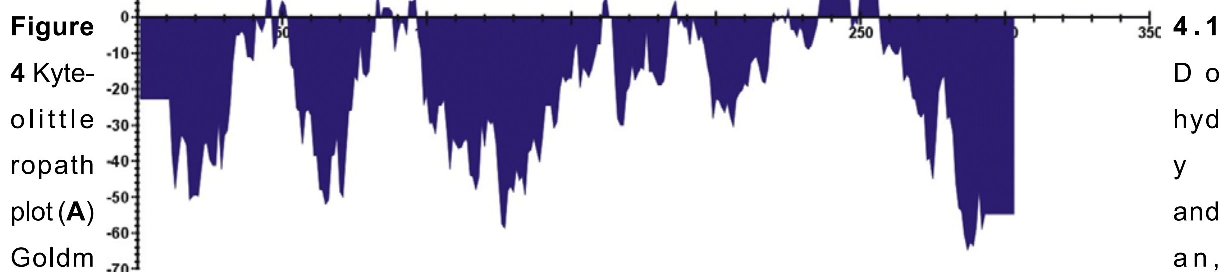


Figure 4.14 Kyte-Doolittle hydropathy plot (**A**) and Goldman, Engelman and Steitz transmembrane prediction plot (**B**) of the *A. tumefaciens* RU-OR *NcaR1* predicted amino acid sequence. In each case, hydrophobic regions deflect upward (positive values) and numbers on the horizontal axis indicate amino acid positions.

A

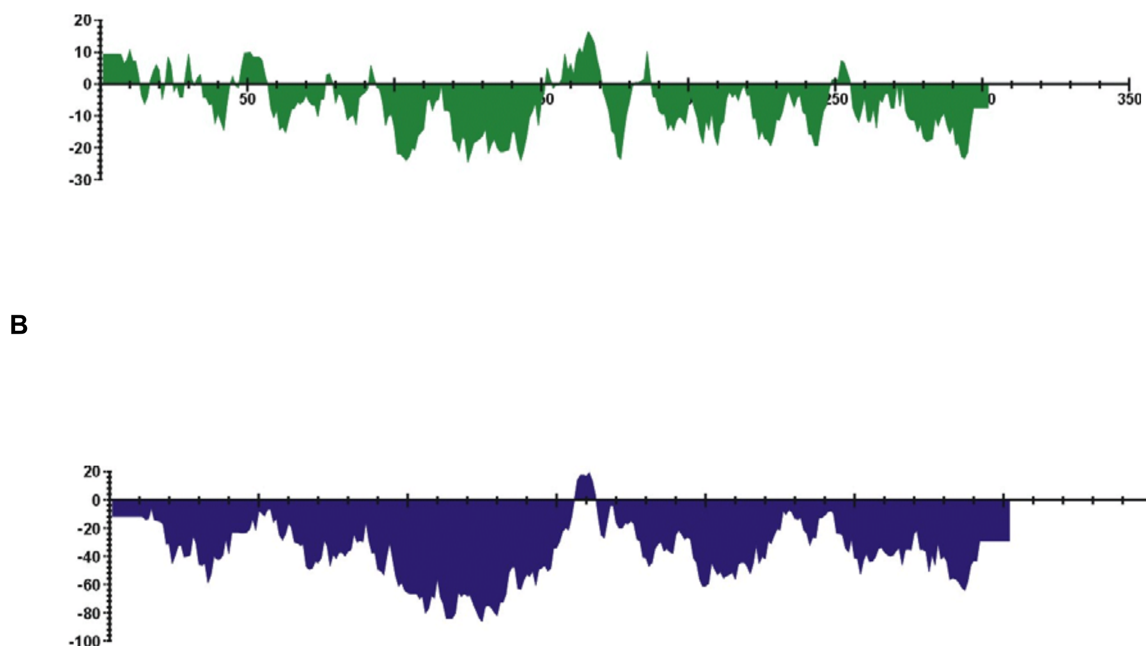


Figure 4.15 Kyte-Doolittle hydropathy plot (**A**) and Goldman, Engelman and Steitz transmembrane prediction plot (**B**) of the *A. tumefaciens* RU-OR *NcaR2* predicted amino acid sequence. In each case, hydrophobic regions deflect upward (positive values) and numbers on the horizontal axis indicate amino acid positions.

4.4 CONCLUSIONS

Purification of the *N*-carbamoylase and hydantoinase from *A. tumefaciens* RU-ORPN1 was severely hampered due to the insoluble nature of the enzymes, described in this chapter. The hydantoinase and *N*-carbamoylase were found to be particulate-associated after investigation of the hydantoinase and *N*-carbamoylase present in the pellet and supernatant of the cell homogenate (enzyme extract). On average, only 14% and 9% of the total hydantoinase and *N*-carbamoylase activities in the enzyme extract, respectively, were present in the supernatant, prepared by removal of the cell debris from the enzyme extract after 5 minutes sonication. This indicated that the enzymes were membrane-associated or present in an insoluble form in the cell homogenate.

Various strategies were investigated in order to promote the solubilisation of the enzymes. At the outset, the effect of various mechanical disruption techniques on the enzyme solubilities were evaluated.

Disruption of *A. tumefaciens* RU-ORPN1 cells by exposing the cells to increasing bead-beating and sonication times and by repeated french-pressing did not release higher levels of the hydantoinase and *N*-carbamoylase activites into the supernatant. Sonication for 15 and 25 minutes produced the highest levels of soluble hydantoinase and *N*-carbamoylase, respectively, in comparison to the other two mechanical disruption techniques. After disruption of the cells using lysozyme, only 14% of the total hydantoinase activity and 0.5% of the total *N*-carbamoylase activity were detected in the supernatant. The addition of a reducing agent and protease inhibitors did not enhance the levels of soluble hydantoinase and *N*-carbamoylase in the supernatant.

Peripheral membrane proteins are bound to membranes by electrostatic or hydrophobic interactions and can therefore be released by changing the pH or ionic strength of the system. Changes in the pH and ionic strength of the enzyme extract had no significant effect on the enzyme solubilities, indicating that the enzymes were not peripheral membrane proteins, and were more strongly associated with the particulate matter than merely reversibly held by electrostatic or non-covalent, hydrophobic interactions. The use of mild proteolysis with chymotrypsin and phospholipase to investigate whether the enzymes were anchored to the membranes was also ineffective for the solubilisation of the enzymes.

The levels of soluble hydantoinase and *N*-carbamoylase in the supernatant were significantly increased after incubation of the enzyme extract in the presence of Triton X-100. After incubation of the enzyme extract in the presence of 2% and 1% Triton X-100, 57% hydantoinase and 55% *N*-carbamoylase were solubilised, respectively. This indicated that the enzymes required disruption of membrane structures in order for solubilisation to take place, indicating that the enzymes were anchored or integrally part of the membranes.

Finally, hydropathy and transmembrane prediction plots of the *A. tumefaciens* RU-OR *NcaR1* and *NcaR2* predicted amino acid sequences showed that each amino acid sequence had a possible transmembrane region. The data generated from the predicted amino acid sequence, together with the results of the preceding solubilisation experiments, therefore suggest that the enzymes may be membrane bound.

In hindsight, some of the results obtained suggest that the very low hydantoinase and *N*-carbamoylase activities in the supernatant may also have been attributed to insufficient cell lysis. The very broad pH optima shown for the enzymes in the enzyme extract in Chapter 3 (Figure 3.6) are very typical of whole cell biocatalysts suggesting that the enzyme extract contained a high proportion of unlysed cells. The SDS-PAGE gel shown in Figure 4.2 should have contained considerably more protein bands if complete lysis had been achieved and the protein concentrations obtained after the cell lysis treatments were generally very low considering the cell concentration used. This could have been investigated further by plating out the lysed cells on an agar plate with growth media. Alternatively, marker proteins known to be soluble or membrane bound could have been used to show the disruption status of the cells.

The hydantoinase and *N*-carbamoylase specific activities measured in the supernatants after the various treatments remained significantly lower than those reported for these enzymes in the literature after cell disruption. This indicated that purification of the enzymes from these enzyme extracts would not be feasible. Purification by cloning and over-expression of the enzymes in *E. coli* is currently being investigated in our laboratory by a co-worker on the Rhodes Hydantoinase Project.

The use of a purified enzyme as a biocatalyst for application in an industrial process would require a relatively quick and simple purification procedure in order for the use of the biocatalyst to be practical and cost-effective. The purification of the *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase would have allowed for the detailed biochemical characterisation of the enzymes and the effects of the various treatments could have been investigated in more detail. For example, the treatments affecting enzyme activity may have been discriminated from those affecting enzyme stability. This type of investigation could be done should further biochemical characterisation be done in the future. The use of the enzymes in a purified form was not essential for the development of a biocatalyst for application in an industrial process. The stabilisation of the enzymes in crude extract form is therefore described in Chapter 5.

CHAPTER 5

BIOCATALYST STABILISATION

5.1 INTRODUCTION

One of the most critical prerequisites for a successful biotechnological process is the stabilisation of the biocatalyst involved. The stability of the *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase enzymes was examined in Chapter 3, and it was shown that the enzymes in whole cells could be stored at -20^{0}C for 21 days without significant loss of activity, while the freeze-dried enzyme extract could be stored at 25^{0}C for 7 days without loss of activity. However, at 25^{0}C and 40^{0}C, the enzymes in the enzyme extract were rapidly inactivated, resulting in almost complete loss of activity after 24 hours.

This section of work was focussed on the stabilisation of the enzyme activities by making use of various immobilisation techniques. The immobilisation methods were evaluated initially in terms of their immediate effect on the enzyme activities and later, the immobilisation methods resulting in the highest enzyme activity yields were evaluated further by examining the effect of the immobilisation on the durability of the enzyme activities during storage. The effects of the immobilisation methods on the enzyme activities in whole cells and enzyme extracts were compared in order to assess which combination of biocatalyst, support and immobilisation method would be most suitable for application in a bioreactor system for the production of amino acids. Whole cells or enzyme extracts were immobilised by adsorption or cross-linking on solid supports, entrapment, encapsulation and by cross-linking. The numerous immobilisation methods described in the literature can be divided into these five different approaches (Klibanov, 1983). Although immobilisation generally results in improved stability of enzymes, a decrease in stability may result in certain instances (Scouten *et al.*, 1995). Therefore, the immobilisation technique and support best suited to an enzyme are frequently identified by comparing a range of immobilisation methods. For this reason, the effects of a variety of immobilisation methods and supports on the hydantoinase and *N*-carbamoylase enzymes were evaluated.

Whole cells and enzyme extracts were immobilised by adsorption or cross-linking on hydrophilic, nylon flat-sheet membranes and two hydrophobic, flat-sheet membranes, *viz.*, polypropylene and polycarbonate. Immobilisation by adsorption relies on the attachment of the enzyme to the support by either an electrostatic attraction of opposite charges on the surface of the enzyme and the support or by hydrophobic

interactions between the enzyme and the support. Immobilisation of enzymes on a support by cross-linking relies on the formation of covalent bonds between the enzyme and the cross-linking agent and the cross-linking agent and the support (Russell, 1998). Enzymes immobilised by cross-linking are therefore more tightly bound to the support.

Immobilisation by cross-linking of whole cells and the enzyme extract using bifunctional reagents was evaluated. The cross-linking may be intermolecular, resulting in insoluble aggregates, or intramolecular. In the case of the former, the enzyme molecules are cross-linked to each other or to other proteins present in solution. Intramolecular cross-linking refers to the introduction of covalent or non-covalent cross-links within protein molecules (Klibanov, 1979; Fernandez-Lafuente *et al.*, 1995; Cheon *et al.*, 2000). The cross-linking agents used for the present study, were glutaraldehyde and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (EDAC). In the case of glutaraldehyde ($OHC-(CH_2)_3-CHO$), enzyme molecules or cells are covalently attached to each other by the formation of covalent bonds between the carboxylic groups on the glutaraldehyde and amino groups on the surfaces of the enzyme molecules or cells. Cross-linking with EDAC relies on the formation of covalent bonds between the cross-linking agent and carboxylic groups on the surfaces of the enzyme molecules or cells.

The effect of immobilisation by entrapment on the hydantoinase and *N*-carbamoylase activities was evaluated by immobilising the whole cells and enzyme extract in polyacrylamide gel and in calcium alginate beads. This method of immobilisation relies on the confinement of biocatalysts within a polymer matrix (Tramper, 1985). The effect of the use of a covalent gel, in the case of the polyacrylamide, was compared with the effect of immobilisation in a non-covalent gel, in the case of the calcium alginate (Klibanov, 1983).

Immobilisation by encapsulation was finally evaluated by immobilising the cells and enzyme extract in alginate-xanthan gum capsules. In this approach, enzymes are encapsulated with matrices which are impermeable for enzymes and other macromolecules but permeable to low molecular weight substrates and products (Klibanov, 1983).

5.2 MATERIALS AND METHODS

5.2.1 Materials

Nylon (47mm diameter, 0.45: m), polypropylene and polycarbonate (47mm diameter, 0.2: m) flat-sheet membranes were obtained from Micron Separations Inc.; alginic acid sodium salt (low viscosity), xanthan gum and EDAC were purchased from Sigma Chemical Co. and glutaraldehyde (25%) was purchased from BDH Chemicals Ltd. All reagents were analytical grade.

5.2.2 Methods

5.2.2.1 Immobilisation of whole cells and enzyme extract on nylon, polypropylene and polycarbonate membranes by adsorption and cross-linking and determination of hydantoinase and *N*-carbamoylase activities

Whole cells (40mg/mL) were resuspended in potassium phosphate buffer (0.1M, pH 9). Enzyme extract was prepared as described in Chapter 3, section 3.2.2.9. Nylon, polypropylene and polycarbonate circular flat-sheet membranes of 47mm diameter were equilibrated by soaking in potassium phosphate buffer (0.1M, pH 9) for 18 h, then allowed to air dry. Whole cells or enzyme extract were immobilised by adsorption by placing the membranes in the whole cell suspension or enzyme extract for 18 h with gentle agitation, at 4^0C. Immobilisation by cross-linking was achieved by placing the membranes (with adsorbed cells or enzyme extract) in 2% glutaraldehyde for a further 60 minutes. Following incubation, the membranes were removed and rinsed with potassium phosphate buffer (0.1M, pH 9). The membranes were then assayed for hydantoinase and *N*-carbamoylase activities by placing them in 5mL volumes of 50mM hydantoin or 25mM NCG and incubating at 40^0C and 200rpm for 6 h. Control experiments were carried out by reacting membranes without immobilised cells or enzyme extract with the substrates. Following incubation, the membranes were removed and the various solutions were assayed for NCG and glycine as described in Chapter 2, section 2.2.2.3. The quantity of active hydantoinase and *N*-carbamoylase immobilised on the membranes was then compared.

5.2.2.2 Effects of immobilisation by cross-linking with glutaraldehyde and EDAC on the hydantoinase and *N*-carbamoylase activities in whole cells and in the enzyme extract

Glutaraldehyde or EDAC was added to whole cells (40mg/mL), resuspended in potassium phosphate buffer (0.1M, pH 9), and to enzyme extract, to give final concentrations of 0, 0.5, 1.0 and 1.5% (v/v). The various samples were assayed for hydantoinase and *N*-carbamoylase activities by reacting the cross-linked

cells and enzyme extract, at 40^0C and 200rpm for 6 h, with hydantoin and NCG to give final concentrations of 50mM and 25mM, respectively, in a 2mL volume. The effect of cross-linking on the cells and enzyme extract was evaluated by comparing the enzyme activities obtained in the presence of the cross-linking agents with the activities obtained in control experiments having no cross-linking agent.

5.2.2.3 Immobilisation of whole cells and enzyme extract by entrapment in polyacrylamide gel and determination of hydantoinase and *N*-carbamoylase activities

Whole cells or enzyme extract were immobilised in polyacrylamide gel according to a method adapted from Yamada *et al.* (1980). Cells or enzyme extract were resuspended in a 10% (w/v) acrylamide solution to give final concentrations equal or equivalent to 40mg cells/mL. The gel was allowed to set by the addition of 150µL 10% (w/v) ammonium persulphate and 10µL N,N,N',N'-tetramethylethylene-diamine, and then cut into cubes of approximately 5 mm^3 volumes. Equivalent quantities of free and immobilised cells or enzyme extract were assayed for hydantoinase and *N*-carbamoylase activities by reacting them with hydantoin or NCG to give final concentrations of 50mM and 25mM, respectively, at 40^0C and 200rpm for 6 h, in a 5mL volume. Following incubation, the reaction supernatants were assayed for NCG and glycine and the percentage hydantoinase and *N*-carbamoylase activities retained after immobilisation were calculated by comparison of the enzyme activities before and after immobilisation.

5.2.2.4 Immobilisation of whole cells and enzyme extract by entrapment in calcium alginate beads and determination of hydantoinase and *N*-carbamoylase activities

Whole cells and enzyme extract were immobilised in calcium alginate beads by a method adapted from Busto *et al.* (1995). Whole cells (3g) or enzyme extract (equivalent to 3g cells) were resuspended in 10mL Tris-HCl buffer (pH 7, 0.1M), added to 50mL sodium alginate (2.5% w/v) and thoroughly mixed. Tris-HCl buffer was used for all assays involving calcium alginate beads since phosphate chelates with calcium ions resulting in the disruption of the bead structure (Fan and Lee, 2001). The solution was placed in a separating funnel and suspended over a beaker containing 200mL ice cold $CaCl_2$ (2% w/v). Calcium alginate beads of approximately 3mm diameter were prepared by dropwise addition of the sodium alginate solution (through a 200µL eppendorf tip) into the gently stirring $CaCl_2$ solution, at a rate of approximately 40 drops/minute. After preparation, the beads were left to harden in the $CaCl_2$ solution for a further 2 h. The beads were then filtered out of the $CaCl_2$ solution and washed thoroughly in Tris-HCl buffer (0.1M, pH 7).

Equivalent quantities of bead-immobilised and non-immobilised cells or enzyme extract were assayed for hydantoinase and *N*-carbamoylase activities by reacting them with hydantoin or NCG to give final concentrations of 50mM and 25mM, respectively, at 40^0C and 200rpm for 6 h, in a 2mL volume. The percentage enzyme activities retained after immobilisation of the cells and enzyme extract were then calculated by comparing the activities of equivalent quantities of cells or enzyme extract before and after immobilisation.

The effect of the separate alginate-bead components was investigated by assaying the enzyme extract in Tris-HCl buffer (0.1M, pH 7) in the presence of 0.5% (w/v) $CaCl_2$, 1% (w/v) $CaCl_2$ or 1% (w/v) sodium alginate, added separately to the enzyme solution. The effect of the Tris-HCl buffer on the enzyme activities was investigated by performing enzyme assays in the presence of potassium phosphate buffer (0.1M, pH 7) and Tris-HCl buffer (0.1M, pH 7).

5.2.2.5 Immobilisation of whole cells and enzyme extract in alginate-xanthan gum capsules and determination of hydantoinase and *N*-carbamoylase activities

Whole cells and enzyme extract were immobilised in alginate-xanthan gum capsules using a method adapted from Chang *et al.* (1996). Cells (2.4g) and enzyme extract (equivalent to 2.4g cells) were resuspended in 50 mL $CaCl_2$ solution (2% w/v) containing 0.26% (w/v) xanthan gum. Capsules were then produced by dropwise addition of the solution into a gently stirring, cold, sodium alginate (1% w/v) solution containing 0.1% Tween-20 (v/v). The capsules were left to harden in the alginate solution for 10 minutes with stirring. The capsules were then washed thoroughly with distilled water for 10 minutes and allowed to harden in a 1.3% (w/v) $CaCl_2$ solution overnight. The immobilised cells and enzyme extract were assayed for hydantoinase and *N*-carbamoylase activity, together with an equivalent quantity of free cells or enzyme extract, by reacting each with hydantoin or NCG to give final concentrations of 50mM and 25mM, respectively, at 40^0C and 200rpm for 6 h, in a 2mL volume.

5.2.2.6 Effects of cross-linking with EDAC, and cross-linking combined with freeze-drying, on the storage stability of the hydantoinase and *N*-carbamoylase activities in the enzyme extract

Samples of cross-linked (0.5% w/v EDAC) and non-cross-linked enzyme extracts were stored at 4^0C for 7 days. Samples of the cross-linked and non-cross-linked enzyme extracts were also freeze-dried and

stored at 4^0C for 7 days. The effect of cross-linking and the combined effect of cross-linking and freeze-drying on the durability of the hydantoinase and *N*-carbamoylase activities was evaluated by assaying the enzyme activities before and after storage as described in section 5.2.2.2.

5.2.2.7 Storage stability of the hydantoinase and *N*-carbamoylase activities in the enzyme extract immobilised on nylon, polypropylene and polycarbonate membranes

Enzyme extracts immobilised on nylon, polypropylene and polycarbonate membranes by adsorption and cross-linking were stored at 4^0C for 14 days. The effects of the immobilisation methods on the durability of the hydantoinase and *N*-carbamoylase activities were evaluated by assaying the enzyme activities before and after 7 and 14 days storage as described in section 5.2.2.1.

5.2.2.8 Storage stability of the hydantoinase and *N*-carbamoylase activities in the enzyme extract immobilised by entrapment in calcium alginate beads

Free and alginate bead-immobilised enzyme extracts were stored at 4^0C for 21 days. The effect of this immobilisation method on the durability of the hydantoinase and *N*-carbamoylase activities during storage was evaluated by assaying the enzyme activities before and after 7, 14 and 21 days storage as described in section 5.2.2.4.

5.3 RESULTS AND DISCUSSION

5.3.1 Immobilisation of whole cells and enzyme extract on nylon, polypropylene and polycarbonate membranes by adsorption and cross-linking

Whole cells and enzyme extract were immobilised on nylon, polypropylene and polycarbonate membranes by adsorption and cross-linking, to assess the effect of these supports and immobilisation methods on the hydantoinase and *N*-carbamoylase activities (Figure 5.1 and 5.2). These membranes were chosen to compare the effects of hydrophilic and hydrophobic supports on the effectiveness of the immobilisation. Glutaraldehyde was used a cross-linking agent in order to prevent leakage of the enzyme extract or whole cells from the membranes. A number of trends were observed in the data generated.

In general, much higher hydantoinase activity was detected on the membranes than *N*-carbamoylase activity, in both the immobilised whole cells and enzyme extract, indicating sensitivity of the *N*-carbamoylase to the immobilisation process. This was not unexpected, since *N*-carbamoylase enzymes are generally reported to be more unstable than hydantoinase enzymes and are highly susceptible to inactivation through oxidation which may have occurred during the immobilisation process. The *N*-carbamoylase activity in the immobilised whole cells was higher than the activities measured in the immobilised enzyme extract which further indicated that the enzyme may have been exposed to oxidation, since the enzymes in the cells would have been exposed to less oxygen. Alternatively, less *N*-carbamoylase activity may have been detected due to changes in the microenvironment of the enzyme affecting substrate transport to the enzyme or the enzyme may have been partially inactivated through attachment to the membranes via its active site, in the case of the enzyme extract.

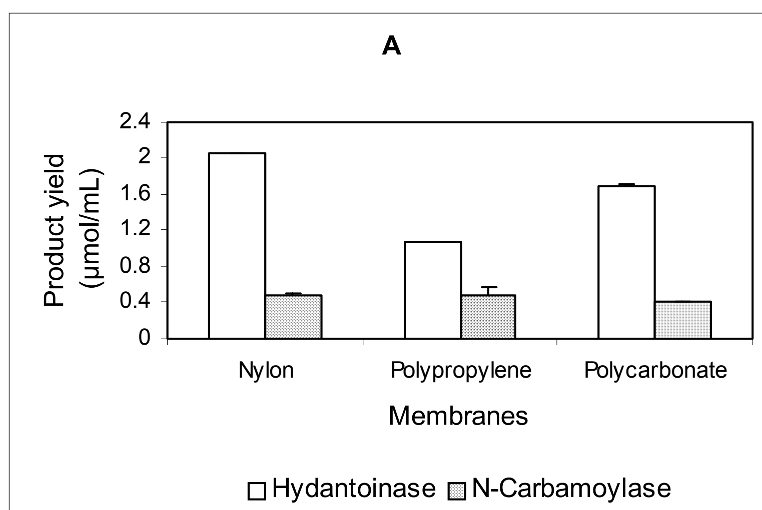
Whole cells and enzyme extract immobilised by cross-linking generally exhibited higher hydantoinase activity than the cells and enzyme extract immobilised by adsorption. In contrast to this, higher *N*-carbamoylase activities were detected when immobilisation by adsorption was used as opposed to immobilisation by cross-linking. This was particularly evident with the immobilisation of the enzyme extract by these two techniques (Figure 5.2). The decreased *N*-carbamoylase activity in the presence of the cross-linking agent indicated that the enzyme was possibly immobilised through covalent attachment of the enzyme to the supports via its active site resulting in the inactivation of the enzyme. This is consistent with the results of the immobilisation of the whole cells by cross-linking which did not have the same effect on the enzyme, since the cross-linking would not have affected the enzyme structure.

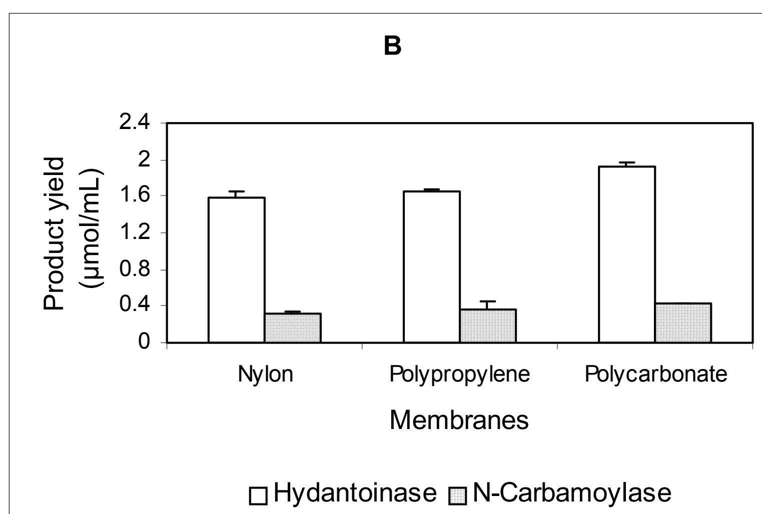
Whole cells and enzyme extract immobilised on the hydrophilic support generally exhibited higher enzyme activities than those immobilised on the hydrophobic supports. With the immobilisation of whole cells, this was more evident when adsorption was used, which produced lower enzyme activities on the polypropylene and polycarbonate in comparison to the nylon. With the immobilisation of the enzyme extract, this was more evident when cross-linking was used, indicating that the hydrophobic supports may have altered the microenvironment of the enzymes in a way unfavourable for the conversion of the substrates.

Similar trends have been reported in the literature on the immobilisation of hydantoinases and *N*-carbamoylases by adsorption and cross-linking on various supports. The hydantoinase and L-*N*-carbamoylase from *Arthrobacter aurescens* were immobilised by cross-linking on Eupergit C, Eupergit C 250L and EAH-sepharose (Ragnitz *et al.*, 2001a). For both enzymes, immobilisation on the hydrophilic EAH-sepharose gave enhanced specific activities in comparison to the enzymes immobilised on the more hydrophobic Eupergit supports. Immobilisation of the crude L-*N*-carbamoylase resulted in very low activity yields, while the immobilisation of the highly purified enzyme resulted in nearly 100% retention of activity (Ragnitz *et al.*, 2001b). The hydrophilic support, DEAE-cellulose, was found to be most effective for the immobilisation of the D-hydantoinase from *Bacillus stearothermophilus* SD-1 in terms of enzyme activity recovery (Lee *et al.*, 1996b). This support was also found to be effective for the immobilisation of a D-hydantoinase from lentil seeds (Rai and Taneja, 1998).

The immobilisation of crude extracts of a native D-*N*-carbamoylase and mutant *N*-carbamoylases with improved thermostability, from *Agrobacterium* sp. KNK712, cloned in *E.coli*, have been reported. The best immobilisation support for the enzymes was a macroporous phenol formaldehyde resin, Duolite A-568. The thermotolerant enzymes showed improved resistance to oxidation and to heat but very low activity yields were obtained after immobilisation, particularly after cross-linking with glutaraldehyde (Nanba *et al.*, 1998b, Nanba *et al.*, 1999). The L-*N*-carbamoylase from *Arthrobacter aurescens* was also found to be sensitive to cross-linking with glutaraldehyde (Ragnitz *et al.*, 2001b).

In this study, the highest hydantoinase and *N*-carbamoylase activities were observed in whole cells immobilised by adsorption on nylon membrane (Figure 5.1). The highest hydantoinase activity in the immobilised enzyme extract was obtained with cross-linking on the nylon membrane, while the highest *N*-carbamoylase activity was obtained by adsorption on the nylon membrane (Figure 5.2). Nylon membrane was therefore the best support for the immobilisation of whole cells and enzyme extract, producing the highest relative enzyme activities and indicating that a hydrophilic support would be more suitable than a hydrophobic support for the immobilisation of these cells or enzyme extracts. Furthermore, immobilisation by adsorption was best in terms of the enzyme activities produced. However, the use of these immobilisation methods and supports resulted in very low *N*-carbamoylase activity, indicating that they would not be suitable for the immobilisation of the biocatalyst for the intended process.


A
Product yield (µmol/mL)
2.4
2
1.6
1.2
0.8
0.4
0
Nylon
Polypropylene
Polycarbonate
Membranes
Hydantoinase
N-Carbamoylase


B
Product yield (µmol/mL)
2.4
2
1.6
1.2
0.8
0.4
0
Nylon
Polypropylene
Polycarbonate
Membranes
Hydantoinase
N-Carbamoylase

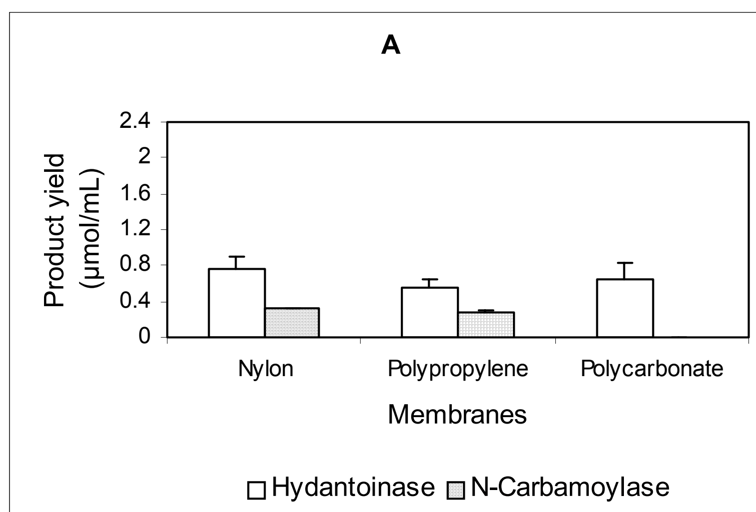


Figure 5.1 Hydantoinase and *N*-carbamoylase activities measured in whole cells after immobilisation on nylon, polypropylene and polycarbonate membranes by adsorption (**A**) and by cross-linking (**B**).

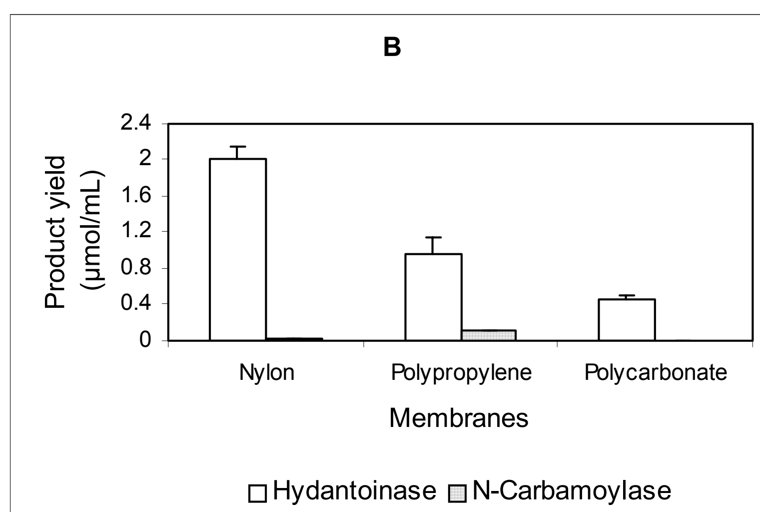


Figure 5.2 Hydantoinase and *N*-carbamoylase activities measured in the enzyme extract after immobilisation on nylon, polypropylene and polycarbonate membranes by adsorption (**A**) and by cross-linking (**B**).

5.3.2 Effect of cross-linking with glutaraldehyde and EDAC on the hydantoinase and *N*-carbamoylase activities in whole cells and in the enzyme extract

Cross-linking whole cells and the enzyme extract was investigated as an alternative method for the immobilisation and stabilisation of the hydantoinase and *N*-carbamoylase enzymes since this method is relatively simple compared with other immobilisation methods. The effect of this immobilisation technique on the enzyme activities was evaluated by assaying whole cells and the enzyme extract in the presence of various concentrations of two different cross-linking agents, glutaraldehyde (Figure 5.3) and EDAC (Figure 5.4). The effects of various degrees of cross-linking were investigated by assaying the enzyme activities in increasing concentrations of the cross-linking agents. Higher concentrations of cross-linking agents are expected to result in the formation of more linkages between an enzyme and the cross-linking agent, resulting in greater stability of the enzyme. The increased number of linkages, however, increases the chances of attachments occurring at the active site of the enzyme which results in the inactivation of the enzyme. The effects of the various concentrations of the cross-linking agents were therefore investigated in order to optimise the degree of cross-linking without compromising enzyme activity.

Cross-linking of whole cells with 0.5% glutaraldehyde resulted in an approximate 50% loss of hydantoinase activity and an almost complete loss of *N*-carbamoylase activity (Figure 5.3A). Cross-linking of the enzyme extract with glutaraldehyde at all the concentrations tested resulted in an almost complete loss of both hydantoinase and *N*-carbamoylase activities (Figure 5.3B). The hydantoinase and *N*-carbamoylase in both the cells and enzyme extract were therefore found to be sensitive to cross-linking with glutaraldehyde. Recombinant *E.coli* cells expressing the *N*-carbamoylase enzyme from *Agrobacterium radiobacter* NRRL B 11291 have been immobilised by cross-linking with glutaraldehyde. Cross-linking using 0.08% glutaraldehyde resulted in a 50% decrease in the *N*-carbamoylase activity of these cells (Fan *et al.*, 2000). The effect of this form of immobilisation on the *A. tumefaciens* RU-ORPN1 *N*-carbamoylase was therefore similar to the effect on this *N*-carbamoylase. As mentioned previously, other researchers have also found *N*-carbamoylases to be sensitive to cross-linking with glutaraldehyde (Ragnitz *et al.*, 2001b; Nanba *et al.*, 1999). Sensitivity of hydantoinases to glutaraldehyde, however, has not been reported previously.

The hydantoinase and *N*-carbamoylase were reduced to 58% and 21% of their original activities, respectively, following cross-linking of whole cells with 0.5% EDAC (Figure 5.4A). Cross-linking of the enzyme extract with 0.5% EDAC reduced the hydantoinase and *N*-carbamoylase to 46% and 48% of their original activities, respectively (Figure 5.4B). In both whole cells and enzyme extracts, the enzyme activities

were further reduced by additional cross-linking with EDAC. The *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase were therefore less susceptible to inactivation by EDAC suggesting that covalent coupling via carboxylic groups was more favourable for enzyme activity retention. This was also reported for the L-*N*-carbamoylase from an *Arthrobacter aurescens* strain by Ragnitz *et al.* (2001b). Intersubunit cross-linking of the D-hydantoinase from *Bacillus stearothermophilus* SD1 using EDAC has been also been described in the literature. The enzyme, purified from an *E. coli* strain expressing the D-hydantoinase gene, retained 85% activity after cross-linking with 10mM (0.2%) EDAC (Cheon *et al.*, 2000). Thus, the effect of cross-linking with EDAC on this hydantoinase was similar to that observed for the *A. tumefaciens* RU-ORPN1 hydantoinase.

The use of glutaraldehyde as a cross-linking agent was found to result in large losses of enzyme activity and was therefore unfavourable for the immobilisation of these enzymes. The use of EDAC, however, resulted in relatively high activity retention, and therefore, the effect of cross-linking using this reagent on the stability of these enzymes was later evaluated further (section 5.3.6).

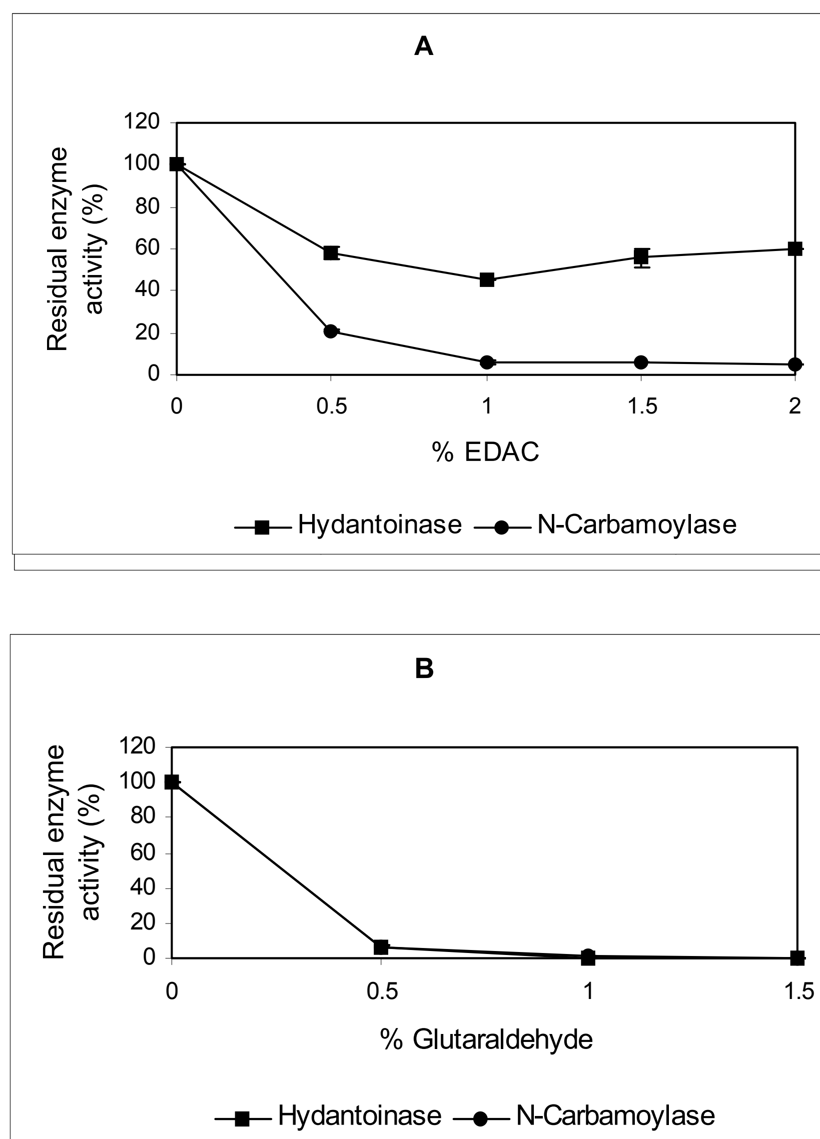


Figure 5.3 The effect of cross-linking with glutaraldehyde on the hydantoinase and *N*-carbamoylase activities in whole cells (**A**) and the enzyme extract (**B**).

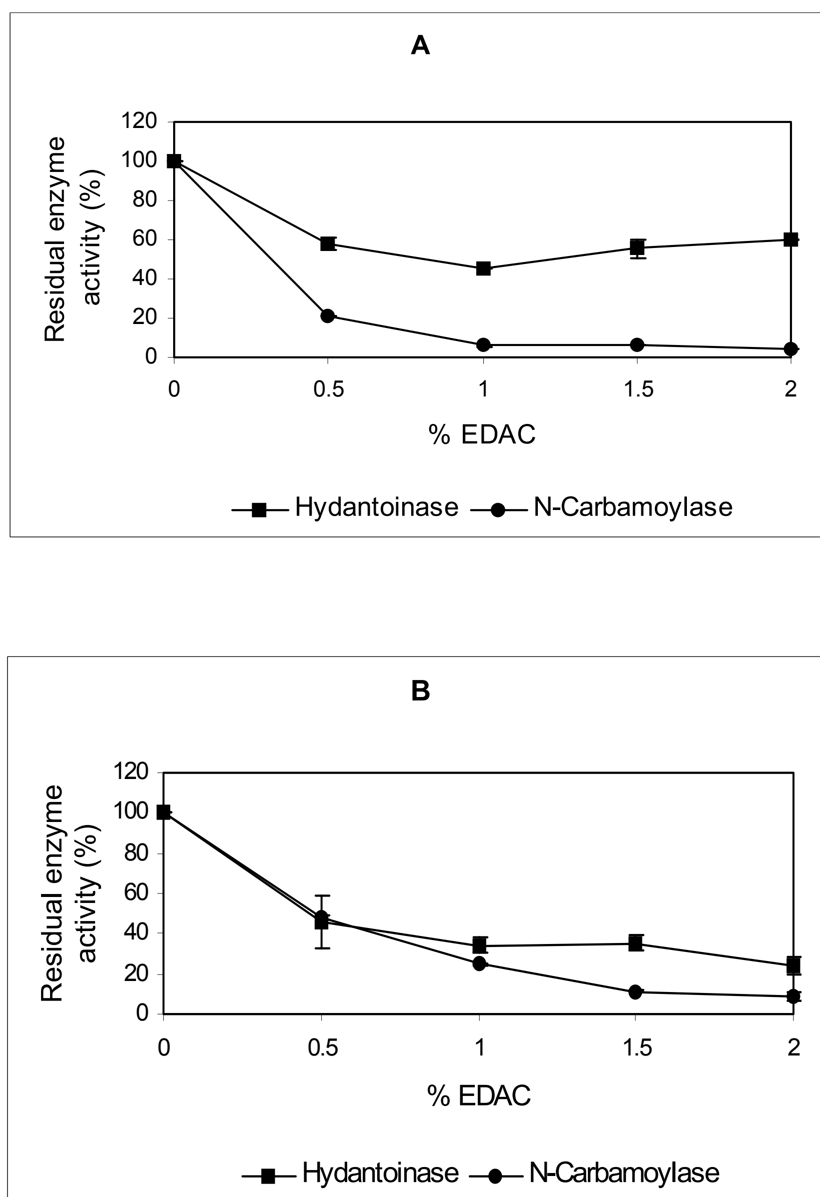


Figure 5.4 The effect of cross-linking with EDAC on the hydantoinase and *N*-carbamoylase activities in whole cells (**A**) and the enzyme extract (**B**).

5.3.3 Immobilisation of whole cells and enzyme extract by entrapment in polyacrylamide gel

Whole cells and enzyme extract were immobilised by entrapment in polyacrylamide gel to investigate the effect of this method of immobilisation on the hydantoinase and *N*-carbamoylase activities. Polyacrylamide was also assessed as a support to determine the effect of a covalently-linked matrix on the enzyme activities. The effect of a non-covalently-linked matrix was evaluated by making use of calcium alginate which is described in the following section. The enzyme activities retained after immobilisation, expressed as percentages of the activities in the extract before immobilisation, are shown in Figure 5.5. The hydantoinase in the whole cells and enzyme extract retained 40% and 26% of its activity, respectively, after immobilisation. The *N*-carbamoylase in the whole cells and enzyme extract retained 5% and 20% of its original activity, respectively, after immobilisation.

The immobilisation of a *Bacillus* sp. whole cells with D-hydantoinase activity in polyacrylamide has been reported, where no effect was observed on the activity of the enzyme (Yamada *et al.*, 1980). The immobilisation of a *Pseudomonas* sp. cells, with D-hydantoinase activity, in polyacrylamide has also been reported. Although the enzyme activity retained after immobilisation was not reported, the immobilised enzyme achieved 99% conversion of D,L-5-HPH in repeated batch reactions (Kim *et al.*, 1994), indicating that high levels of enzyme activity were retained. No reports have been published of the immobilisation of whole cells or enzyme extracts having *N*-carbamoylase activity in polyacrylamide.

By comparison of the results obtained with those reported in the literature for the immobilisation of these enzymes by this method, the hydantoinase activities retained after immobilisation of the whole cells and enzyme extract were very low. The *N*-carbamoylase activity decreased significantly after immobilisation of the cells and enzyme extract in polyacrylamide. A component of the gel may have had an inhibitory effect on the enzymes or the mass transport of the substrates to the enzymes may have been restricted by the gel. This support was therefore not considered suitable for the immobilisation of the enzymes.

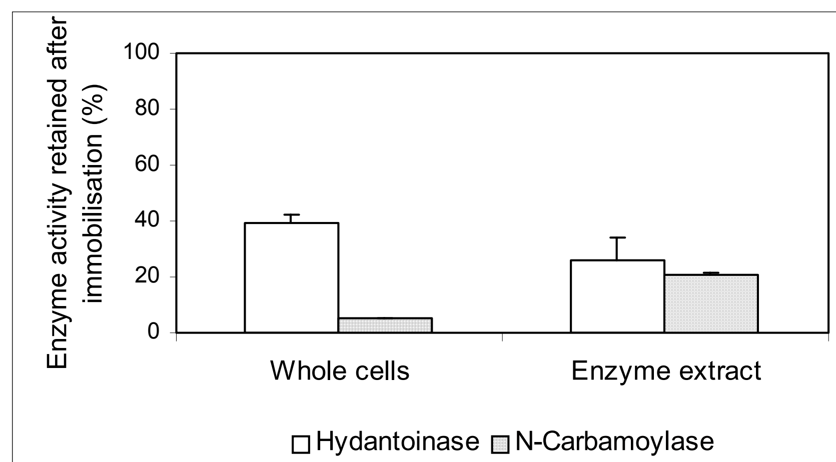


Figure 5.5 Percentage hydantoinase and *N*-carbamoylase activities retained after immobilisation of whole cells and the enzyme extract in polyacrylamide gel.

5.3.4 Immobilisation of whole cells and enzyme extract by entrapment in calcium alginate beads

Whole cells and enzyme extract were immobilised in calcium alginate beads in order to determine the effect of entrapment in a non-covalent gel on the hydantoinase and *N*-carbamoylase activities. The hydantoinase enzyme in the whole cells and enzyme extract immobilised by this method retained 47% and 55% activity after immobilisation, respectively (Figure 5.6). The *N*-carbamoylase in the immobilised whole cells retained 39% activity, while this enzyme in the immobilised enzyme extract increased to a remarkable 727% of the original activity (Figure 5.6). This result was confirmed in numerous subsequent experiments and published as novel (Foster *et al.*, 2002).

The possible role of any of the calcium alginate bead components in the large increase in the *N*-carbamoylase activity was investigated further by individually assaying the enzyme extract in Tris-HCl buffer and in the presence of $CaCl_2$ and sodium alginate (Figure 5.7). The replacement of the potassium phosphate buffer with Tris-HCl buffer, which was used for all reactions involving the calcium alginate beads, resulted in a 16% increase in the hydantoinase activity and a 28% decrease in the *N*-carbamoylase activity. Decreased *N*-carbamoylase activity in Tris-HCl buffer compared with phosphate buffer has also been reported in the literature (Fan *et al.*, 2000). The addition of $CaCl_2$ did not significantly affect the enzyme activities. The addition of sodium alginate to the enzyme extract slightly lowered the hydantoinase product yield from 6: mol/mL to 4.8: mol/mL and increased the *N*-carbamoylase product yield from

8.5: mol/mL to 12: mol/mL. This 41% increase in the *N*-carbamoylase activity was significant but was much less than the 7-fold increase in the activity of the immobilised *N*-carbamoylase. The increase in the *N*-carbamoylase activity could therefore not be attributed to any of the individual alginate bead components.

A number of factors could be considered in formulating an explanation for the increased *N*-carbamoylase activity. The steric stabilisation of enzyme structures by the immobilisation process is a recognised factor contributing to the stabilisation of enzyme activities in immobilised enzymes by preventing the unfolding and subsequent denaturing of the enzyme. This could have contributed to the stabilisation of the immobilised *N*-carbamoylase and therefore increased the enzyme productivity. Absorption of NCG by the alginate beads and the resulting enhanced concentration of the substrate in the microenvironment of the enzyme could be another possible factor favouring increased enzyme activity. It is also generally known that *N*-carbamoylases are particularly susceptible to oxidation in the presence of oxygen. The immobilised enzyme would be exposed to lowered oxygen levels, resulting in the possible stabilisation of the enzyme activity. Limited oxygen diffusion into calcium alginate beads has been demonstrated in the literature using strict aerobic microorganisms (Chevalier *et al.*, 1989). Furthermore, a number of oxygen-labile enzymes, stabilised against oxygen inactivation through immobilisation, have been reported in the literature (Klibanov, 1979). Research has also shown that some D-*N*-carbamoylases are susceptible to inhibition by ammonium ions (Chao *et al.*, 1999a) and that the removal of ammonium ions from the hydantoin-hydrolysing enzyme reaction results in increased conversion yields (Kim and Kim, 1994). The possible diffusion of ammonium ions out of the alginate beads during the enzyme reaction with the substrates could also have contributed to the increased productivity of the enzyme. The increased activity was in most likelihood a result of some or all of these contributing factors.

The immobilisation of a number of hydantoin-hydrolysing enzymes in calcium alginate beads has been reported in the literature. The immobilisation of *Pseudomonas putida* cells having D-hydantoinase activity has been described. Although the percentage enzyme activity retained after immobilisation was not reported, sufficient activity was retained for the application of the immobilised enzyme in various bioreactor systems (Chevalier *et al.*, 1989). The immobilisation of recombinant *E.coli* cells expressing the gene for the D-hydantoinase from a *Pseudomonas putida* strain has also been reported (Chen *et al.*, 1999; Yin *et al.*, 2000). These researchers reported a large decrease in the activity of the hydantoinase after

immobilisation in the alginate beads which was attributed to the possible formation of cell or enzyme aggregates within the beads with cores inaccessible to substrate (Chen *et al.*, 1999) or to high substrate mass-transfer resistance (Yin *et al.*, 2000). The decreased activity of the immobilised hydantoinase due to high mass transfer resistance in the beads was later confirmed by the immobilisation of permeabilised cells, which led to a 60% increase in the activity of the immobilised enzyme (Yin *et al.*, 2000). A partially purified D-hydantoinase from adzuki bean immobilised in calcium alginate beads retained 84% of its activity after immobilisation (Fan and Lee, 2001). The immobilisation of *Pseudomonas* sp. cells having both hydantoinase and L-*N*-carbamoylase activity in calcium alginate beads has also been reported. The activity yields were not reported but these immobilised cells were applied in a bioreactor system for the continuous production of L-cysteine.

To date, no reports of D-*N*-carbamoylases immobilised in calcium alginate beads have been published. However, the immobilisation of *E.coli* cells expressing the D-hydantoinase and D-*N*-carbamoylase from *Agrobacterium radiobacter* NRRL B11291 in κ-carrageenan has been described. The immobilised cells retained high hydantoinase and *N*-carbamoylase activity and were applied to the production of D-HPG from D,L-5-HPH. Although high conversion of D,L-5-HPH was reported, very low yields of D-HPG were obtained due to the low *N*-carbamoylase activity produced in the strain.

Immobilisation in calcium alginate beads was thus found to be an effective method for the immobilisation of the *A. tumefaciens* RU-ORPN1 enzyme extract resulting in high yields of enzyme activity compared with reports in the literature for other bacterial hydantoinases and *N*-carbamoylases immobilised by this method. The increase in the *N*-carbamoylase activity after immobilisation was a novel result, since no other reports have been made of increased *N*-carbamoylase activity on immobilisation.

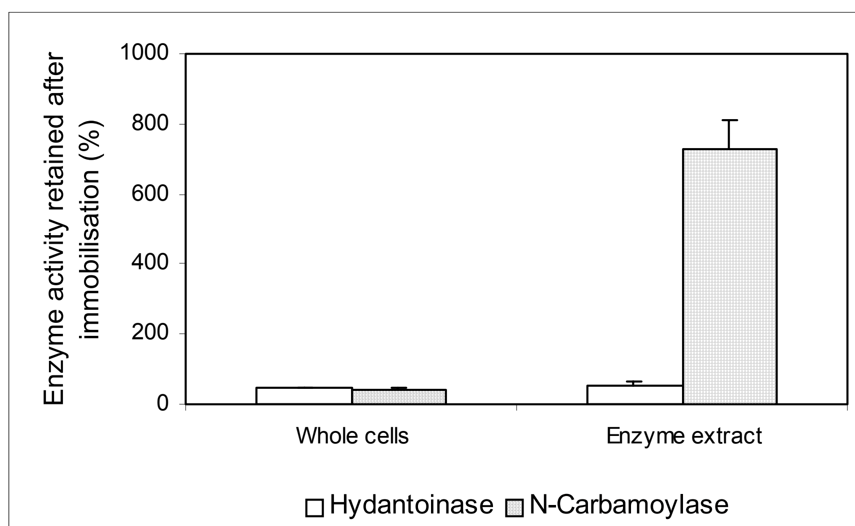


Figure 5.6 Percentage hydantoinase and *N*-carbamoylase activities retained after immobilisation of whole cells and the enzyme extract in calcium alginate beads. Error bars represent SEM (n=3 for whole cells; n=15 for enzyme extract).

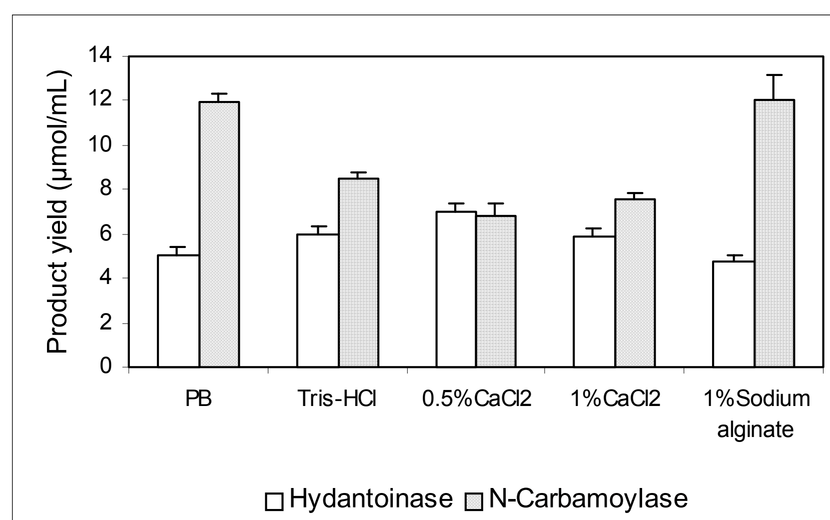


Figure 5.7 Determination of the effects of the separate alginate-bead components on the hydantoinase and *N*-carbamoylase activities in the enzyme extract (PB = potassium phosphate buffer).

5.3.5 Immobilisation of whole cells and enzyme extract in alginate-xanthan gum capsules

The effect of immobilisation by encapsulation was investigated by immobilising whole cells and the enzyme extract in alginate-xanthan gum capsules, which were also expected to exhibit higher physical resilience in an industrial process. Encapsulation has been introduced as an alternative to entrapment since higher cell loads can be immobilised, mass transfer resistance is reduced and less leakage of cells or enzymes immobilised by this method is experienced (Park and Chang, 2000). The percentage activities retained by the hydantoinase and *N*-carbamoylase from *A. tumefaciens* RU-ORPN1, after immobilisation by this method, are shown in Figure 5.8. The hydantoinase activity in the whole cells increased to 136% of the original activity after immobilisation, while the *N*-carbamoylase retained 40% activity. The increased hydantoinase activity in the immobilised cells may have been due to reduced mass transfer resistance in the capsules. The *N*-carbamoylase, however, was not affected by reduced mass-transfer and retained similar activity levels to those retained in the whole cells immobilised in calcium alginate. The hydantoinase and *N*-carbamoylase in the immobilised enzyme extract retained 14% and 31% of their original activities, respectively. The large decrease in the hydantoinase activity in the encapsulated enzyme extract was surprising since reduced mass transfer resistance was expected to increase the enzyme activity. The decreased activity could be due to the possible inactivation of the enzyme due to presence of an inhibitor of the enzyme in the enzyme extract or support. The decrease in the *N*-carbamoylase activities in the encapsulated cells and extract could be attributed to higher exposure to oxygen which would have diffused more easily into the capsules. The xanthan gum and Tween-20 which were used in the production of the capsules may also have affected the enzyme activities. No reports have been published of the immobilisation of hydantoinase or *N*-carbamoylase enzymes by encapsulation. The large losses of activity observed with the immobilisation of the cells and enzyme extract made this method unsuitable for the immobilisation of the enzymes.

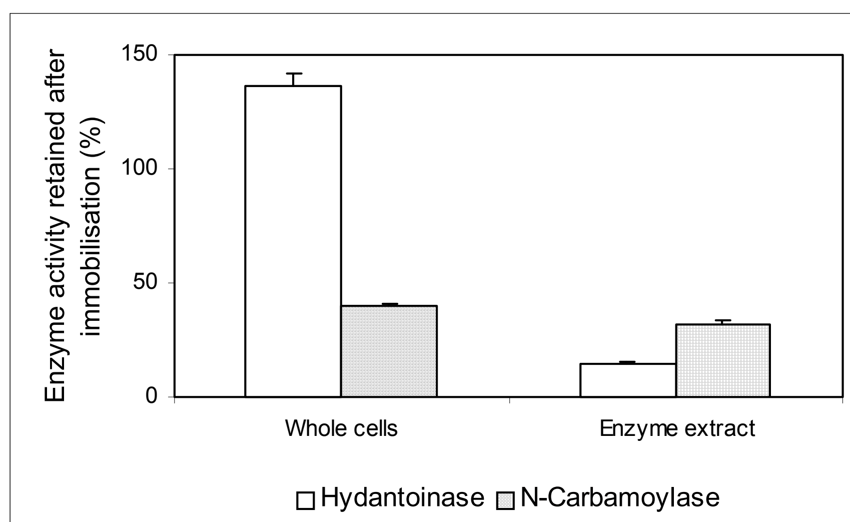


Figure 5.8 Percentage hydantoinase and *N*-carbamoylase activities retained after immobilisation of whole cells and the enzyme extract in alginate-xanthan gum capsules.

5.3.6 Effect of cross-linking, and cross-linking combined with lyophilisation, on the storage stability of the hydantoinase and *N*-carbamoylase activities in the enzyme extract

After the investigation of immobilised whole cells and enzyme extracts using various supports and methods, the most successful of the immobilisation methods, in terms of activity retained, were evaluated further by determining the residual activities of the immobilised biocatalysts during storage. The most successful immobilisation procedure, in terms of enzyme activity retention, was achieved by the immobilisation of the enzyme extract in calcium alginate beads. The effect of this immobilisation method on the stability of the enzymes was therefore assessed. The immobilisation of the enzyme extract on nylon, polypropylene and polycarbonate membranes yielded high levels of hydantoinase activity and the stability of the enzymes immobilised on these membranes was also evaluated. This method of immobilisation was also of interest due to the novelty of the immobilisation supports, since no reports have been published of immobilised hydantoin-hydrolysing enzyme extracts on these membranes. The effect of cross-linking with EDAC on the stability of the hydantoinase and *N*-carbamoylase activities was evaluated since this immobilisation method yielded relatively high hydantoinase and *N*-carbamoylase activities. This section presents the results of the investigation of the durability of the enzyme activities in the extract cross-linked with 0.5% EDAC.

The stabilities of the cross-linked hydantoinase and *N*-carbamoylase enzymes in the enzyme extract (0.5% EDAC) were evaluated by storing cross-linked and non-cross-linked (free) enzyme extracts at 4^0C and comparing the enzyme activities in each before and after 7 days storage (Figure 5.9). The combined effect of cross-linking and lyophilisation was investigated by storing the two extracts in lyophilised form (Figure 5.9). The free hydantoinase retained 74% activity, while the cross-linked enzyme increased to 236% of its original activity after the storage period. Thus, the cross-linked enzyme showed improved stability in comparison to the free enzyme. Similar increases in hydantoinase activity during storage have been reported for an immobilised hydantoinase (Ragnitz *et al.*, 2001a) and a free hydantoinase (May *et al.*, 1998b) but no explanation for the increase in activity was given. The free *N*-carbamoylase retained 83% activity while the cross-linked enzyme retained only 51% activity indicating that the cross-linked *N*-carbamoylase was less stable than the free enzyme.

In the lyophilised extracts, the free and cross-linked hydantoinases retained 82% and 43% activity, respectively, while the free and cross-linked *N*-carbamoylases retained 53% and 31% activity, respectively, after 7 days storage. Cross-linking with EDAC therefore improved the stability of the hydantoinase enzyme but decreased the stability of the *N*-carbamoylase enzyme, making this immobilisation method unsuitable for the development of a stable biocatalyst.

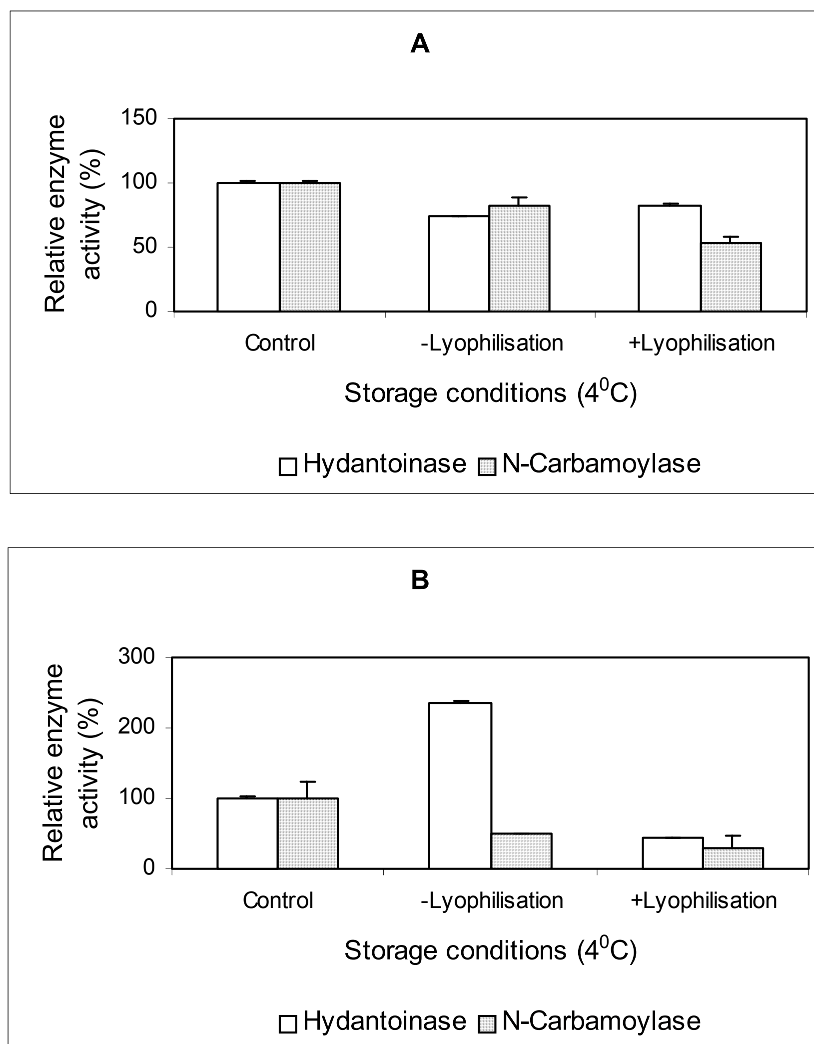


Figure 5.9 Hydantoinase and *N*-carbamoylase activities in non-cross-linked (**A**) and cross-linked (**B**) enzyme extracts after storage at 4^0C for 7 days with (+) and without (-) lyophilisation. Enzyme activities in the stored extracts are expressed as percentages of the enzyme activities in the extracts before storage.

5.3.7 Storage stability of the hydantoinase and *N*-carbamoylase activities in the enzyme extract immobilised on nylon, polypropylene and polycarbonate membranes

The effect of immobilisation by adsorption and cross-linking on solid supports on the hydantoinase and *N*-carbamoylase activities was evaluated further by assessing the durability of the immobilised enzyme activities during storage. The residual activities of the hydantoinase and *N*-carbamoylase enzymes in the enzyme extract immobilised on nylon, polypropylene and polycarbonate membranes after 14 days storage at 4^0C are shown in Figures 5.10, 5.11 and 5.12, respectively. The hydantoinase enzyme immobilised on nylon decreased to 24% activity when immobilised by adsorption and 5% when immobilised by cross-linking after storage (Figure 5.10). The *N*-carbamoylase immobilised by adsorption on nylon retained 90% activity after storage, while no activity was detected for the enzyme immobilised by cross-linking (Figure 5.10). The hydantoinase immobilised by adsorption on polypropylene retained 46% activity after the storage period, while the enzyme immobilised by cross-linking retained only 10% activity (Figure 5.11). The *N*-carbamoylase immobilised on this membrane by adsorption and cross-linking decreased to 14% and 26% activity, respectively, after 14 days storage (Figure 5.11). The hydantoinase enzyme immobilised on polycarbonate by adsorption and cross-linking retained only 7% and 2% activity, respectively, after the 14-day storage period. *N*-carbamoylase activity could not be detected after 14 days storage of the enzyme immobilised on polycarbonate by adsorption or by cross-linking (Figure 5.12).

By comparison of the stabilities of the immobilised hydantoinase and *N*-carbamoylase with the stabilities of the free enzymes during storage at 4^0C (Chapter 3, Figure 3.5B), improved stability as a result of immobilisation was only observed for the hydantoinase immobilised by adsorption on polypropylene and for the *N*-carbamoylase immobilised by adsorption on nylon and polypropylene. These immobilised enzymes retained 92%, 99% and 98% activity, respectively, after 7 days storage at 4^0C, while the free hydantoinase and *N*-carbamoylase retained 74% and 83% activity, respectively. The best immobilisation support for the hydantoinase enzyme in terms of storage stability was polypropylene, on which the hydantoinase, immobilised by adsorption, retained 46% activity after 14 days. The storage stability of the *N*-carbamoylase was best on nylon, on which the enzyme retained 90% activity when immobilised by adsorption after 14 days. However, the low levels of hydantoinase and *N*-carbamoylase activities immobilised on these particular membranes were not sufficient for the application of these immobilised enzymes in a bioreactor system.

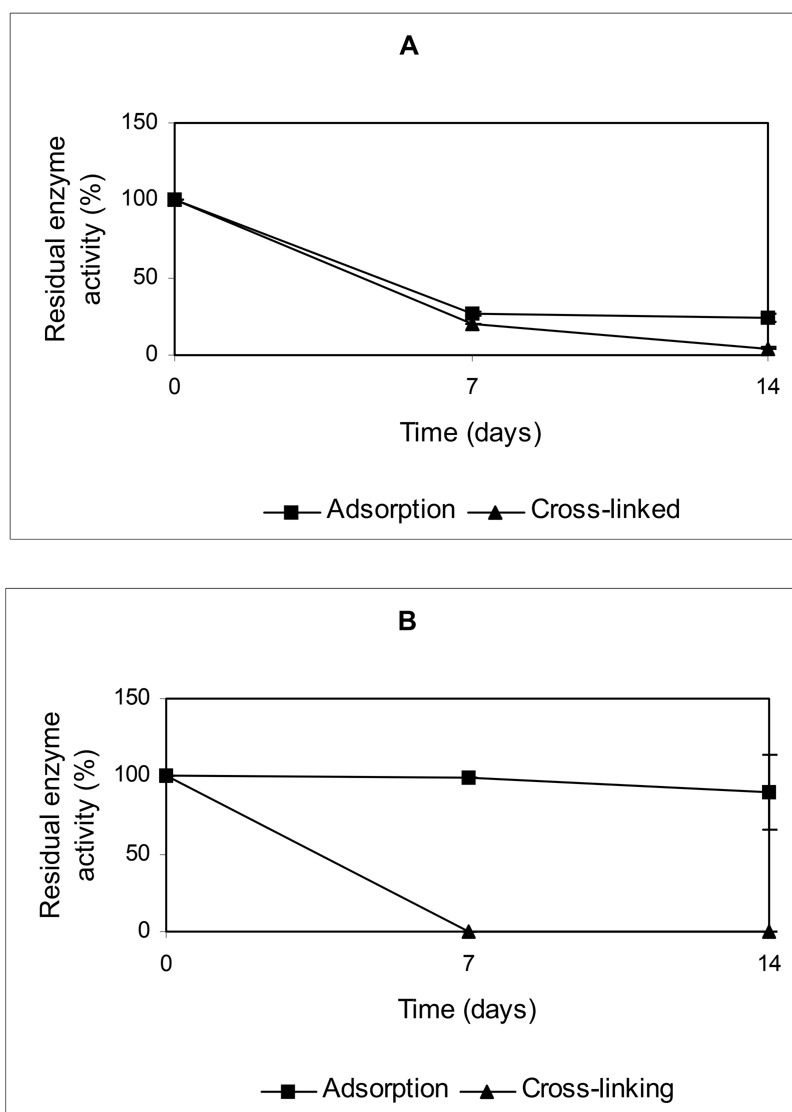


Figure 5.10 Residual hydantoinase (**A**) and *N*-carbamoylase (**B**) activities in the enzyme extract immobilised by adsorption and cross-linking on nylon membranes during storage at 4^0C for 14 days.

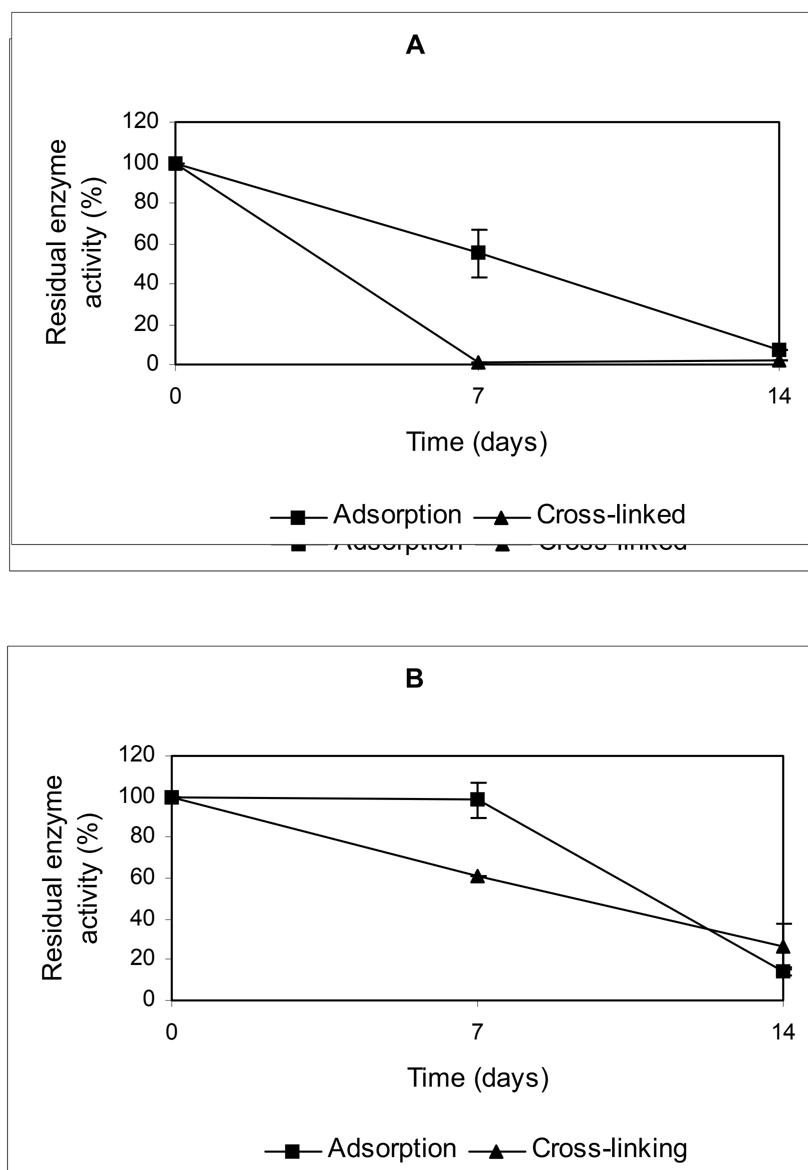


Figure 5.11 Residual hydantoinase (**A**) and *N*-carbamoylase (**B**) activities in the enzyme extract immobilised by adsorption and cross-linking on polypropylene membranes during storage at 4^0C for 14 days.

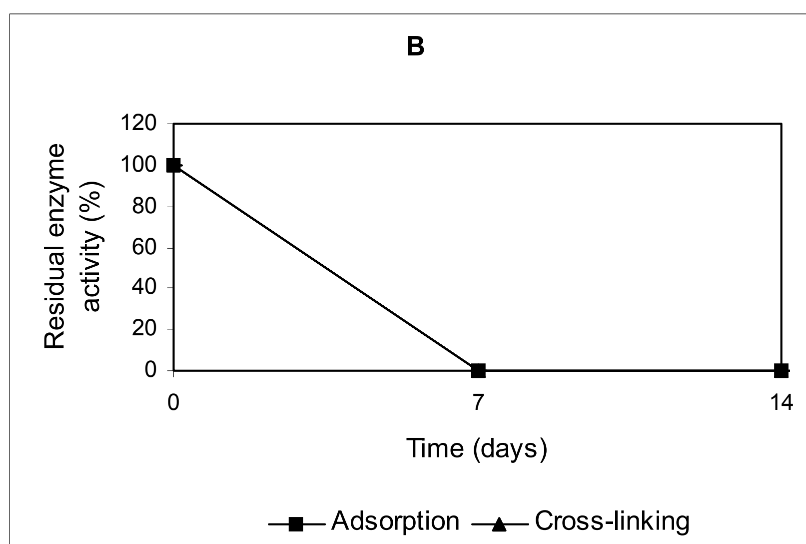


Figure 5.12 Residual hydantoinase (**A**) and *N*-carbamoylase (**B**) activities in the enzyme extract immobilised by adsorption and cross-linking on polycarbonate membranes during storage at 4^0C for 14 days.

5.3.8 Storage stability of the hydantoinase and *N*-carbamoylase activities in the enzyme extract immobilised by entrapment in calcium alginate beads

This section reports the results of the investigation of the durability of the hydantoinase and *N*-carbamoylase activities in the enzyme extract immobilised in calcium alginate beads, the most successful of the immobilisation methods in terms of hydantoinase and *N*-carbamoylase activity retained. The stabilities of the immobilised enzymes were evaluated by comparing the residual activities of the free and immobilised enzymes during storage at 4^0C (Figure 5.13). The free hydantoinase decreased to 83% activity over the 21-day storage period, while the immobilised enzyme showed a remarkable increase in activity to 546% of the original activity (Figure 5.13A). Increased hydantoinase activity after storage was observed a number of times throughout this research and has been reported by other researchers. A similar increase in the activity of a hydantoinase immobilised by covalent attachment to Eupergit® during storage has been reported by Ragnitz *et al.* (2001). These researchers also reported a 5-fold increase in the immobilised hydantoinase activity after storage at 4^0C. Although the reason for the increase in the hydantoinase activity has not been determined, it could be attributed to an indirect effect on an inhibitor of the enzyme which is inactivated under certain conditions, allowing for increased enzyme activity. The immobilised *N*-carbamoylase retained 30% activity after 21 days storage, while *N*-carbamoylase activity could not be detected for the free enzyme after the 21-day storage period (Figure 5.13B). Thus, the stabilities of the hydantoinase and *N*-carbamoylase both increased after immobilisation of the enzyme extract in the calcium alginate beads.

Immobilisation of the enzyme extract in calcium alginate beads was the best immobilisation method investigated in terms of hydantoinase and *N*-carbamoylase activities retained after immobilisation, and in terms of the durability of the enzyme activities during storage. Therefore, this immobilisation method was selected as most suitable for application of the enzyme extract in a bioreactor system. Most immobilisations reported in the literature using calcium alginate beads involve the immobilisation of whole cells, usually having only hydantoinase activity *e.g.* Chevalier *et al.* (1989). One report of the immobilisation of whole cells having both hydantoinase and L-*N*-carbamoylase activity has been published by Ryu *et al.* (1997). Therefore, the immobilisation of an enzyme extract with both hydantoinase and D-*N*-carbamoylase activity is novel.

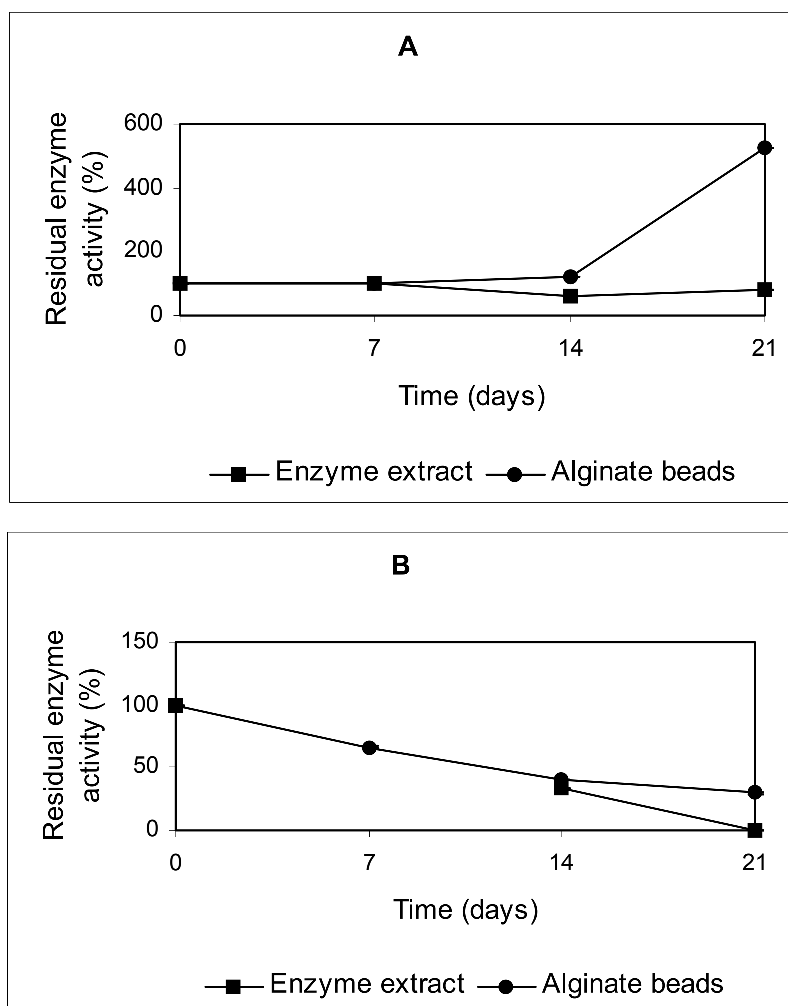


Figure 5.13 Residual hydantoinase (**A**) and *N*-carbamoylase (**B**) activities in the enzyme extract and alginate bead-immobilised enzyme extract during storage at 4^0C for 21 days.

5.4 CONCLUSIONS

The effects of a variety of immobilisation methods and supports were evaluated in terms of their effects on the hydantoinase and *N*-carbamoylase activities in *A. tumefaciens* RU-ORPN1 whole cells and enzyme extract. Immobilisation of the enzymes on flat sheet membranes both by adsorption and by cross-linking resulted in high levels of hydantoinase activity but greatly reduced levels of *N*-carbamoylase activity. The *N*-carbamoylase activity was particularly reduced in the presence of glutaraldehyde, a characteristic which arose again in experiments involving cross-linking of the whole cells and enzyme extract with glutaraldehyde. Cross-linking of whole cells and enzyme extract with glutaraldehyde was unsuccessful as an immobilisation method since large losses in enzyme activity resulted at low concentrations of glutaraldehyde. Cross-linking of the whole cells and enzyme extracts with EDAC was more successful resulting in higher levels of activity retention. Immobilisation of the enzymes by entrapment in polyacrylamide resulted in low hydantoinase and *N*-carbamoylase activities. By far the most successful immobilisation method was entrapment of the enzyme extract in calcium alginate beads, which yielded 55% hydantoinase activity and a remarkable increase in *N*-carbamoylase activity to seven times the original activity immobilised. Encapsulation of whole cells resulted in an increase in the hydantoinase activity but low *N*-carbamoylase activity. Low hydantoinase and *N*-carbamoylase activities were also obtained after encapsulation of the enzyme extract.

The immobilisation of the hydantoinase enzyme in the enzyme extract by cross-linking with EDAC increased the stability of the enzyme during storage in comparison to the free enzyme, but the *N*-carbamoylase immobilised by this method showed decreased stability during storage. A combination of cross-linking and lyophilisation also decreased the stability of both enzymes. The hydantoinase and *N*-carbamoylase in the enzyme extract immobilised on flat-sheet membranes were found to be most stable during storage when immobilised by adsorption on polypropylene and nylon, respectively. However, the enzyme activities when immobilised on these membranes were low.

The calcium alginate bead-immobilised enzymes showed enhanced storage stability in comparison to the free enzymes, with the hydantoinase increasing in activity during the storage period and the *N*-carbamoylase retaining 30% activity after 21 days storage. The enzyme extract immobilised in calcium alginate beads was therefore chosen as the most suitable biocatalyst in terms of the enzyme activity levels retained after immobilisation and storage durability for application in a bioreactor system. The further

characterisation of this biocatalyst, and its conversion efficiency in various bioreactors, is described in Chapter 6.

CHAPTER 6

BIOCATALYST CHARACTERISATION AND APPLICATION

6.1 INTRODUCTION

In this final section of work, the alginate bead biocatalyst, the best biocatalyst identified from the previous experimental studies, in terms of enzyme activity retained and durability of activity during storage, was characterised further in order to determine the optimum operating conditions for the biocatalyst. The biocatalyst reaction time, temperature and pH optima, substrate specificity, thermostability and reusability were evaluated. It should be noted here that as in Chapter 3, the use of the term “biocatalytic activity” in this chapter refers to measurements of product yields and should not be understood as measurements of amounts of enzyme. The biocatalyst was then applied in various packed-bed bioreactor systems and the productivity of the biocatalyst was evaluated under the optimal conditions. The production of glycine from hydantoin was assessed initially and then the conversion of D,L-5-HPH to D-HPG was evaluated, since this is a product of commercial interest. The suitability of the biocatalyst for application in an industrial process was assessed based on the conversion yields obtained in the various bioreactors.

The *A. tumefaciens* RU-ORPN1 biocatalyst was initially applied in a packed-bed bioreactor with hydantoin as substrate, under the optimised conditions for the biocatalyst. The conversion yield obtained in this bioreactor was low, possibly as a result of the low hydantoinase activity of the *A. tumefaciens* biocatalyst. In order to improve the conversion yields for a more complete enzymatic conversion of hydantoin to glycine, one approach was to combine the biocatalyst with a second biocatalyst having high hydantoinase activity. Thus, the *A. tumefaciens* RU-ORPN1 biocatalyst was used in combination with a *Pseudomonas* sp., strain KM1, biocatalyst, also produced by the immobilisation of enzyme extract in alginate beads. The *Pseudomonas* sp. biocatalyst had been developed and characterised by a co-worker in the research group and was found to have high hydantoinase activity (Burton *et al.*, 1998). The productivity of the two biocatalysts, used in combination, was assessed in the packed-bed bioreactor and compared with the productivity of the *A. tumefaciens* biocatalyst alone. Finally, the conversion of D,L-5-HPH to D-HPG in the packed-bed bioreactor containing the two biocatalysts combined was evaluated. The research presented here is the first report of a bioreactor system utilising the hydantoinase and *N*-carbamoylase from different bacterial species for the complete conversion of D,L-5-HPH to D-HPG.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Hydantoin was purchased from Aldrich. D,L-5-HPH and NC-HPG were synthesized by CSIR, South Africa. D-HPG and alginic acid, sodium salt (low viscosity) were purchased from Sigma Chemical Co. All reagents were analytical grade.

6.2.2 Methods

6.2.2.1 Determination of the alginate bead-immobilised hydantoinase and *N*-carbamoylase reaction product yields

Alginate beads containing immobilised *A. tumefaciens* RU-ORPN1 at a concentration equivalent to 20 mg wet cell mass/mL were assayed during optimisation experiments, in Tris-HCl buffer (0.1M, pH 7) containing 50mM hydantoin or 25mM NCG, in a 2 mL final volume. Hydantoinase product yield is expressed as the total concentration (: mol/mL) of NCG and glycine produced after 24 h at 40^{0}C with shaking at 200rpm. *N*-carbamoylase product yield is expressed as the total concentration (: mol/mL) of glycine produced from NCG after 24 h at 40^{0}C with shaking at 200rpm. NCG and glycine concentrations were assayed colorimetrically using Ehrlich's reagent and Ninhydrin assays, respectively, as described in Chapter 2, section 2.2.2.3.

6.2.2.2 Specification of a standard biocatalyst reaction time for determination of the alginate bead-immobilised enzyme product yields

The NCG and glycine concentrations produced by the alginate bead-immobilised hydantoinase and *N*-carbamoylase enzymes were measured after reacting the biocatalyst (equivalent to 20mg wet cell mass/mL reaction solution) in 50mM hydantoin or 25mM NCG for 0, 1, 2, 3, 6, 12 and 24 h, in a 2 mL final volume, at 40^{0}C and 200rpm. The biocatalyst reaction time after which the highest concentrations of NCG and glycine were measured was then selected as the standard biocatalyst reaction time for measuring the immobilised enzyme product yields.

6.2.2.3 Temperature and pH optima for the alginate bead-immobilised hydantoin-hydrolysing biocatalytic reaction

The pH optima for the immobilised enzymes were determined by performing enzyme reactions as described in section 6.2.2.1, except the following buffers were used: 100mM potassium phosphate buffer, pH 5-8; 100mM Tris-HCl buffer, pH 8-9 and 100mM carbonate buffer pH 9-10. The temperature optima

of the immobilised enzymes were determined by performing enzyme reactions as described in section 6.2.2.1, except incubation temperatures of 30, 40, 50 and 60^0C were used.

6.2.2.4 Investigation of the thermostability of the alginate bead-immobilised enzymes

Free and alginate bead-immobilised enzyme extracts were stored at 40^0C for 24 h in sealed bottles. Residual enzyme activities were determined after 0, 6, 12 and 24 h in order to compare the thermostabilities of the free and immobilised enzymes. Enzyme product yields were measured as described in section 6.2.2.1.

6.2.2.5 Investigation of the reusability of the alginate bead-immobilised enzymes

The alginate bead-immobilised hydantoinase and *N*-carbamoylase product yields were measured after 7 consecutive biocatalytic reactions. Enzyme product yields were measured as described in section 6.2.2.1, except enzyme reaction times of 3 h were used.

6.2.2.6 Substrate specificity of the alginate bead-immobilised enzymes

The NCG and glycine produced by the alginate bead-immobilised enzymes from hydantoin and NCG were compared with the NC-HPG and D-HPG produced by the immobilised enzymes from D,L-5-HPH and NC-HPG in order to investigate the substrate specificities of the enzymes. Enzyme reactions were carried out in 12.5mM substrate concentrations over 24 h at 40^0C and 200rpm. The hydantoinase product yield is expressed as the total concentration (: mol/mL) of NC-HPG and D-HPG produced from 12.5mM D,L-5-HPH after 24 h. The *N*-carbamoylase product yield is expressed as the total concentration (: mol/mL) of D-HPG produced from 12.5mM NC-HPG after 24 h. NC-HPG and D-HPG concentrations were determined colorimetrically using Ehrlich's reagent and Ninhydrin assays, respectively, and interpolated from standard curves as described in Chapter 2, section 2.2.2.3.

6.2.2.7 Productivity of the *A. tumefaciens* RU-ORPN1 alginate-bead biocatalyst in a packed-bed bioreactor operated in recycle mode

A. tumefaciens RU-ORPN1alginate-bead biocatalyst (15g) was reacted with 50mL 50mM hydantoin in Tris-HCl buffer (0.1M, pH 7) containing 100: M $CaCl_2$ in a packed-bed bioreactor (working volume: 20mL) at 37^0C. A photograph of the bioreactor configuration is shown in Figure 6.1. The substrate (and products)

were recycled in upflow mode at a constant flow rate of 0.3mL/min using an Ismatic peristaltic pump (Glattbrugg-Zürich, Switzerland). Samples were taken from the substrate reservoir daily over the operation time and assayed for NCG and glycine using Ehrlich's reagent and Ninhydrin assay, respectively. The hydantoin converted was measured by calculating the increase in the product concentrations in the substrate reservoir every 24 hours, and is presented as a percentage of the total hydantoin originally present in the system.

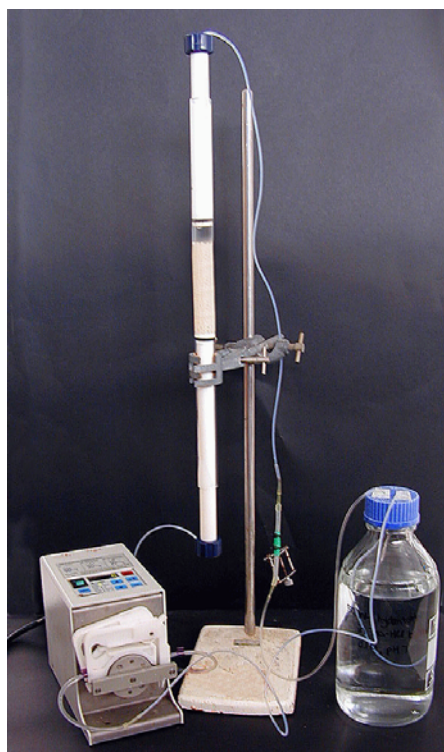


Figure 6.1 Photograph of the alginate bead packed-bed bioreactor configuration.

6.2.2.8 Productivity of the *A. tumefaciens* RU-ORPN1 biocatalyst combined with the *Pseudomonas* sp. KM1 biocatalyst in a packed-bed bioreactor operated in recycle mode

The productivity of the packed-bed bioreactor containing the combined *A. tumefaciens* RU-ORPN1 and *Pseudomonas* sp. strain RU-KM1 biocatalysts was evaluated under the same conditions as described in section 6.2.2.7, except 100mL substrate solution was used and the pH of the Tris-HCl buffer was pH 8. Equal masses

(15g) of each biocatalyst (total mass of biocatalysts: 30g) were used. Samples were taken from the substrate reservoir daily over the operation time and assayed for NCG and glycine using Ehrlich's reagent and Ninhydrin assay, respectively. The hydantoin converted was measured by calculating the increase in the product concentrations in the substrate reservoir every 24 hours and is presented as a percentage of the total hydantoin originally present in the system.

6.2.2.9 Productivity of a scaled-up continuous bioreactor system containing the combined *Agrobacterium tumefaciens* RU-ORPN1 and *Pseudomonas* sp. KM1 biocatalysts

The *A. tumefaciens* RU-ORPN1 and *Pseudomonas* sp. KM1 biocatalysts were applied in a continuous bioreactor system consisting of four packed-bed columns linked in series (total working volume: 80mL). A schematic representation of the bioreactor configuration is shown in Figure 6.2. Columns 1 and 3 contained 15g each of the *Pseudomonas* sp. strain KM1 biocatalyst, and columns 2 and 4 contained 15g each of the *A. tumefaciens* RU-ORPN1 biocatalyst. Daily samples were taken from the eluent and NCG and glycine concentrations were determined. Substrate solution (50 mM hydantoin in Tris-HCl buffer, 0.1M, pH 8) was fed through the bioreactor in upflow mode (without recycling) at a constant flow rate of 0.3mL/min (dilution rate: $0.23h^{-1}$) using an Ismatic peristaltic pump (Glattbrugg-Zürich, Switzerland). The hydantoin converted was measured by assaying the product concentrations in the eluent every 24 hours and is presented as a percentage of the hydantoin concentration in the substrate solution.

6.2.2.10 D-HPG production in a packed-bed bioreactor containing the combined *A. tumefaciens* RU-ORPN1 and *Pseudomonas* sp. KM1 biocatalysts operated in recycle mode

The conversion of D,L-5-HPH to D-HPG in the packed-bed bioreactor (working volume: 20mL) containing the combined *A. tumefaciens* and *Pseudomonas* sp. biocatalysts was evaluated using 20mM D,L-5-HPH in Tris-HCl buffer (0.1M, pH 8) fed at a flow rate of 0.3mL/min in upflow mode under the same conditions described in section 6.2.2.7, except 100mL substrate solution was used. NC-HPG and D-HPG concentrations were determined colorimetrically and interpolated from standard curves (Appendices B and C). The D,L-5-HPH converted was measured by calculating the increase in the product concentrations in the substrate reservoir every 24 hours and is presented as a percentage of the total D,L-5-HPH originally present in the system.

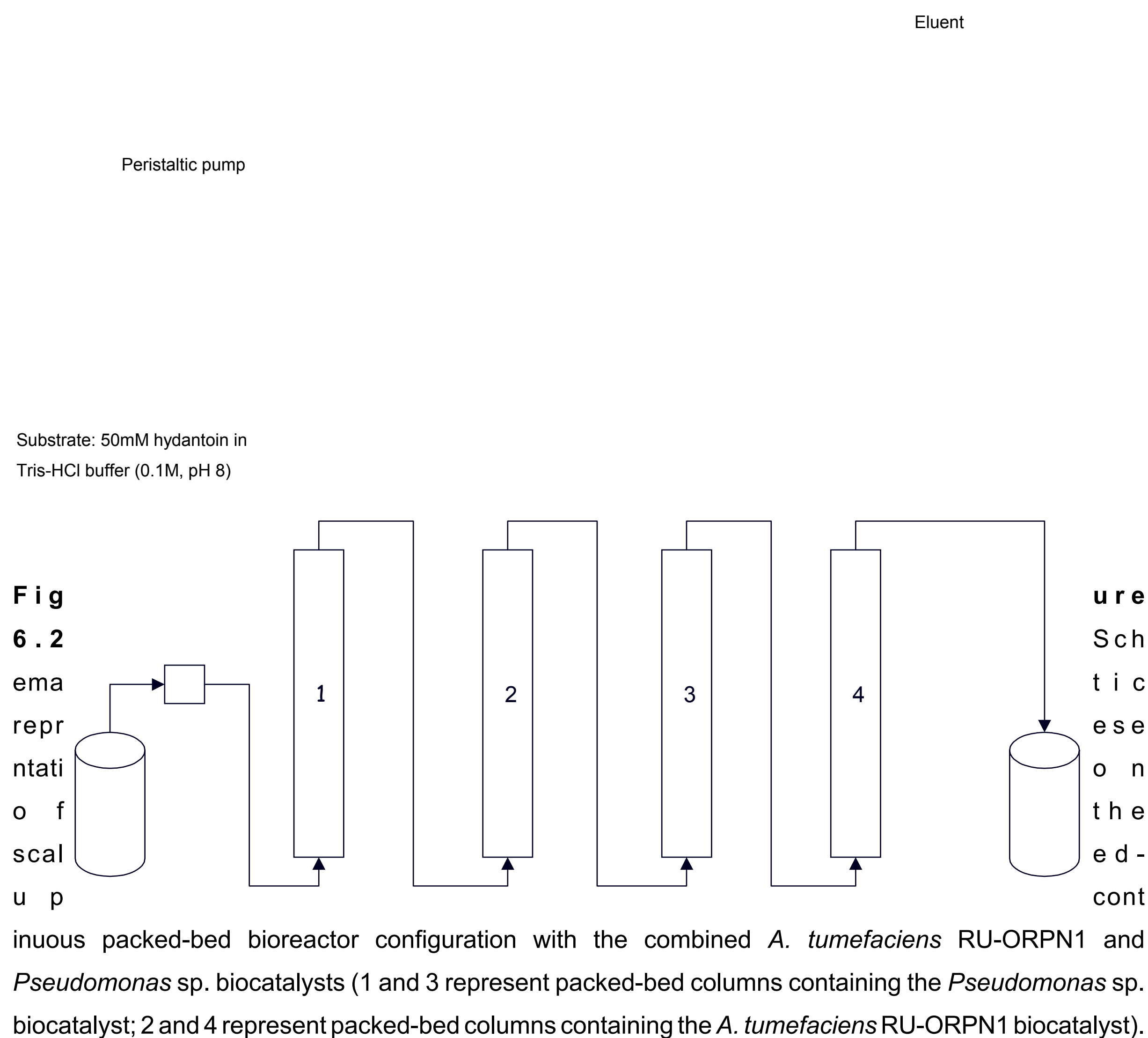


Figure 6.2 Schematic representation of the scaled-up continuous packed-bed bioreactor configuration with the combined *A. tumefaciens* RU-ORPN1 and *Pseudomonas* sp. biocatalysts (1 and 3 represent packed-bed columns containing the *Pseudomonas* sp. biocatalyst; 2 and 4 represent packed-bed columns containing the *A. tumefaciens* RU-ORPN1 biocatalyst).

6.3 RESULTS AND DISCUSSION

6.3.1 Specification of a standard biocatalyst reaction time for determination of the alginate bead-immobilised enzyme product yields

The alginate-immobilised hydantoinase and *N*-carbamoylase product yields were measured after various reaction times, in order to standardise the biocatalytic reaction time for determination of the immobilised enzyme product yields. The immobilised hydantoinase and *N*-carbamoylase product yields measured after 0, 3, 6, 12 and 24 h are shown in Figure 6.3. Although the maximum reaction rate took place over the first 3 h of the incubation period, the highest product concentrations were measured after 24 hours. The increase in hydantoinase product between 3 and 24 hours was significant, increasing from 0.18: mol/mL to 0.38: mol/mL. The *N*-carbamoylase product increased from 4.3: mol/mL to 5.8 : mol/mL between 3 and 24 hours. The biocatalyst product yields were therefore assayed after 24 hours in subsequent experiments.

In the case of the *N*-carbamoylase, the enzyme reaction levelled off at only 25% conversion of the substrate. This was probably due to inactivation of the enzyme during the incubation period. The initial reaction rate of the immobilised *N*-carbamoylase was slightly reduced to 28.6 : mol/mL/h/g cells in comparison to 30.5 : mol/mL/h/g cells calculated for the free enzyme from the data generated in Chapter 3, section 3.3.4. The *N*-carbamoylase activity therefore did not appear to be hampered by the immobilisation process. Immobilisation of the hydantoinase resulted in a significant reduction in the initial enzyme reaction rate, from 9.2 : mol/mL/h/g cells for the free enzyme, to 1.41 : mol/mL/h/g cells. A similar reduction in the initial enzyme reaction rate of a hydantoinase enzyme immobilised in calcium alginate beads was reported by Chen *et al.* (1999). The initial reaction rate of this enzyme for the conversion of D,L-5-HPH to NC-HPG was reduced, from 24.5 : mol/mL/h/g cells for the free enzyme, to 3.2 : mol/mL/h/g cells after immobilisation. The reduced reaction rates after immobilisation could be due to retention of the product in the calcium alginate beads resulting in less being detected in the liquid phase or due to an inhibitory effect. The low hydantoinase activity produced by the *A. tumefaciens* biocatalyst justified combining the biocatalyst with a biocatalyst having high hydantoinase activity in order to develop a fully enzymatic process for the conversion of hydantoin substrates to the corresponding amino acid.

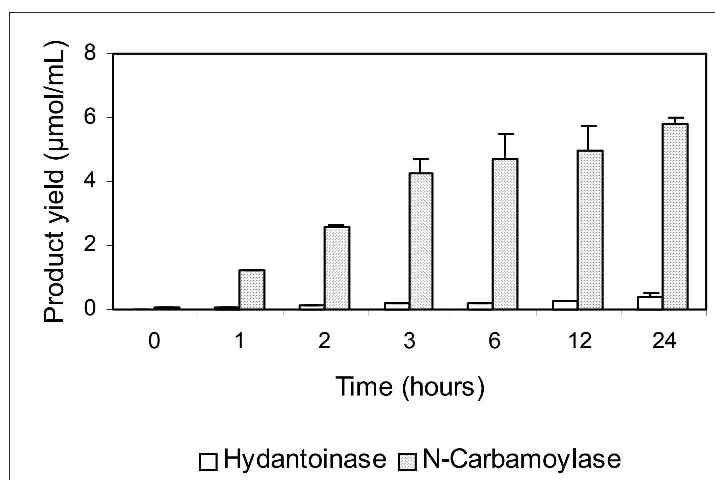


Figure 6.3 Product yields for the alginate bead-immobilised hydantoinase and *N*-carbamoylase after increasing enzyme reaction times (batch experiment).

6.3.2 Effect of immobilisation on the hydantoinase and *N*-carbamoylase apparent pH optima

Immobilisation may result in changes in the pH and temperature optimum profiles of enzymes due to slight changes in the conformation of the protein structure or due to slight changes in the microenvironment of the enzyme as a result of the immobilisation process. The effect of immobilisation in calcium alginate beads on the apparent pH optima of the hydantoinase and *N*-carbamoylase was therefore determined. The apparent pH optimum of the hydantoinase enzyme decreased by 2 pH units to pH 7 after immobilisation (Figure 6.4A). Similarly, the apparent pH optimum of the *N*-carbamoylase enzyme decreased by one pH unit to pH 8 on immobilisation (Figure 6.4B). Nanba *et al.* (1999) report a decrease in the pH optimum of an *N*-carbamoylase from pH 7 to pH 6.5 after immobilisation on DEAE-Sepharose. Increased pH optima (8 to 9.5) have been reported for a DEAE-sepharose-immobilised L-*N*-carbamoylase (Ragnitz *et al.*, 2001) and a polyacrylamide-immobilised hydantoinase (9 to 10) (Kim and Kim, 1993).

The physical properties of the alginate beads was also affected by pH and the buffer used. In potassium phosphate buffer (0.1M) at pH 7 the beads dissolved, while Tris-HCl (0.1M) buffer at this pH had no effect on the beads. The beads also disintegrated in sodium carbonate buffer (0.1M) at pH 11. The disintegration of the beads in potassium phosphate buffer at pH 7 could have been due to the chelating of phosphate in the buffer and calcium ions in the beads resulting in leaching of the calcium from the beads and disruption

of the bead structure (Fan and Lee, 2001). Similarly, the disruption of the beads in the case of the sodium carbonate buffer may have been due to an interaction between the carbonate and calcium ions. The use of the different buffers did not appear to significantly effect the activities of the immobilised enzymes. In the case of both enzymes, immobilisation appeared to decrease the resistance of the enzymes to changes in pH as the enzyme activities decreased sharply below and above the pH optima of the enzymes. This was an unexpected result, since generally, immobilisation results in greater resistance of enzymes to changes in pH (Lee *et al.*, 1996b; Chao *et al.*, 1999b).

The decrease in the apparent pH optima of the immobilised hydantoinase and *N*-carbamoylase enzymes would be advantageous for an industrial process where milder reaction conditions are favoured, and importantly, reactions carried out at lower pH have also been shown to result in less chemical racemisation of the products (Drauz and Waldman, 1995). At the same time, the apparent pH optima of the immobilised enzymes were still in the alkaline range which would allow for some chemical racemisation of hydantoin substrates, which takes place under alkaline conditions, and is important for productivity (Sharma and Vohra, 1997). The optimal pH for the *Pseudomonas* sp. hydantoinase biocatalyst was pH 8 (S. Kirchmann, *pers. comm.*) and therefore an operational pH of 8 was chosen for the bioreactor systems, favouring the *Pseudomonas* sp. hydantoinase activity and the *A. tumefaciens* sp. *N*-carbamoylase activity.

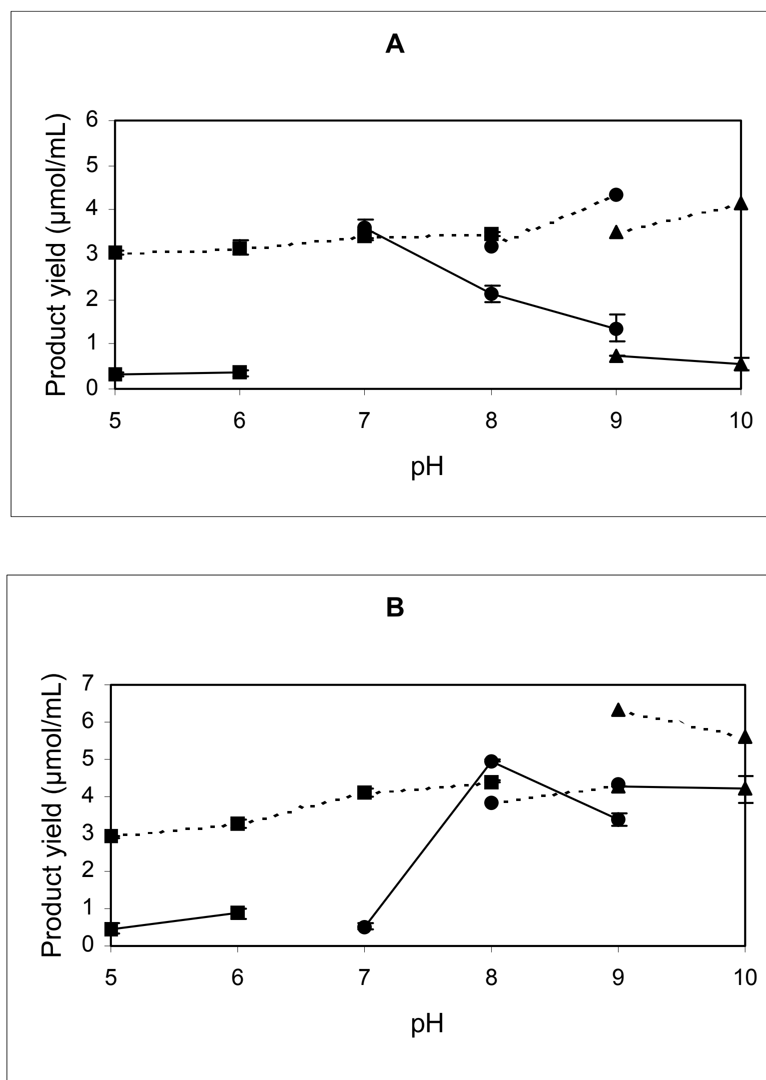


Figure 6.4 Comparison of the apparent pH optima for the free (**---**) and immobilised ( ) hydantoinase (**A**) and *N*-carbamoylase (**B**). The following buffers were used: 100mM potassium phosphate buffer (■), 100mM Tris-HCl buffer (●) and 100mM carbonate buffer (▲).

6.3.3 Effect of immobilisation on the apparent temperature optima of the hydantoinase and *N*-carbamoylase

The apparent temperature optima of the free and immobilised hydantoinase and *N*-carbamoylase enzymes were compared in order to investigate the effect of the immobilisation process on the temperature optima of the enzymes (Figure 6.5). The apparent temperature optimum for the immobilised hydantoinase increased to 50^{0}C (Figure 6.5A), compared with the highest activity at 30^{0}C previously observed. The apparent temperature optimum of the immobilised *N*-carbamoylase remained at 40^{0}C (Figure 6.5B). The immobilised *N*-carbamoylase appeared to be more thermally sensitive than the free enzyme and showed a sharp loss in activity at temperatures above 40^{0}C. This was unusual as, immobilisation generally results in increased resistance of enzymes to denaturation at higher temperatures (Rai and Taneja, 1998). Increases in the temperature optima of a Eupergit-immobilised hydantoinase from 40^{0}C to 60^{0}C and a sepharose-immobilised L-*N*-carbamoylase from 50^{0}C to 60^{0}C have been reported (Ragnitz *et al.*, 2001). The temperature optimum for an immobilised recombinant hydantoinase from *Pseudomonas putida* increased from 20^{0}C to 60^{0}C on immobilisation in calcium alginate beads (Chen *et al.*, 1999). However, immobilisation of the hydantoinase from *Bacillus stearothermophilus* SD-1 had no effect on the temperature optimum of the enzyme (Lee *et al.*, 1996b).

The higher apparent temperature optimum of the immobilised *A. tumefaciens* RU-ORPN1 hydantoinase enzyme would be advantageous for the application of the biocatalyst in an industrial process, since D,L-5-HPH has a solubility of less than 1% (w/v) (Lee *et al.*, 2001b) and higher temperatures would improve the solubility of this and other poorly soluble substrates. The hydantoinase activity of the *Pseudomonas* sp. biocatalyst was found to be optimal at 70^{0}C (S. Kirchmann, *pers. comm.*) but an operating temperature of 40^{0}C was chosen for the operation of the bioreactor systems in order to optimise the activity of the *A. tumefaciens* RU-ORPN1 *N*-carbamoylase.

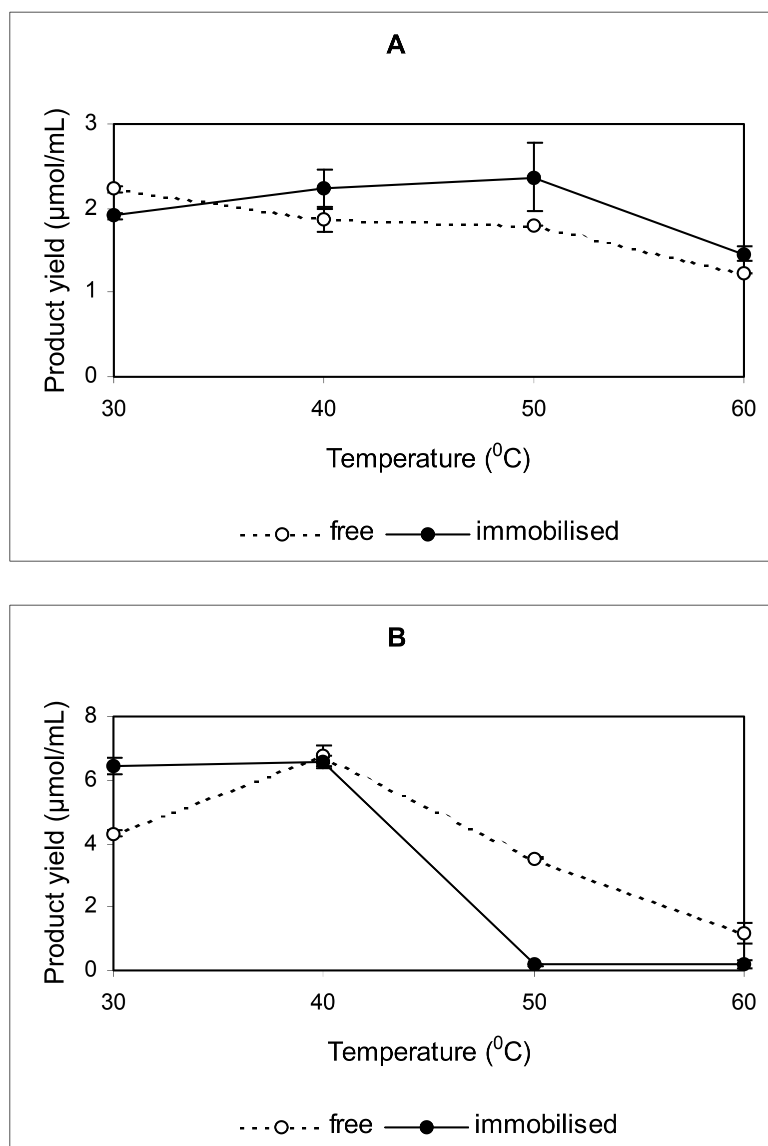


Figure 6.5 Apparent temperature optima for the free and immobilised hydantoinase (**A**) and *N*-carbamoylase (**B**).

6.3.4 Effect of immobilisation on the thermostability of the enzymes

The thermostabilities of the immobilised *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase were evaluated at 40^0C as this was chosen as the operating temperature for the application of the enzymes in the bioreactor systems. The residual activities of the immobilised and free enzymes were compared over 24 hours in order to investigate whether the immobilised enzymes showed enhanced thermostabilities in comparison to the free enzymes. The alginate bead-immobilised hydantoinase retained 67% of its original activity after 12 hours incubation and then decreased to 24% of the original activity after 24 hours (Figure 6.6A). As observed in Chapter 3 (Figure 3.7), the free enzyme showed a remarkable increase in activity after 24 hours incubation at 40^0C to over 4 times its original activity (Figure 6.6A). A large increase in hydantoinase activity was also described in Chapter 5 for the alginate bead-immobilised enzyme incubated at 4^0C (Figure 5.16). The increase in activity could be due to the possible inactivation of an inhibitor of the enzyme under these conditions. Increases in activity have been reported for the Sepharose-immobilised hydantoinase (Ragnitz *et al.*, 2001) and free hydantoinase (May *et al.*, 1998b) from *Arthrobacter aurescens* sp. DSM 3745 during storage. However, an explanation for this phenomenon was not put forward by these authors.

The immobilisation of the *A. tumefaciens* RU-ORPN1 hydantoinase did not increase the thermostability of the enzyme which was, again, an unexpected result since immobilisation generally increases the thermostability of enzymes. Improved thermostability of hydantoinases on immobilisation has been reported by Rai and Taneja (1998) and Chen *et al.* (1999). However, the immobilised *A. tumefaciens* RU-ORPN1 *N*-carbamoylase did show improved thermostability relative to the free enzyme (Figure 6.6B). The immobilised enzyme retained 82% of its original activity after 3 hours incubation while the activity of the free enzyme decreased to 33% of its original activity after the same length of time. Despite the improved thermostability of the immobilised *N*-carbamoylase, the enzyme retained only 20% of its original activity after 24 hours incubation at 40^0C.

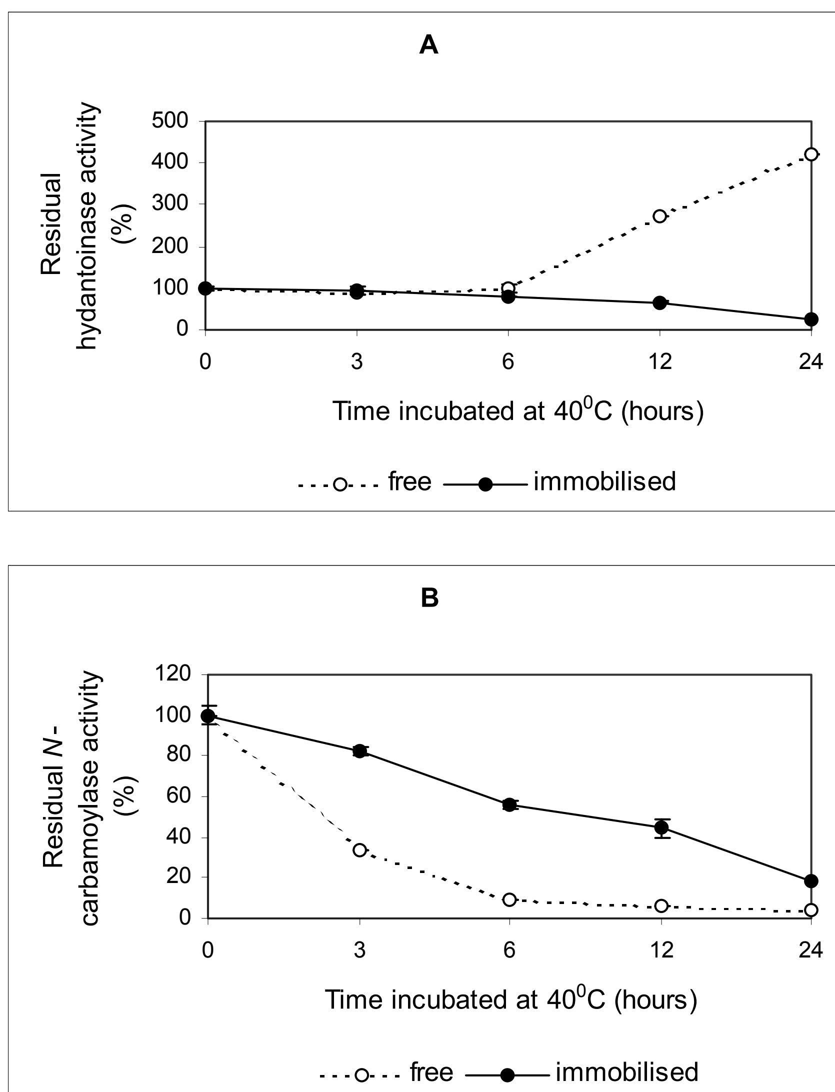


Figure 6.6 Comparison of the thermostability of the free and immobilised hydantoinase (**A**) and *N*-carbamoylase (**B**) at 40^0C.

6.3.5 Investigation of the reusability of the alginate bead-immobilised hydantoinase and *N*-carbamoylase

The reusability of the alginate bead-immobilised enzymes in repeated biocatalytic reactions was assessed in order to further evaluate the stability of the biocatalyst (Figure 6.7). The immobilised hydantoinase retained over 90% of its original activity, converting hydantoin at a rate of 5.03 : mol/mL/h/g wet cell weight, for 3 reactions and then gradually decreased to 10% of its original activity after 7 reactions. Similar reusability of an immobilised hydantoinase from lentil was reported which retained 85% activity after three reactions (Rai and Taneja, 1998). Recombinant *E.coli* cells expressing D-hydantoinase immobilised in calcium alginate beads retained 70% activity after 6 reactions (Chen *et al.*, 1999). Generally, immobilised hydantoinases reported in the literature retain more than 60% activity for at least 5 batch reactions (Durham and Weber, 1995; Fan and Lee, 2001). The most efficient biocatalyst reported in terms of reusability was a DEAE-cellulose-immobilised hydantoinase from *B. stearothermopilus* SD-1 which retained almost 100% activity, at a production rate of 14g NC-HPG/h/kg biocatalyst, for 9 successive batch reactions at 55^0C (Lee *et al.*, 1996b).

The immobilised *A. tumefaciens* RU-ORPN1 *N*-carbamoylase showed remarkable reusability relative to the immobilised hydantoinase, retaining 100% activity, and producing glycine at a rate of 36.6 : mol/mL/h/g wet cell weight, for 4 repeated reactions and then decreasing progressively to 50% of its original activity after 7 reactions. In comparison, recombinant *E.coli*, immobilised in κ-carrageenan expressing the hydantoinase and *N*-carbamoylase from *A. tumefaciens* NRRL B11291, maintained a 93% conversion rate of 100mM D,L-5-HPH, producing D-HPG at a rate of 5.6 : mol/mL/h/g DCW, for only two batch reactions (Chao *et al.*, 1999b). A recombinant, thermostable *N*-carbamoylase from *Agrobacterium* sp. KNK712, immobilised on Duolite A-568 retained 80% activity, producing D-HPG at a rate of 0.69 : mol/mL/h/g biocatalyst, after 15 batch reactions. The activity of this mutant enzyme was reported to be low in comparison to the wild-type enzyme (Nanba *et al.*, 1999). The high activity achieved by the immobilised *A. tumefaciens* RU-ORPN1 *N*-carbamoylase over the 5 repeated batch reactions indicates good stability of this enzyme compared with the *N*-carbamoylases reported in the literature.

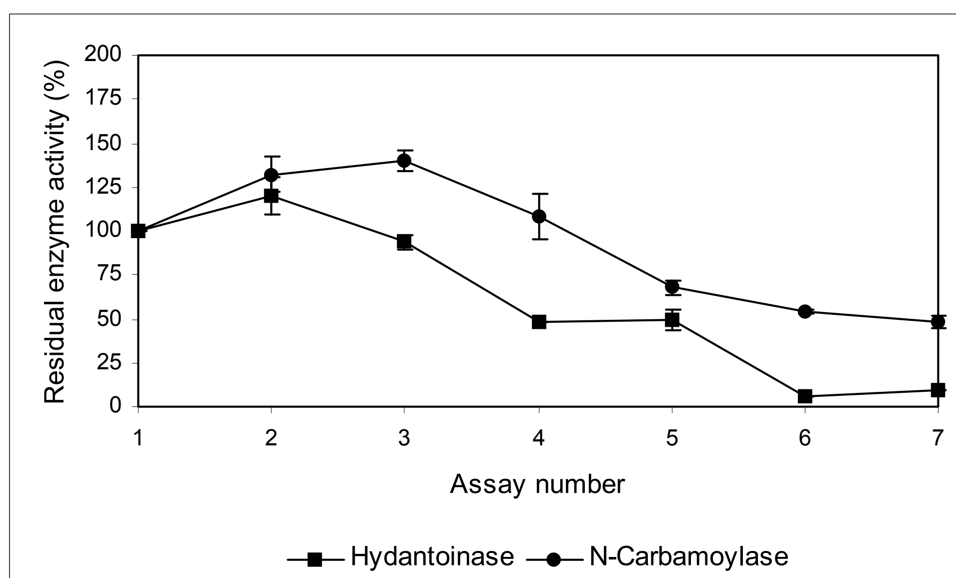


Figure 6.7 Reusability of the alginate bead-immobilised hydantoinase and *N*-carbamoylase.

6.3.6 Investigation of the substrate specificities of the alginate bead-immobilised enzymes for D,L-5-HPH and NC-HPG

The substrate specificities of the immobilised hydantoinase and *N*-carbamoylase were investigated in order to determine the effect of the immobilisation on the interaction of the enzymes with D,L-5-HPH and NC-HPG compared with hydantoin and NCG. Hydantoin and NCG were used throughout the project as model substrates; however, D,L-5-HPH and NC-HPG are the substrates of industrial interest for the production of D-HPG. It was therefore important to establish that the immobilised enzymes would be able to produce D-HPG from D,L-5-HPH before applying the biocatalyst in the bioreactor systems. The enzyme product yields obtained for the immobilised hydantoinase using hydantoin and D,L-5-HPH as substrates and for the immobilised *N*-carbamoylase using NCG and NC-HPG as substrates are shown in Figure 6.8. The immobilised hydantoinase product yields decreased from 2.4: mol/mL to 0.7: mol/mL when hydantoin and D,L-5-HPH were used as substrates, respectively. The rate of D,L-5-HPH conversion for the immobilised hydantoinase was 0.63: mol/mL/h/g wet weight cells. In comparison, a conversion rate of 2.9: mol/mL/h/g DCW for the conversion of 1.5mM D,L-5-HPH to NC-HPG for calcium alginate-immobilised recombinant *E.coli* whole cells expressing D-hydantoinase activity was reported (Chen *et al.*, 1999). Although the conversion of D,L-5-HPH by the *A. tumefaciens* RU-ORPN1 biocatalyst was low, the conversion rate was comparable with the conversion rate reported by these authors. The low activity obtained for the

hydantoinase with D,L-5-HPH can be attributed to poor mass transport of the substrate to the enzyme in the calcium alginate bead and the low specificity of the enzyme for D,L-5-HPH described in Chapter 3, section 3.3.9. The immobilised *N*-carbamoylase product yields increased from 5.0: mol/mL to 7.8: mol/mL when NCG and NC-HPG were used as substrates, respectively.

These results showed that D,L-5-HPH and NC-HPG could be used as substrates for the immobilised enzymes, and the biocatalyst was therefore applied in developing bioreactor systems where the hydantoinase activity in the bioreactors was increased by combining the *A. tumefaciens* sp. biocatalyst with the *Pseudomonas* sp. biocatalyst which displayed high hydantoinase activity in the presence of D,L-5-HPH (Burton *et al.*, 1998).

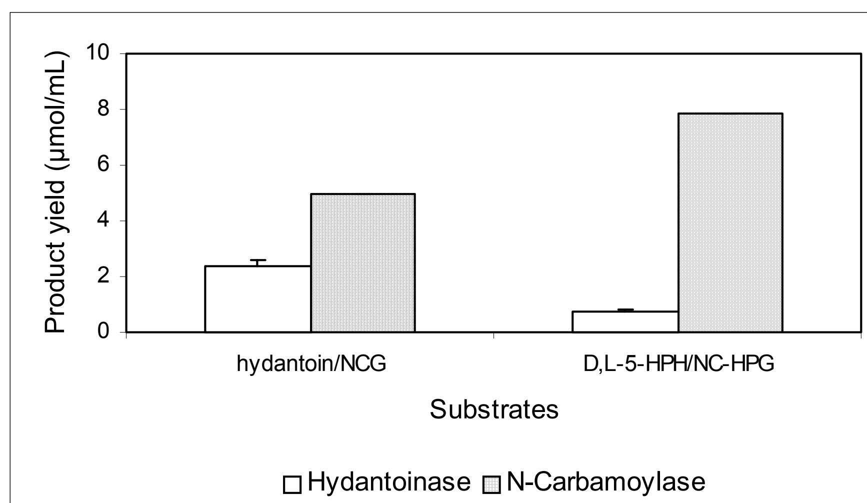


Figure 6.8 Comparison of hydantoin/NCG and D,L-5-HPH/NC-HPG substrates for the immobilised *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase.

6.3.7 Productivity of the *A. tumefaciens* RU-ORPN1 alginate bead biocatalyst in a packed-bed bioreactor operated in recycle mode

The productivity of the *A. tumefaciens* biocatalyst was initially evaluated by applying it in a packed-bed bioreactor system. The packed-bed bioreactor configuration was chosen in order to limit agitation which may have altered the physical integrity of the alginate beads. The unconverted substrate (and products) were recycled in order to evaluate the total conversion yield in the bioreactor over time and 100: M $CaCl_2$ was added to the substrate solution to minimise decalcification of the beads. The yields of NCG and glycine produced in the bioreactor over 18 days of operation, and the total conversion yield, are shown in Figure 6.9. The percentage of the total hydantoin converted in the bioreactor on each day of the operation is shown in Figure 6.10. In total, only 37% of the total hydantoin was converted after 18 days of operation at 37^0C (Figure 6.9). The percentage of the total hydantoin converted in the bioreactor each day was less than 5% (Figure 6.10) and the average rate of hydantoin conversion was 0.21mg/L/h. The average productivity of the bioreactor was calculated at 0.12mg glycine/L/h. This productivity is less than the productivity (0.86mg/L/h) of a continuous system reported by Ryu *et al.* (1997) for the production of L-cysteine. The low conversion yield obtained in this bioreactor may have been due to the presence of the products in the system. However, complete conversion of substrates was observed in subsequent bioreactors and therefore the enzymes did not appear to be susceptible to product inhibition. The low conversion yield obtained in this bioreactor was thus attributed to the low hydantoinase activity produced by the *A. tumefaciens* biocatalyst, and this was confirmed by the observation that throughout the operation of the bioreactor, the glycine concentration remained higher than the NCG concentration. The *A. tumefaciens* biocatalyst was therefore used in combination with the *Pseudomonas* sp. biocatalyst in subsequent bioreactors. The biochemical characteristics of the *Pseudomonas* sp. (*pers. comm.*, S. Kirchmann) and *A. tumefaciens* biocatalysts are summarised in Table 6.1.

The data generated from this bioreactor demonstrates that the *A. tumefaciens* biocatalyst was stable under the bioreactor operating conditions. In fact, the percentage hydantoin converted in the bioreactor gradually increased over the operation time (Figure 6.10), suggesting an increase in the enzyme activities. In contrast to this, the enzymes lost activity during incubation at 40^0C in the thermostability study (section 6.3.4). A possible explanation for the apparent increase in the enzyme activities is that the enzymes may have been stabilised in the presence of their substrates (O'Fagain *et al.*, 1988). The stability of the *A.*

tumefaciens RU-ORPN1 enzymes in the bioreactor indicated that the biocatalyst could be applied in a bioreactor system for the production of amino acids.

Table 6.1 Biochemical characteristics of the *Pseudomonas* sp. and *A. tumefaciens* sp. biocatalysts.

Biocatalyst	Enzyme	Activity* (μmol/mL)#	Activity/g bead (μmol/mL/g)*	pH optima	Temperature optima (^{0}C)
***A. tumefaciens* RU-ORPN1**	Hydantoinase	1.6 ± 1.4	2.4 ±1.4	7	50
	N-carbamoylase	6.6 ± 1.2	9.7 ±1.2	8	40
***Pseudomonas* sp. KM1**	Hydantoinase	14 ± 0.01	20.6 ±0.01	8	70
	N-carbamoylase	7.3 ± 0.2	10.7 ±0.2	8	60

* Enzyme activities presented as product concentrations produced after 24 hours

Errors represent the SEM (n=6)

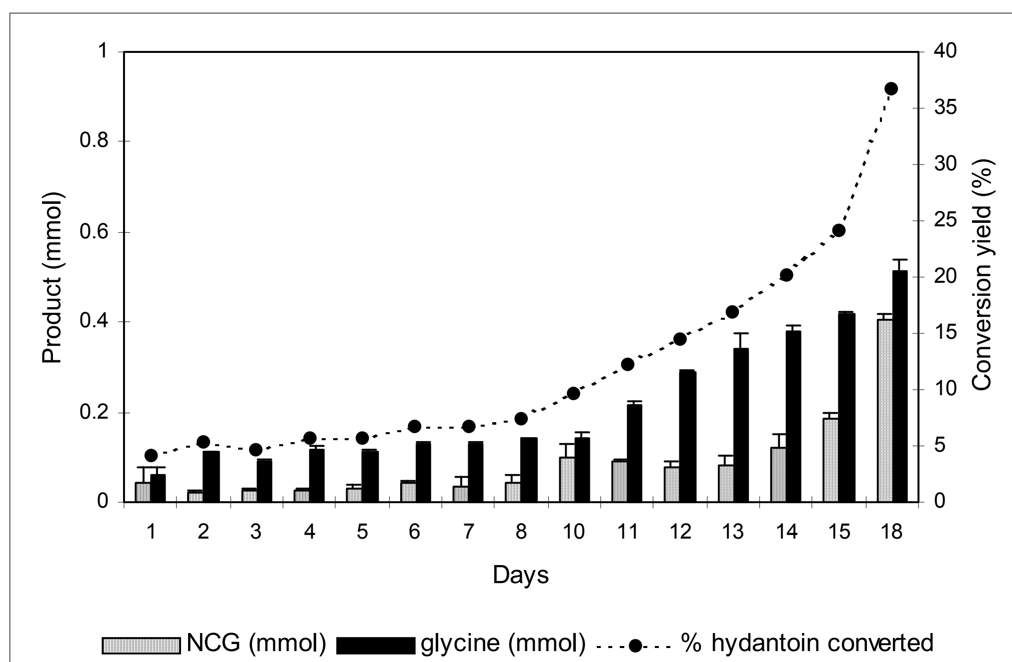


Figure 6.9 Product yield of the *A. tumefaciens* RU-ORPN1 biocatalyst in a packed-bed bioreactor over 18 days, operated in recycle mode.

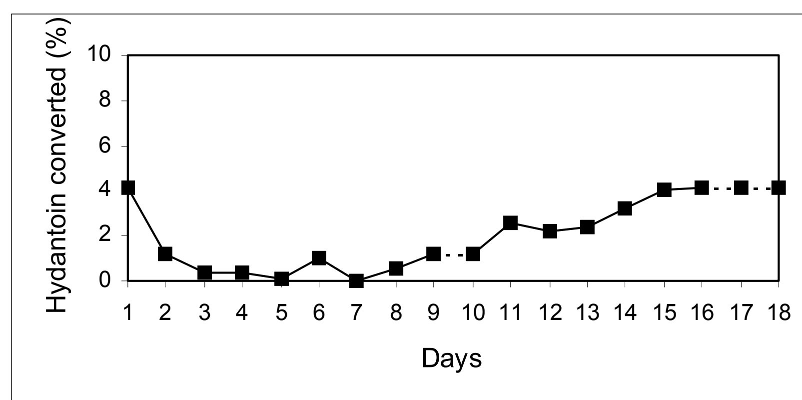


Figure 6.10 Hydantoin converted each day in a packed-bed bioreactor, operated in recycle mode, containing the *A. tumefaciens* biocatalyst, expressed as a percentage of the total hydantoin originally present in the system. Broken lines indicate mean values.

6.3.8 Productivity of the *A. tumefaciens* RU-ORPN1 biocatalyst combined with the *Pseudomonas* sp. biocatalyst in a packed-bed bioreactor operated in recycle mode

The yields of NCG and glycine produced, and the conversion yield, in the packed-bed bioreactor with the *A. tumefaciens* and *Pseudomonas* sp. biocatalysts combined in equal quantities are shown in Figure 6.11. The average rate of hydantoin conversion in this bioreactor was 1.4mg/L/h and the average productivity increased to 0.57mg glycine/L/h compared with 0.12 mg glycine/L/h in the bioreactor with the *A. tumefaciens* biocatalyst alone. The total biocatalyst loads in these two bioreactors were the same and therefore, the productivity in the bioreactor with the combined biocatalysts was greatly enhanced in comparison to the bioreactor containing the A. *tumefaciens* biocatalyst alone. A conversion yield of 93% was achieved in this bioreactor after 14 days operation (Figure 6.11). The percentage of the total hydantoin converted on each day of the operation more than doubled, varying between 8 and 18% for the majority of the operation time (Figure 4.12). A sharp increase in the hydantoin converted was observed on days 9 and 10 of the operation. This may have been due to improved mass transfer of substrate into the beads, as a result of possible weakening of the integrity of the beads or due to possible leakage of the enzyme extract out of the beads.

A potential advantage of having the majority of the hydantoinase and *N*-carbamoylase activities as separate biocatalysts would be that the levels of the two enzymes could be optimised for optimal productivity. It was observed that the NCG levels were higher than the glycine levels throughout the operation of this bioreactor, indicating that the *N*-carbamoylase was rate-limiting in this system. Optimisation of the relative amounts of the two biocatalysts for maximum productivity of the system was therefore needed. The accumulation of NCG in the system suggested that more of the *A. tumefaciens* biocatalyst was needed. Research investigating optimal ratios of hydantoinase to *N*-carbamoylase for optimal productivity in other systems has been described in the literature. The optimal ratio of D-hydantoinase to *N*-carbamoylase, identified by numerical simulation of the sequential reaction and experimental confirmation, using the enzymes partially purified from *Agrobacterium* sp. IP-671, was 3:1 for conversion of D,L-5-HPH to D-HPG (Kim and Kim, 1995). The overall reaction was thought not to be limited by either D-hydantoinase or *N*-carbamoylase at this ratio and accumulation of the reaction intermediate was minimised, resulting in an increased conversion yield of D-HPG. Chao *et al.* (2000b) reported an optimal productivity of D-HPG when the ratio of D-carbamoylase to D-hydantoinase ranged between 1 and 2 for the enzymes cloned separately from *Agrobacterium radiobacter* NRRL B11291 in

E.coli. This optimal ratio was identified by measuring D-HPG production with different proportions of the two recombinants producing the individual enzymes.

Despite the increase in the productivity of this bioreactor, the percentage hydantoin converted on each day was low. A number of parameters needed consideration in order to improve the productivity of the system. The low conversion yield may have been improved by increasing the biocatalyst load in the bioreactor or by altering the concentration of the substrate. The enzyme load within the beads could also have been optimised for maximum productivity as described by Chen *et al.* (1999). Finally, the flow rate for maximum conversion yield could have been optimised. The effect of increasing the biocatalyst load in the bioreactor was subsequently investigated.

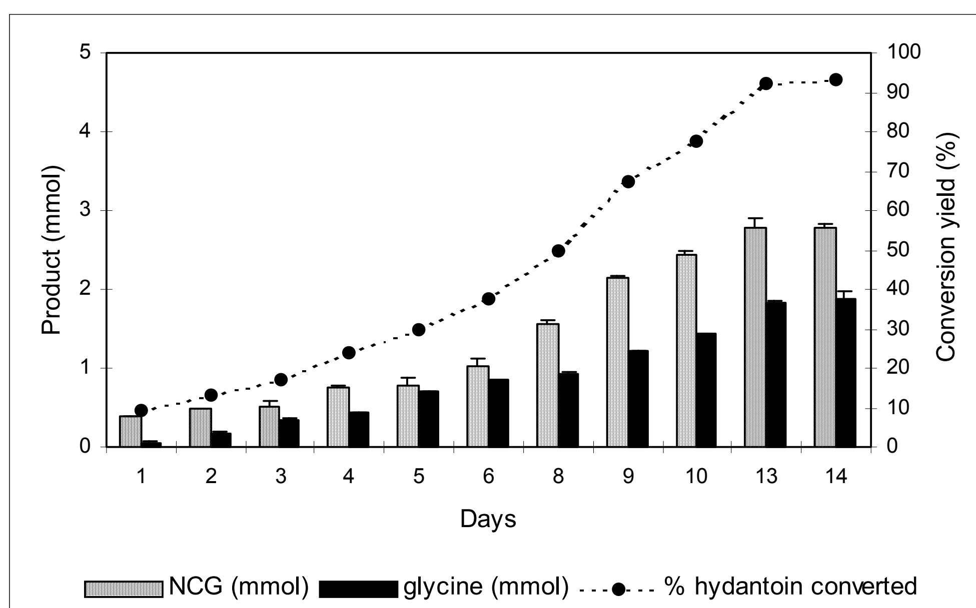


Figure 6.11 Product yield of the combined *A. tumefaciens* RU-ORPN1 and *Pseudomonas* sp. biocatalysts in a packed-bed bioreactor operated in recycle mode over 14 days.

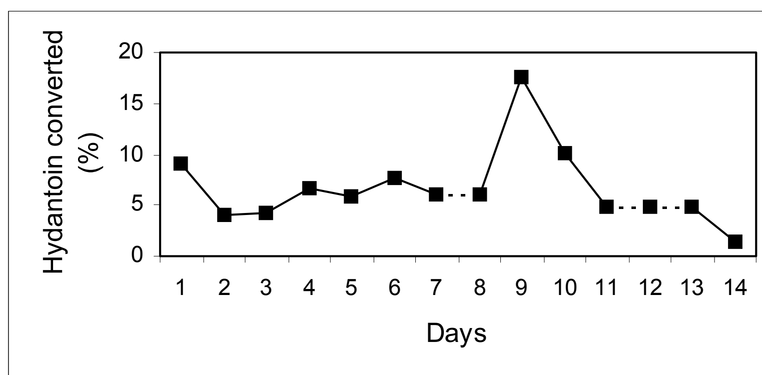


Figure 6.12 Hydantoin converted each day in a packed-bed bioreactor, operated in recycle mode, containing the combined *A. tumefaciens* and *Pseudomonas* sp. biocatalysts, expressed as a percentage of the total hydantoin originally present in the system. Broken lines indicate mean values.

6.3.9 Productivity of a scaled-up continuous bioreactor system containing the combined *A. tumefaciens* RU-ORPN1 and *Pseudomonas* sp. biocatalysts

The biocatalyst load in the packed-bed bioreactor was doubled in order to further improve the productivity of the bioreactor. The bioreactor was therefore scaled-up to a 80mL working volume by connecting 4 packed-bed columns in series as described in section 6.2.2.9. One of the problems with the two-step enzymatic production of amino acids is finding the optimal operating conditions with respect to activity and stability of both enzymes, if their properties differ. This problem could be overcome by using separately immobilised hydantoinase and *N*-carbamoylase in a two step process. Separate modules would allow for changes in pH to be made between modules, and the modules could be operated at different temperatures, in the case of biocatalysts having different pH and temperature optima. The separation of the biocatalysts could also be advantageous in an industrial process as a single module could easily be replaced if necessary. No industrial processes utilising free or immobilised hydantoinase and *N*-carbamoylase in a two step process have been reported (Syldatk *et al.*, 1999). Although, complete separation of the hydantoinase and *N*-carbamoylase enzymes was not achieved because the biocatalysts produced both enzyme activities, the respective biocatalysts had high hydantoinase and *N*-carbamoylase components. The modules in this bioreactor system were all operated under the same conditions in order to evaluate the effect of the increased biocatalyst load in the system.

The average rate of conversion of hydantoin in the bioreactor was 22mg/L/h and the productivity of the bioreactor increased to, on average, 14mg glycine/L/h with the increased biocatalyst load. The percentage of the total hydantoin converted on each day gradually decreased from 17% to 6% (Figure 6.13). The bioreactor was only operated over 6 days as, after this period, a significant degree of swelling of the alginate beads was observed. The swelling of the beads, however, was confined to the beads in the first two columns of the bioreactor only. The swelling resulted in the breaking and eventual disintegration of the beads. Swelling of the beads was not observed in any of the other bioreactor systems evaluated but swelling of calcium alginate beads in a continuous packed-bed bioreactor has been described by Chevalier *et al.* (1989).

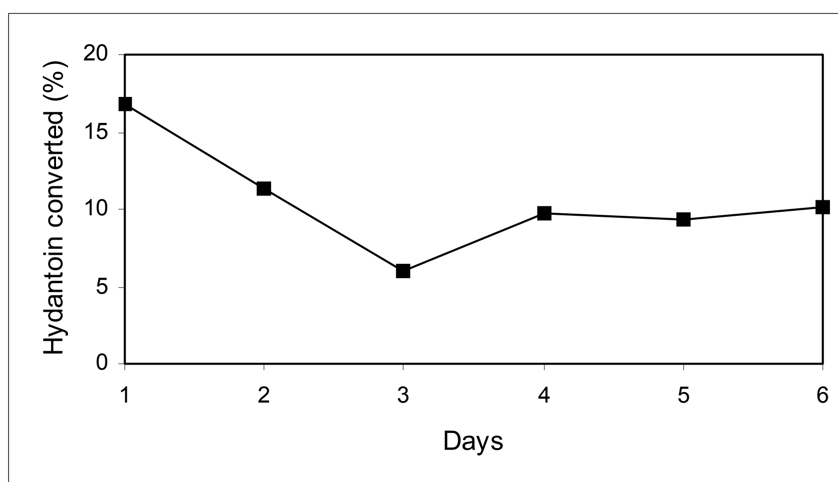


Figure 6.13 Hydantoin converted each day in a continuous bioreactor system with the *A. tumefaciens* and *Pseudomonas* sp. biocatalysts in separate packed-bed columns linked in series.

6.3.10 D-HPG production in a packed-bed bioreactor containing the combined *A. tumefaciens* RU-ORPN1 and *Pseudomonas* sp. biocatalysts operated in recycle mode

The production of D-HPG from D,L-5-HPH in the packed-bed bioreactor (working volume:20mL) with the combined *A. tumefaciens* and *Pseudomonas* sp. biocatalysts is shown in Figure 6.14. The average rate of D,L-5-HPH conversion in this bioreactor was 3.3mg/L/h and the productivity was, on average, 2.8mg D-HPG/L/h. The productivity of this bioreactor was therefore higher than the 0.86mg/L/h productivity reported for L-cysteine production in the continuous system reported by Ryu *et al.* (1997). The percentage of D,L-5-HPH converted in this bioreactor on each day is shown in Figure 6.15. The smaller volume configuration, having a single packed-bed column containing the biocatalysts, was chosen to avoid swelling and disruption of the beads during the operation. The system was further optimised by the use of *A. tumefaciens* RU-ORPN1 cells grown in PP2 medium in the Bioflow fermenter (see Chapter 2, section 2.2.2.2), which exhibited much higher *N*-carbamoylase activity than the cells used in the previous bioreactor experiments. In this bioreactor, almost complete conversion of D,L-5-HPH (99%) was achieved after 5 days operation with 30% of the total D,L-5-HPH being converted on the first day, decreasing to 22% on the final day (Figure 6.15). Again, in this bioreactor, the D-HPG concentration was much higher than the NC-HPG concentration, indicating that the hydantoinase enzyme was rate-limiting in this system and the productivity of the bioreactor could therefore be further improved by increasing the levels of the hydantoinase biocatalyst in the system.

The percentage conversion achieved in this bioreactor over the operation time is comparable to most of the percentage conversions achieved in the literature for continuous bioreactor systems utilising hydantoin-hydrolysing enzymes. Some of these are listed in Table 6.2. No continuous systems for the production of D-HPG or NC-HPG from D,L-5-HPH have been reported. A stirred tank, batch bioreactor system for the conversion of D,L-5-HPH to D-HPG using recombinant *E. coli* cells expressing the D-hydantoinase and *N*-carbamoylase genes from *Agrobacterium tumefaciens* NRRL B11291 has been described. The cells were immobilised in κ-carrageenan beads. A conversion of 93% D,L-5-HPH was achieved in this system after 14 hours, however, the yield of D-HPG from this reaction reached a maximum of only 25% after 4 hours, indicating a severe loss of *N*-carbamoylase activity after this length of time. The immobilised cells could only achieve a conversion of more than 90% for two batch cycles (Chao *et al.*, 1999b).

Some very efficient batch bioreactor systems have been developed for the conversion of D,L-5-HPH to NC-HPG. Kim and Kim (1993) describe a 300mL stirred-tank batch bioreactor system using polyacrylamide-immobilised whole cells for the production of NC-HPG from D,L-5-HPH. A 99% conversion of 1% D,L-5-HPH was achieved for 4 batch reactions varying from 10 to 30 hours in duration. More recently, a pressure swing batch reactor for the conversion of solid suspensions of D,L-5-HPH to NC-HPG has been reported. Concentrations of D,L-5-HPH as high as 15% (w/v) were converted in this bioreactor by recombinant D-hydantoinase covalently immobilised on porous polymer particles (Lee *et al.*, 2001b).

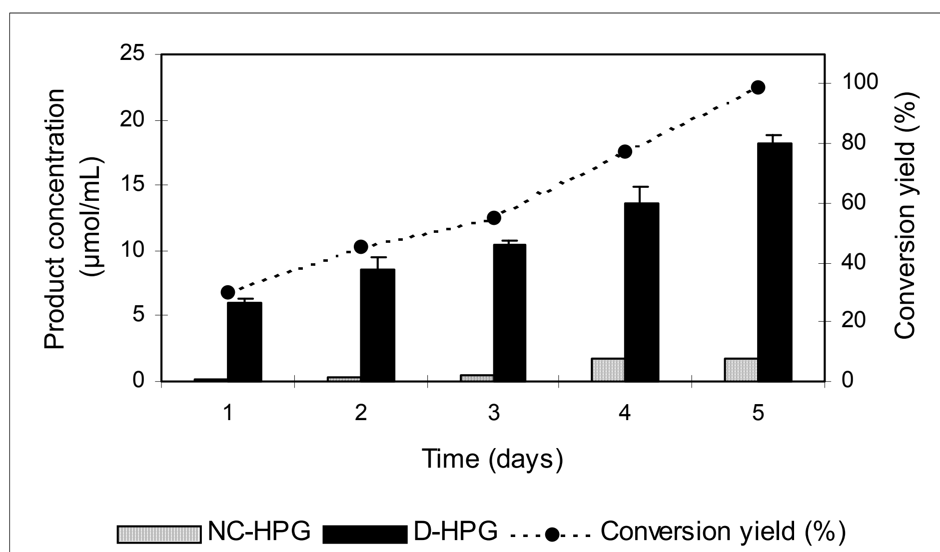


Figure 6.14 Conversion of D,L-5-HPH to D-HPG in a packed-bed bioreactor system containing the combined *A. tumefaciens* sp. and *Pseudomonas* sp. biocatalysts operated in recycle mode.

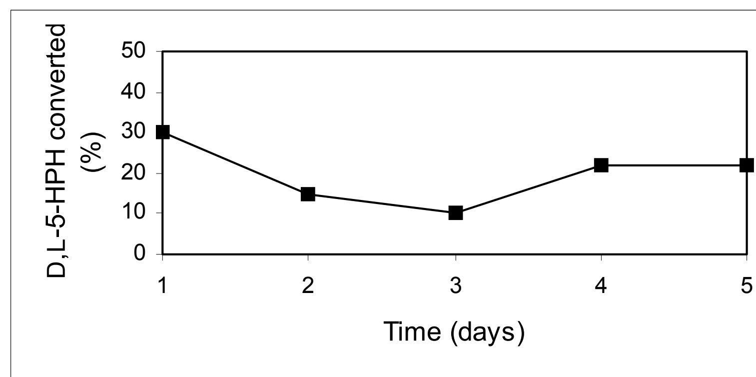


Figure 6.15 D,L-5-HPH converted each day in a packed-bed bioreactor, operated in recycle mode, containing the combined *A. tumefaciens* sp. and *Pseudomonas* sp. biocatalysts, expressed as the percentage of the total D,L-5-HPH originally present in the system.

Table 6.2 Percentage conversions achieved in some continuous bioreactor systems reported in the literature for the production of amino acids.

Organism	Biocatalyst configuration	Bioreactor type	Substrate	Product	Flow rate	% conversion
Pseudomonas flourescens DSM 84 (Morin *et al.*, 1986)	partially purified hydantoinase (non-immobilised)	membrane (M_r cut-off 5000) (10mL)	isopropylhydantoin (50mM)	*N*-carbamyl-D-valine	4.4mL/h	30
Pseudomonas putida DSM 84 (Chevalier *et al.*, 1989)	whole cells immobilised in calcium alginate beads	packed-bed (45mL)	dihydrouracil (50mM)	*N*-carbamyl-$-alanine	23mL/h	40-45
Peptococcus anaerobius (Morin and Lafond, 1992)	whole cells adsorbed on activated charcoal	packed bed with percolation (8mL)	methylhydantoin (100mM)	*N*-carbamyl-D-alanine	0.5mL/h	30
		fluidised-bed (25mL)				20
Pseudomonas sp. M-38 (Ryu *et al.*, 1997)	whole cells immobilised in calcium alginate beads	packed-bed columns (total volume: 60mL) combined with product extractors	D,L-amino-A^2-thiazoline-4-carboxylic acid (30mM)	L-cysteine	23mL/h	40-53
Arthrobacter aurescens (Ragnitz *et al.*, 2001)	crude hydantoinase immobilised on Eupergit C250 L and purified L-*N*-carbamoylase immobilised on EAH-Sepharose	packed-bed	DL-5-indolylmethyl hydantoin (0.4g/L) or NC-L-tryptophan (1g/L)	NC-L-tryptophan or L-tryptophan	not specified	over 90% for hydantoinase; 10-80% for L-*N*-carbamoylase
		stirred tank (150mL)			0.3mL/min	0ver 90% for hydantoinase; 10-70% for L-*N*-carbamoylase

6.4 CONCLUSIONS

The alginate bead-immobilised *A. tumefaciens* RU-ORPN1 hydantoinase and *N*-carbamoylase were biochemically characterised in order to establish the optimal operating conditions for the biocatalyst in a bioreactor system. The enzyme reaction time required for optimal hydantoinase and *N*-carbamoylase product yields was found to be 24 hours. The hydantoinase activity, however, was low and the enzyme had low substrate specificity for D,L-5-HPH. The apparent pH optima of the alginate bead-immobilised hydantoinase and *N*-carbamoylase were pH 7 and 8, respectively. The apparent temperature optimum of the alginate bead-immobilised hydantoinase was 50^0C, while the apparent temperature optimum for the immobilised *N*-carbamoylase was 40^0C. Immobilisation of the hydantoinase did not increase the thermostability of the enzyme in comparison to the free enzyme, while the immobilised *N*-carbamoylase showed significantly enhanced thermostability in comparison to the free enzyme.

Both immobilised enzymes could be reused in repeated batch reactions with the hydantoinase retaining over 90% activity after three cycles and the *N*-carbamoylase retaining 100% activity for 4 cycles. In general, the immobilisation of the enzymes had a favourable effect on the stability of the enzymes and the productivity of the biocatalyst was evaluated in various bioreactor systems. The productivity of the *A. tumefaciens* RU-ORPN1 biocatalyst in a packed-bed bioreactor appeared to be low due to the low hydantoinase activity produced by the *A. tumefaciens* strain and the average conversion of hydantoin was less than 5%. The productivity of the system was improved by using the *A. tumefaciens* biocatalyst in combination with the *Pseudomonas* sp. biocatalyst which had high hydantoinase activity. The average conversion of hydantoin in this bioreactor approximately doubled, at an average of 10%, showing that enhanced productivity could be achieved by combining the two biocatalysts from different bacterial strains. The productivity of the bioreactor with the combined biocatalysts was improved further by increasing the load of each biocatalyst in the system. The calcium alginate beads in this, larger-scale bioreactor, however, were unstable and swelling resulted in the disintegration of the beads. Finally, by optimising the *N*-carbamoylase activity in the cells used for the *A. tumefaciens* biocatalyst, and by using this biocatalyst in combination with the *Pseudomonas* sp. biocatalyst, a conversion of 22 to 30% D,L-5-HPH was achieved in a packed-bed bioreactor operated for 5 days. The average productivity of this bioreactor was 2.8mg D-HPG/L/h. In many instances, the effects of changes in the product yields due to changes in the enzyme

stabilities could not be distinguished from effects resulting from changes in enzyme activity. This was due to the complexity of the system, particularly in the various bioreactors. Such a study of the biocatalyst could be addressed further in possible future work.

To our knowledge, this is the first report of the application of a D-*N*-carbamoylase in a continuous bioreactor system, and the use of a hydantoinase and *N*-carbamoylase from two different bacterial species for the complete enzymatic production of D-HPG from D,L-5-HPH is novel. One of the most important results generated from the various bioreactor systems was the demonstration of the stability of the *A. tumefaciens* RU-ORPN1 biocatalyst, particularly the stability of the *N*-carbamoylase activity under the various operating systems. In fact, the *N*-carbamoylase showed greater stability under the bioreactor operating conditions than in the preliminary thermostability and storage experiments. In addition, the conversion rate achieved for the production of D-HPG from D,L-5-HPH in the packed-bed bioreactor operated in recycle mode compared favourably with the conversion rates reported in the literature for the continuous production of other amino acids. Furthermore, optimisation of the bioreactor system developed here in terms of relative biocatalyst loads, substrate concentration and flow rates could further improve the productivity of the system. The data generated here demonstrates that the hydantoin-hydrolysing enzymes from *A. tumefaciens* RU-ORPN1 could be stabilised and, in combination with a biocatalyst with high hydantoinase activity, could be applied to the complete enzymatic conversion of D,L-5-HPH to D-HPG.

This thesis describes the development and evaluation of a hydantoin-hydrolysing biocatalyst for the production of D-HPG, an amino acid of commercial interest to the pharmaceutical industry. *Agrobacterium tumefaciens* RU-ORPN1, a regulatory mutant of *A. tumefaciens* RU-OR, was chosen as the source of the hydantoin-hydrolysing enzymes, based on certain characteristics found in this bacterial strain which were favourable for the development of an industrial biocatalyst. The first of these was the relatively rare characteristic of having both hydantoinase and *N*-carbamoylase activities present in a single strain which made the development of a fully enzymatic process for the production of D-HPG possible. Secondly, the strain was found to produce high levels of *N*-carbamoylase activity relative to other reported bacterial strains (Burton *et al.*, 1998) which offers the potential for high productivity. A third favourable characteristic was the inducer-independence of the mutant strain which also offered the potential for the development of a more cost-effective process, since the addition of inducers increases the costs of industrial processes.

One of the most important prerequisites for the development of a successful biotechnological process is a stable biocatalyst. The development of a fully enzymatic process thus required the stabilisation of the hydantoinase and *N*-carbamoylase enzymes, and stabilisation of the enzymes was a major emphasis of the research. The general instability of *N*-carbamoylases, in particular, has hampered the introduction of these enzymes into other industrial processes. As a result, the majority of industrial processes for the production of amino acids are currently carried out using a hydantoinase enzyme for the conversion of the hydantoin substrate to the *N*-carbamyl intermediate, followed by a chemical conversion step to generate the amino acid. The stabilisation of the *N*-carbamoylase was therefore a particular challenge to be considered in this research.

A further criterion for determining the feasibility of a commercial biotransformation process is the availability of inexpensive starting materials (Fotheringham, 2000). Thus, the cost of the growth medium for the production of the biocatalyst had to be considered. This aspect of the research was investigated by collaborators with the Rhodes Hydantoinase Research project, at CSIR, South Africa. The PP2 medium used for the growth of *A. tumefaciens* RU-ORPN1 was developed in the later stages of the research by these researchers, for optimal production of hydantoin-hydrolysing enzyme activity. This growth medium, containing sunflower oil as a carbon source, was developed as a cheap growth medium for production of large yields of bacterial biomass with high levels of hydantoinase and *N*-carbamoylase activities. The

medium was found to be favourable for the production of high yields of biomass with particularly high *N*-carbamoylase activity.

The hydantoinase reaction product yields obtained throughout the research were relatively low in comparison to the *N*-carbamoylase product yields and in comparison to the levels reported by previous researchers working on the strain (Hartley *et al.*, 2001). It therefore became apparent during the initial studies that the hydantoinase enzyme reaction would be the rate limiting step in the production of D-HPG, restricting the productivity of the process. This was confirmed later by the application of the enzymes in a bioreactor system which resulted in a very low conversion yield. The levels of hydantoinase activity in the strain would therefore need to be optimised either by adjusting the growth conditions of the strain or by the cloning and over-expression of the gene for the enzyme in *E.coli*, in order to develop a successful one-step process using this strain alone. The effects of various sources and levels of carbon and nitrogen in the PP2 medium on the hydantoinase activity are currently being investigated by co-workers on the project in order to optimise the hydantoinase activity in the cells.

One of the objectives of this research was to identify the optimal reaction conditions for the hydantoinase and *N*-carbamoylase from *A. tumefaciens* RU-ORPN1 with the aim of process development. The biochemical characterisation of the enzymes was not achieved as the enzymes were not purified. This type of characterisation would have given valuable information regarding the intrinsic properties of the enzymes and their stabilities. However, sufficient data was collected using the enzymes in the form of a crude enzyme extract. The apparent pH optima of the enzymes were amongst the highest reported for bacterial hydantoinases and *N*-carbamoylases in the literature. These high apparent pH optima were advantageous for the production of D-HPG since spontaneous racemisation of the substrates occurs at alkaline pH allowing for the 100% conversion of a racemic mixture of substrate. The apparent temperature optima for the enzymes were amongst the lowest reported for bacterial hydantoinase and *N*-carbamoylases. High temperatures increase reaction rates and are therefore favoured for industrial processes. The solubility of D,L-5-HPH is also increased at higher temperatures, promoting the conversion of D,L-5-HPH to D-HPG. The *N*-carbamoylase was found to be unstable at 40^0C and was gradually inactivated at this temperature. However, the *N*-carbamoylase from a currently used industrial strain *Agrobacterium radiobacter* NRRL B 11291 was also rapidly inactivated at this temperature (Fan *et al.*, 2000). At 4^0C, the *A. tumefaciens* RU-

ORPN1 *N*-carbamoylase showed greater stability, decreasing to 34% residual activity after 14 days storage, in comparison to 20% residual in the case of the industrial enzyme (Buson *et al.*, 1996). The *N*-carbamoylase from *A. tumefaciens* RU-ORPN1 thus showed good stability in comparison to this industrial enzyme indicating that it would be suitable for application in an industrial process.

The hydantoinase and *N*-carbamoylase in the enzyme extract appeared to be insoluble or membrane-bound and therefore required solubilisation to facilitate purification of the enzymes. The specific enzyme activities in the supernatant of the enzyme extract were significantly lower than those reported for these enzymes in the literature in supernatants after cell lysis. Solubilisation of the enzymes could not be achieved without further decreasing the specific enzyme activities in the supernatants. However, although the purification of the enzymes would have been beneficial for the biochemical characterisation of the enzymes and examination of the enzyme reaction kinetics, the use of purified enzymes as industrial biocatalysts is usually not cost-effective. The purification procedures described in the literature for the purification of *N*-carbamoylase enzymes, in particular, generally involve multiple steps and result in low yields of active enzyme due to its innate susceptibility to oxidising conditions (Sareen *et al.*, 2001). The enzymes are generally considered to be more stable within cells due to the reducing nature of the intracellular environment (Fan *et al.*, 2000).

Various immobilisation methods were investigated to improve the stability of the enzymes. Immobilisation not only has the advantage of allowing the reuse of the biocatalyst, thereby reducing costs, but has been shown to improve the stability of some enzymes (Klibanov, 1983). The best method of immobilisation, in terms of activity retention and durability during storage, was entrapment of the enzyme extract in calcium alginate beads. The marked increase in the *N*-carbamoylase product yield achieved with this form of immobilisation was an important result, since no other reports of increased *N*-carbamoylase activity on immobilisation have been made. The alginate bead-immobilised hydantoinase and *N*-carbamoylase showed improved stabilities at 4^0C and, importantly, the immobilised *N*-carbamoylase showed improved thermostability at 40^0C relative to the free enzyme. The apparent pH optima of the immobilised enzymes decreased to pH 7 and 8 for the hydantoinase and *N*-carbamoylase, respectively. The differing pH optima for the enzymes would not be ideal for the development of a fully enzymatic process for D-HPG since the conditions could not be optimised for both enzymes. A pH of 8 was chosen for the operation of the

bioreactor systems, favouring the *N*-carbamoylase activity. The higher apparent temperature optimum of the hydantoinase enzyme, however, allowed for the operation of the bioreactor systems at 40^0C, the optimal temperature for the *N*-carbamoylase.

The use of an immobilised cell homogenate, including the cell debris, as described in this research, for the production of D-HPG, was novel. To date, all but one industrial process, which uses purified hydantoinase from calf liver (Dinelli *et al.*, 1976, Cecere *et al.*, 1977), use free or immobilised resting microbial cells. The use of free and immobilised whole cells in industrial processes has the disadvantage of possible contamination occurring with the use of viable cells. Furthermore, the formation of extracellular metabolites may lead to product contamination (Yin *et al.*, 2000). In the case of whole cell biocatalysts immobilised by entrapment, mass transport problems are often experienced since substrates and products have to be transported through the support matrix and cell membrane. Cell membranes have been found to be impermeable to *N*-carbamyl intermediates, in particular (Lee *et al.*, 2001a). On the other hand, the use of purified enzymes has the disadvantage of increasing the costs of an industrial process and, in the case of entrapped or encapsulated enzymes, leaching of the purified enzymes may be experienced resulting in the loss of enzyme activity and lowered productivity. The lack of solubility of the *A. tumefaciens* RU-ORPN1 enzymes therefore may be advantageous, since with the immobilisation of the enzymes which appeared to be membrane-bound in the cell debris, leaching of the enzymes may occur less readily. Furthermore, the use of an enzyme extract would improve the mass transfer of the substrates due to the disruption of the cell membrane. The use of the enzyme extract could thus avoid some of the shortcomings of whole cell- and purified enzyme-immobilisation.

The low conversion yield obtained with the application of the *A. tumefaciens* RU-ORPN1 biocatalyst in the packed-bed bioreactor system indicated that the hydantoinase activity levels of the biocatalyst were insufficient for the efficient conversion of D,L-5-HPH to D-HPG. This was confirmed by the enhanced productivity of the bioreactor with the use of the *A. tumefaciens* RU-ORPN1 biocatalyst in combination with the *Pseudomonas* sp. biocatalyst which had high hydantoinase activity. The two biocatalysts combined in the packed-bed bioreactor produced D-HPG from D,L-5-HPH at a conversion rate comparable to continuous systems reported in the literature for the production of amino acids. The conversion of D,L-5-HPH over 5 days operation demonstrated that the biocatalysts were stable under the operating conditions.

Furthermore, the bioreactor system developed here was novel, since no other bioreactor systems have been described using hydantoinase and *N*-carbamoylase enzymes from two different bacterial species.

In order to apply this system to a scaled-up, cost-effective industrial process, certain draw-backs in the system would need to be addressed. The growth of two different bacterial strains with potentially different optimal growth conditions and growth media would elevate the costs of an industrial process enormously. The cloning and over-expression of the genes for the *Pseudomonas* sp. hydantoinase and *A. tumefaciens* *N*-carbamoylase in *E. coli,* or in a homologous host, would be favourable as a recombinant strain could be grown up in a single fermentation, cutting costs. This has been done for the thermostable hydantoinase from *Bacillus stearothermophilus* SD-1 and the *N*-carbamoylase from *Agrobacterium tumefaciens* NRRL B11291 which have been cloned and expressed in *E.coli* (Park *et al.*, 2000). A further advantage of the recombinant expression of the enzymes is that the level of expression of each enzyme could be controlled and optimised for maximum productivity. The *A. tumefaciens* RU-ORPN1 enzymes could also be purified by cloning and expression of the genes for the hydantoinase and *N*-carbamoylase enzymes in *E.coli* in a soluble form. This has been accomplished for the enzymes from *Agrobacterium tumefaciens* NRRL B11291 (Chao *et al.*, 2000a). This approach to the purification of the *A. tumefaciens* RU-ORPN1 *N*-carbamoylase is currently being researched by a co-worker on the Rhodes Hydantoinase Project. Cloning of the hydantoinase and *N*-carbamoylase genes would also allow purification of the enzymes by expression of tagged proteins and isolation using immobilised metal affinity chromatography (Pietzsch *et al.*, 2000; Pasquinelli *et al.*, 2000). The control of the expression of all three enzymes, hydantoin racemase, hydantoinase and *N*-carbamoylase, has been demonstrated by Wilms *et al*., (2001).
The preparation of large quantities of calcium alginate beads may also be logistically complex and time-consuming and the robustness of the beads in a large scale operation is questionable. Alternative immobilisation methods more suitable for a large-scale industrial process would need to be investigated. Immobilisation on the macroporous bead carrier, Eupergit®, has been found to be very efficient, resulting in high retention of catalytic activity (Hernaiz and Crout, 2000) and half-lives of 14000 and 900 hours for a hydantoinase and *N*-carbamoylase immobilised on this support has been reported (Ragnitz *et al.*, 2001). However, partially purified hydantoinase and highly purified *N*-carbamoylase were required for immobilisation on this support.

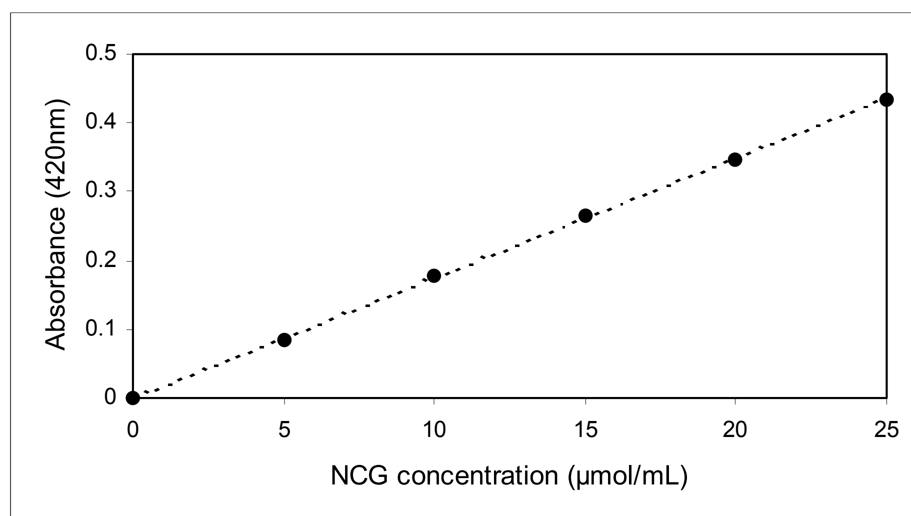
In conclusion, this research has demonstrated that the product yields of the hydantoin-hydrolysing enzymes from *A. tumefaciens* RU-ORPN1 could be enhanced and the enzymes stabilised by optimisation of the culture and enzyme reaction conditions, and by immobilisation of the enzymes in crude extract form. The improved stability of the hydantoin-hydrolysing enzymes was further demonstrated by their application in bioreactor systems where the enzymes were shown to retain catalytic activity for prolonged periods of time. A novel bioreactor system containing the *A. tumefaciens* RU-ORPN1 biocatalyst in combination with a *Pseudomonas* sp. biocatalyst, showed that the *A. tumefaciens* biocatalyst, in combination with a biocatalyst with high hydantoinase activity, could be applied in an industrial process for the completely enzymatic conversion of D,L-5-HPH to D-HPG.

APPENDIX A

Constituents of the PP2 growth medium

1g hydantoin
10g yeast extract
10g sunflower oil (autoclaved separately)
0.8g citric acid
1.4g $MgSO_4.7H_2O$
4g KH_2PO_4
3.5mL trace elements*
1000mL dH_2O

* Trace elements solution (1L) :
10g $FeSO_4.7H_2O$
2.6g $CaCl_2.2H_2O$
2.2g $ZnSO_4.7H_2O$
0.5g $MnSO_4.4H_2O$
1g $CuSO_4.5H_2O$
0.1g $(NH_4)MO_7O_{24}.4H_2O$
0.02g $Na_2B_4O_7.10H_2O$
160mL HCl
840mL dH_2O

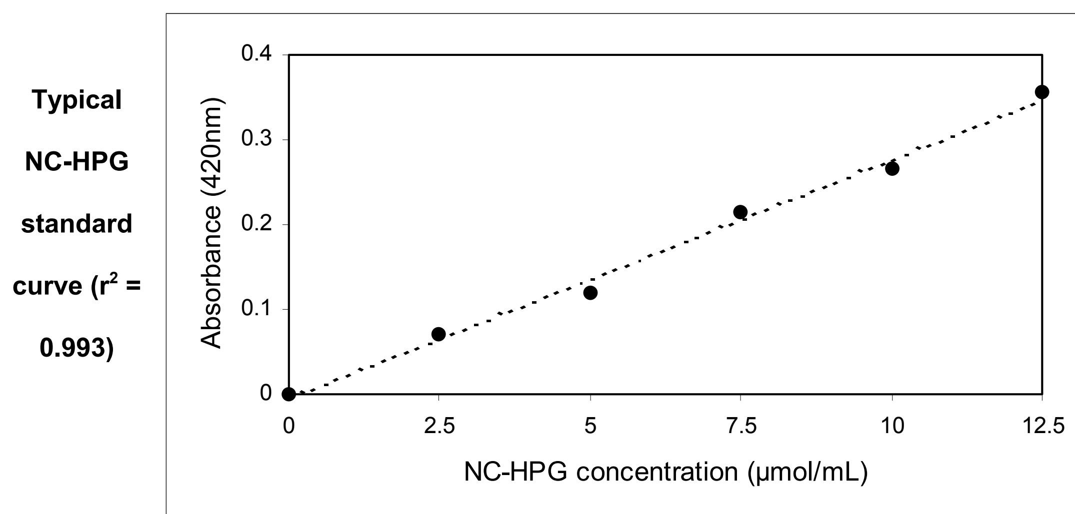


APPENDIX B

Typical NCG and NC-HPG standard curves

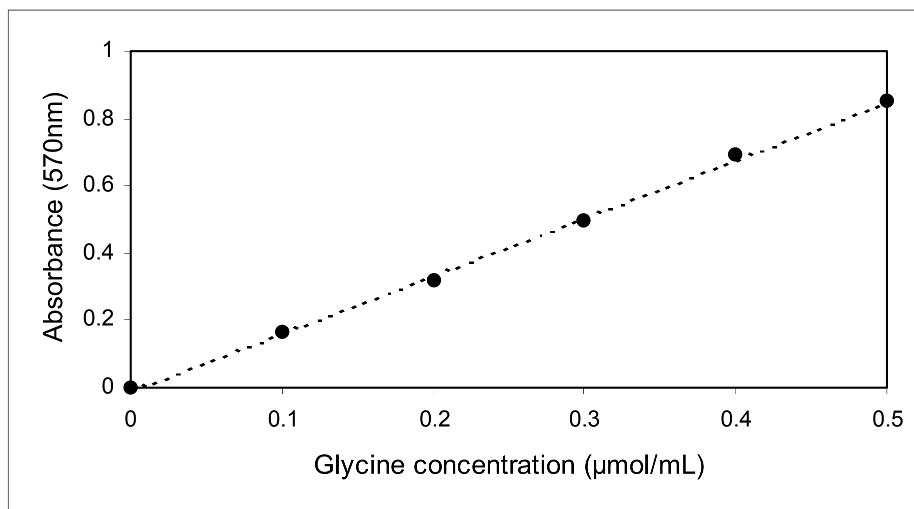
Typical NCG standard curve (r^2 = 0.999)

Typical NC-HPG standard curve (r^2 = 0.993)

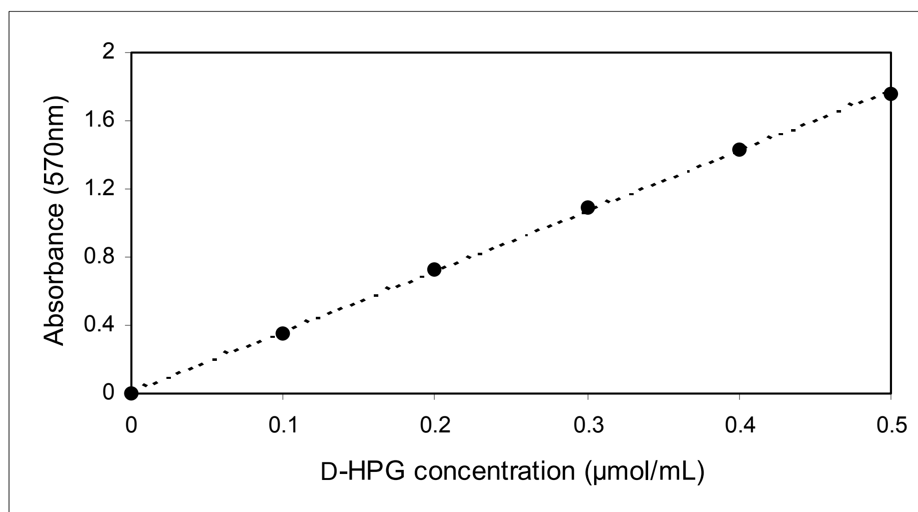


APPENDIX C

<u>Typical glycine and D-HPG standard curves</u>



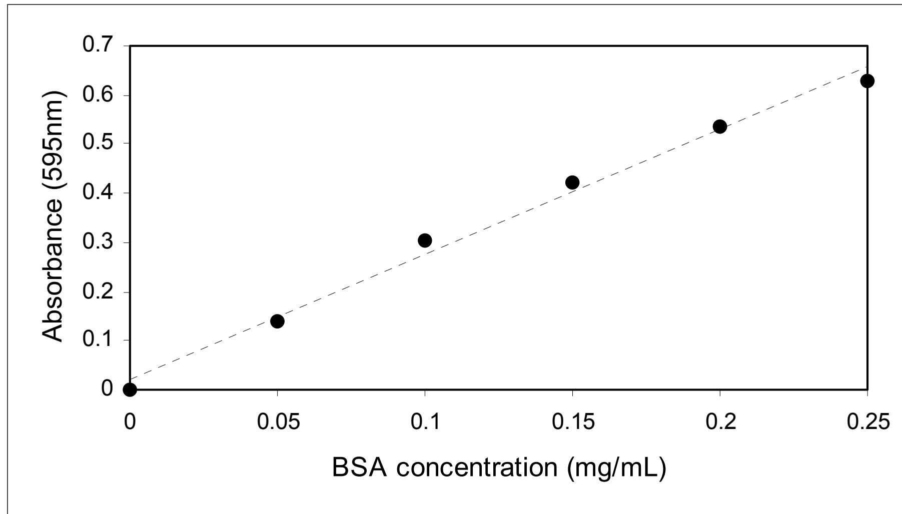
Typical glycine standard curve (r^2 = 0.999)



Typical D-HPG standard curve (r^2 = 0.999)

APPENDIX D

Typical protein standard curve



Typical BSA Standard Curve (r^2 = 0.992)

APPENDIX E

***A. tumefaciens* RU-OR *N*-carbamoylase genes (*NcaR1* and *NcaR2*) predicted amino acid sequences**

***NcaR1* predicted amino acid sequence:**

1 MTRQMILAVG QQGPIARAET REQVVGRLLD MLTNAASRGV NFIVFPELAL
51 TTFFPRWHFT DEAELDSFYE TEMPGPVVRP LFETAAELGI GFNLGYAELV
101 VSGGVKRRFN TSILVDKSGK IVGKCRKIHL PGHKEYEAYR PFQLLKRYFE
151 PGDLGFPVYN VDAAKMGMFI CNDRRWPETW RVMGLKGAEI ICGGYNTPTH
201 NPPVPQHDHL TSFHHLLSMQ AGSYQNGAWS AAAGKVGMEE GCMLLGHSCI
251 VAPTGEIVAL TTTLEDEVIT AAVDLDRCRE LREHIFNFKA HRQPQHYGLI
301 KEF*

***NcaR2* predicted amino acid sequence:**

1 MTRQMILAVA QQGPISRALA TANRLVSRLR ALAKMAEQRG VRTSSVFPEL
51 ALTTFFPRSP DEAELDSFYP KCPARWSVPL FRRPPNSGIG LQSGLRELRE
101 GGRRRRFNTS LQHVKSGGVR QKRRKVSHPG HKEPEAYRPH QHLEKRYFFP
151 GDLGFRGLPA VPASMAMFIC NDRRWPELRR VMGLGDVELQ RSGYNTPAGD
201 PPVGRRDHLR LQHADLSMQA GPYQNSRWTS DAAKMGMEEG CDRRGPECIV
251 APRGVKGARS DSLEKLRIPS SSDLDCKKYW RSTRFNFKAH REPDHYRLIA
301 EL*

REFERENCES

Abendroth J, Chetterjee S and Schomburg D (2000a) Purification of a D-hydantoinase using a laboratory-scale Streamline phenyl column as the initial step. J. Chromatography B **737**:187-194

Abendroth J, Niefind K, Chatterjee S and Schomburg D (2000b) Crystallization, preliminary X-ray analysis of a native and selenomethionine D-hydantoinase from *Thermus* sp. Acta Cryst. Section D, Biol. Cryst. **56**:1166-1169

Abendroth J, Niefind K, May O, Siemann M, Syldatk C and Schomburg D (2002a) The structure of L-hydantoinase from *Arthrobacter aurescens* leads to an understanding of dihydropyrimidinase substrate and enantiospecificity. Biochemistry **41(27)**:8589-8597

Abendroth J, Niefind K and Schomburg D (2002b) X-ray structure of a dihydropyrimidinase from *Thermus* sp. at 1.3D resolution. J. Mol. Bio. **320(1)**:143-156

Achary A, Hariharan KA, Bandhyopadhyaya S, Ramachandran R and Jayaraman K (1997) Application of numerical modeling for the development of optimized complex medium for D-hydantoinase production from *Agrobacterium radiobacter* NRRL B 11291. Biotechnol. Bioeng. **55(1)**:148-154

Altenbuchner J, Siemann-Herzberg M and Syldatk C (2001) Hydantoinases and related enzymes as biocatalysts for the synthesis of unnatural chiral amino acids. Curr. Opin. Biotechnol. **12**:559-563

Arcuri MB, Antunes OAC, Sabino SJ, Pinto GF and Oestreicher EG (2000) Resolution of DL-hydantoins by D-hydantoinase from Vigna angularis: Production of highly enantioenriched *N*-carbamoyl-D-phenylglycine at 100% conversion. Amino acids **19(2)**:477-482

Azerad R (2001) Chemical biotechnology: better enzymes for green chemistry. Curr. Opin. Biotechnol. **12**:533-534

Bernheim F and Bernheim MLC (1946) The hydrolysis of hydantoin by various tissues. J. Biol. Chem. **163**:683-685

Bollag DM and Edelstein SJ (1991) Protein methods. Wiley-Liss, New York

Bommarius AS, Schwarm M and Drauz K (1998) Biocatalysis to amino acid-based chiral pharmaceuticals-examples and perspectives. J. Mol. Catal. B **5(1-4)**:1-11

Boshoff A (2001) The biotransformation of phenolic pollutants using polyphenol oxidase. PhD thesis, Rhodes University

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254

Buchanan K, Burton SG, Dorrington RA, Matcher GF and Skepu Z (2001) A novel *Pseudomonas putida* strain with high levels of hydantoin-converting activity, producing L-amino acids. J. Mol. Catal. B **11**:397-406

Burton SG, Dorrington RA, Hartley C, Kirchmann S, Matcher G and Phehane V (1998) Production of enantiomerically pure amino acids: characterisation of South African hydantoinases and hydantoin-producing bacteria. J. Mol. Catal. B **5**:301-305

Buson A, Negro A, Grassato L, Tagliaro M, Basaglia M, Grandi C, Fontana A and Nuti MP (1996) Identification, sequencing and mutagenesis of the gene for a D-carbamoylase from *Agrobacterium radiobacter*. FEMS Microbiol. Lett. **145**:55-62

Busto MD, Ortega N and Perez-Mateos M (1995) Studies on microbial $-D-glucosidase immobilized in alginate gel beads. Process Biochem. **30(5)**:421-426

Cecere F, Galli G, Della Penna G and Rappuoli B (1977) Method for the preparation of D-carbamyl amino acids and the corresponding D-amino acids. US Patent 4065353, Snamprogetti, S.p.A.

Chang HN, Seong GH, Yoo IK, Park JK and Seo JH (1996) Microencapsulation of recombinant *Saccharomyces cerevisiae* cells with invertase activity in liquid-core alginate capsules. Biotechnol. Bioeng. **51**:157-162

Chao Y-P, Chiang C-J and Chen PT (2000b) Optimum ratio of D-carbamoylase to D-hydantoinase for maximizing D-*p*-hydroxyphenylglycine productivity. Biotechnol. Lett. **22(1)**:99-103

Chao Y-P, Chiang C-J, Lo TE and Fu H (2000a) Overproduction of D-hydantoinase and carbamoylase in a soluble form in *Escherichia coli*. Appl. Microbiol. Biotechnol. **54**:348-353

Chao Y-P, Fu H, Lo T-E, Chen PT and Wang J-J (1999b) One-step production of D-*p*-hydroxyphenylglycine by recombinant *Escherichia coli* strains. Biotechnol. Prog. **15**:1039-1045

Chao Y-P, Juang T-Y, Chern J-T and Lee C-K (1999a) Production of D-*p*-hydroxyphenylglycine by *N*-carbamoyl-D-amino acid amidohydrolase-overproducing *Escherichia coli* strains. Biotechnol. Prog. **15**:603-607

Chen Y-C, Yin B-D, Lin S-C and Hsu W-H (1999) Production of *N*-carbamoyl-D-hydroxyphenylglycine by D-hydantoinase activity of a recombinant *Escherichia coli*. Process Biochem. **35**:285-290

Cheon Y-H, Kim G-J and Kim H-S (2000) Stabilization of D-hydantoinase by intersubunit cross-linking. J. Mol. Catal. B **11**:29-35

Cheon Y-H, Kim H-S, Han K-H, Abendroth J, Niefind K, Schomburg D, Wang J and Kim Y (2002) Crystal structure of D-hydantoinase from *Bacillus stearothermophilus*: insight into the stereochemistry of enantioselectivity. Biochemistry **41(30)**:9410-9417

Chevalier P, Roy D and Morin A (1989) Hydantoinase activity of immobilized non-growing *Pseudomonas putida* cells. Appl. Microbiol. Biotechnol. **30**:482-486

Chien HR, Jih Y-L, Yang W-Y and Hsu W-H (1998) Identification of the open reading frame for the *Pseudomonas putida* D-hydantoinase gene and expression of the gene in *Escherichia coli*. Biochim. Biophys. Acta **1395**:68-77

Chung JH, Back JH, Lim J-H, Park YI and Han YS (2002) Thermostable hydantoinase from a hyperthermophilic archeon, *Methanococcus jannaschii*. Enzyme Microb. Technol. **30(7)**:867-874

Deepa S, Sivasankar B and Jayaraman K (1993) Enzymatic production and isolation of D-amino acids from the corresponding 5-substituted hydantoins. Process Biochem. **28**:447-452

De Lima Santos H and Ciancaglini P (2000) A practical approach to the choice of a suitable detergent and optimal conditions for solubilizing a membrane protein. Biochemical Education **28**:178-182

Dinelli D, Morisi F and Zaccardelli D (1976) Process for the preparation of L-carbamyl-amino acids and of the corresponding L-amino acids. US Patent 3964970, Snamprogetti, S.p.A.

Drauz K (1997) Chiral amino acids: a versatile tool in the synthesis of pharmaceuticals and fine chemicals. Chimia **51**:310-314

Drauz K, Kottenhahn M, Makryaleas K, Klenk H and Bernd M (1991) Chemoenzymatic syntheses of Ͳ-ureido D-amino acids. Angew. Chem. Int. Ed. Engl. **30(6)**:712-714

Drauz K and Waldman H (1995) Hydrolysis and formation of hydantoins. In: Enzyme catalysis in organic synthesis- a comprehensive handbook. VCH Publishers Inc., Weinheim

Dressman BA, Spangle LA and Laldor SW (1996) Solid phase synthesis of hydantoins using a carbamate linker and a novel cyclization/cleavage step. Tetrahedron Lett. **37(7)**:937-940

Durham DR and Weber JE (1995) Properties of D-hydantoinase from *Agrobacterium tumefaciens* and its

use for the preparation of *N*-carbamyl D-amino acids. Biochem. Biophys. Res. Commun. **216(3)**:1095-1100

Eadie GS, Bernheim F and Bernheim MLC (1949) The partial purification and properties of animal and plant hydantoinases. J. Biol. Chem. **181**:449-458

Engelman DM, TA Steitz and Goldman A (1986) Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. Ann. Rev. Biophys. Chem. **15**:321-353

Fan C-H and Lee C-K (2001) Purification of D-hydantoinase from adzuki bean and its immobilization for *N*-carbamoyl-D-phenylglycine production. Biochem. Eng. J. **8**:157-164

Fan C-H, Lee C-K and Chao Y-P (2000) Recombinant *Escherichia coli* cell for D-*p*-hydroxyphenylglycine production from D-*N*-carbamoyl-p-hydroxyphenylglycine. Enzyme Microb. Technol. **26**:222-228

Fernandez-Lafuente R, Rosell CM, Rodriguez V and Guisan JM (1995) Strategies for enzyme stabilization by intramolecular crosslinking with bifunctional reagents. Enzyme Microb. Technol **17**:517-523

Foster IM, Dorrington RA and Burton SG (2002) Enhanced hydantoinase and *N*-carbamoylase activity on immobilisation of *Agrobacterium tumefaciens*. Biotechnol. Lett. In press.

Fotheringham I (2000) Engineering biosynthetic pathways: new routes to chiral amino acids. Curr. Opin. Chem. Bio. **4**:120-124

George S and Sadhukhan AK (1996) Technical communication: A simple and economically viable medium for the growth of *Agrobacterium radiobacter* for the production of D-amino acid. World J. Microbiol. Biotechnol. **12**:657-659

Gojkovic Z, Jahnke K, Schnackerz KD and Piskur J (2000) PYD2 encodes 5,6-dihydropyrimidine amidohydrolase, which participates in a novel fungal catabolic pathway. J. Mol. Bio. **295(4)**:1073-1087

Gokhale DV, Bastawde KB, Patil SG, Kalkote UR, Joshi RR, Joshi RA, Ravindranathan T, Gaikwad BG, Jogdand VV and Nene S (1996) Chemoenzymatic synthesis of D(-)phenylglycine using hydantoinase of *Pseudomonas desmolyticum* resting cells. Enzyme Microb. Technol. **18**:353-357

Govardhan CP (1999) Crosslinking of enzymes for improved stability and performance. Curr. Opin. Biotechnol. **10**:331-335

Grifantini R, Galli G, Carpani G and Grandi G (1998a) Process for the preparation of D-" -amino acids. US Patent 5 834 258, Eniricerche S.p.A

Grifantini R, Galli G, Carpani G, Pratesi C, Frascotti G and Grandi G (1998b) Efficient conversion of 5-substituted hydantoins to D-" -amino acids using recombinant *Escherichia coli* strains. Microbiology **144**:947-954

Grifantini R, Pratesi C, Galli G and Grandi G (1996) Topological mapping of the cysteine residues of *N*-carbamyl-D-amino acid amidohydrolase and their role in enzymatic activity. J. Biol. Chem. **271(16)**:9326-9331

Gross C, Syldatk C, Mackowiak V and Wagner F (1990) Production of L-tryptophan from D,L-5-indolylmethylhydantoin by resting cells of a mutant of *Arthrobacter* species (DSM 3747). J. Biotechnol. **14**:363-376

Harris ELV and Angal S (1994) Protein purification methods: a practical approach. Oxford University Press, England

Hartley CJ (2001) Elucidation and manipulation of the hydantoin-hydrolysing enzyme system of *Agrobacterium tumefaciens* RU-OR for the biocatalytic production of D-amino acids. PhD Thesis, Rhodes University, Grahamstown, South Africa

Hartley CJ, Kirchmann S, Burton SG and Dorrington RA (1998) Production of D-amino acids from D,L-5-substituted hydantoins by an *Agrobacterium tumefaciens* strain and isolation of a mutant with inducer-independent expression of hydantoin-hydrolysing activity. Biotechnol. Lett. **20(7)**:707-711

Hartley CJ, Manford F, Burton SG and Dorrington RA (2001) Overproduction of hydantoinase and *N*-carbamoylase enzymes by regulatory mutants of *Agrobacterium tumefaciens*. Appl. Microbiol. Biotechnol. **57**:43-49

Hernaiz MJ and Crout DHG (2000) Immobilization on Eupergit C of the $-galactosidase from *B. circulans* and an " -galactosidase from *Aspergillus oryzae*. Enzyme Microb. Technol. **27**:26-32

Ikenaka Y, Nanba H, Yajima K and Yamada Y (1998a) Increase in the thermostability of *N*-carbamyl-D-amino acid amidohydrolase an amino acid substitutions. Biosci. Biotechnol. Biochem. **62(9)**:1668-1671

Ikenaka Y, Nanba H, Yajima K, Yamada Y, Takano M and Takahashi S (1998c) Relationship between an increase in thermostability and amino acid substitutions in *N*-carbamyl-D-amino acid amidohydrolase. Biosci. Biotechnol. Biochem. **62(9)**:1672-1675

Ikenaka Y, Nanba H, Yajima K, Yamada Y, Takano M and Takahashi S (1999) Thermostability reinforcement through a combination of thermostability-related mutations of *N*-carbamyl-D-amino acid amidohydrolase. Biosci. Biotechnol. Biochem. **63(1)**:91-95

Ikenaka Y, Nanba H, Yamada Y, Yajima K, Takano M and Takahashi S (1998b) Screening, characterisation, and cloning of the gene for *N*-carbamyl-D-amino acid amidohydrolase from thermotolerant soil bacteria. Biosci. Biotechnol. Biochem. **62(5)**:882-886

Ishikawa T, Mukohara Y, Watabe K, Kobayashi S and Nakamura H (1994) Microbial conversion of DL-5-substituted hydantoins to the corresponding L-amino acids by *Bacillus stearothermophilus* NS1122A. Biosci. Biotechnol. Biochem. **58(2)**:265-270

Ishikawa T, Watabe K, Mukohara Y and Nakamura H (1996) *N*-Carbamyl-L-amino acid amidohydrolase of *Pseudomonas* sp. Strain NS671: purification and some properties of the enzyme expressed in *Escherichia coli*. Biosci. Biotechnol. Biochem. **60**:612-615

Kamphuis J, Boesten WHJ, Broterman QB, Hermes HFM, van Balken EM, Meijer EM and Schoemaker HE (1990) New developments in the chemo-enzymatic production of amino acids. Adv. Biochem. Eng./Biotechnol. **42**:133-186

Katchalski-Katzir E and Kraemer DM (2000) Eupergit® C, a carrier for immobilization of enzymes of industrial potential. J. Mol. Catal. B **10**:157-176

Keil O, Schneider MP and Rasor JP (1995) New hydantoinases from thermophilic microorganisms - synthesis of enantiomerically pure D-amino acids. Tetrahedron: Assymetry **6(6)**:1257-1260

Kim D-M, Kim, G-J and Kim H-S (1994) Enhancement of operational stability of immobilised whole cell D-hydantoinase. Biotechnol. Lett. **16(1)**:11-16

Kim D-M and Kim H-S (1993) Enzymatic synthesis of D-*p*-hydroxyphenylglycine from DL-*p*-hydroxyphenylhydantoin in the presence of organic cosolvent. Enzyme Microb. Technol. **15**:530-534

Kim G-J, Cheon Y-H and Kim H-S (2000b) Directed evolution of a novel *N*-carbamylase/D-hydantoinase fusion enzyme for functional expression with enhanced stability. Biotechnol. Bioeng. **68(2)**:211-217

Kim G-J and Kim H-S (1994) Adsorptive removal of inhibitory by-product in the enzymatic production of optically active D-*p*-hydroxyphenylglycine from 5-substituted hydantoin. Biotechnol. Lett. **16(1)**:17-22

Kim G-J and Kim H-S (1995) Optimisation of the enzymatic synthesis of D-*p*-hydroxyphenylglycine from DL-5-substituted hydantoin using D-hydantoinase and *N*-carbamoylase. Enzyme Microb. Technol. **17**:63-67

Kim G-J and Kim H-S (1998) Identification of the structural similarity in the functionally related amidohydrolases acting on the cyclic amide ring. Biochem. J. **330**:295-302

Kim G-J and Kim H-S (2002) A microbial D-hydantoinase is stabilized and overexpressed as a catalytically active dimer by truncation and insertion of the C-terminal region. J. Microbiol. Biotechnol. **12(2)**:242-248

Kim G-J, Lee D-E and Kim H-S (2000a) Functional expression and characterization of the two cyclic amidohydrolase enzymes, allantoinase and a novel phenylhydantoinase, from *Escherichia coli*. J. Bacteriol. **182**:7021-7028

Kim G-J, Lee D-E and Kim H-S (2000c) Construction and evaluation of a novel bifunctional *N*-carbamylase-D-hydantoinase fusion enzyme. Appl. Environ. Microbiol. **66(5)**:2133-2138

Kim G-J, Lee S-G, Park J-H and Kim H-S (1997) Direct detection of the hydantoinase activity on solid agar plates and electrophoretic acrylamide gels. Biotechnol. Tech. **11**:511-513

Kim G-J, Park J-H, Lee D-C and Kim H-S (1999) Engineering the thermostable D-hydantoinases from two thermophilic *Bacilli* based on their primary structures. Ann. N. Y. Acad. Sci. **864**:332-336

Klibanov AM (1979) Enzyme stabilization by immobilisation. Anal. Biochem. **93**:1-25

Klibanov AM (1983) Immobilized enzymes and cells as practical catalysts. Science **219**:722-727

Kyte J and Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Bio. **157**:105-132

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature **227**:680-685

LaPointe G, Viau S, LeBlanc D, Robert N and Morin A (1994) Cloning, sequencing, and expression in *Escherichia coli* of the D-hydantoinase gene from *Pseudomonas putida* and distribution of homologous genes in other microorganisms. Appl. Environ. Microbiol. **60(3)**:888-895

Lee B and Vasmatzis G (1997) Stabilization of protein structures. Curr. Opin. Biotechnol. **8**:423-428

Lee C-K and Fan C-H (1999) Pressure swing reactor for converting suspension of solid substrate p-hydroxyphenylhydantoin by immobilised D-hydantoinase. Bioprocess Eng. **21**:341-347

Lee C-K, Fan C-H and Yang P-F (2001b) Modeling and simulation of a pressure-swing reactor for the conversion of poorly soluble substrate by immobilised enzyme: the case of D-hydantoinase reaction. Biochem. Eng. J. **7**:233-239

Lee C-K, Lee Z-S and Yang P-F (2001a) Effect of cell membrane of *Agrobacterium radiobacter* on enhancing D-amino acids production from racemic hydantoins. Enzyme Microb. Technol. **28**:806-814

Lee C-K and Lin K-C (1996) *N*-carbamoyl-D-*p*-hydroxyphenylglycine production using immobilized D-hydantoinase from recombinant *E.coli.* Enzyme Microb. Technol. **19**:623-627

Lee D-C, Kim G-J, Cha Y-K and Lee C-Y (1997b) Mass production of thermostable D-hydantoinase by batch culture of recombinant *Escherichia coli* with a constitutive expression system. Biotechnol. Bioeng. **56**:449-455

Lee D-C and Kim H-S (1998) Optimization of a heterogeneous reaction system for the production of optically active D-amino acids using thermostable D-hydantoinase. Biotechnol. Bioeng. **60(6)**:729-738

Lee D-C, Lee S-G, Hong S-P, Sung M-H and Kim H-S (1996a) Cloning and overexpression of thermostable D-hydantoinase from thermophile in *E. coli* and its application to the synthesis of optically active D-amino acids. Ann. N. Y. Acad. Sci. **799**:401-405

Lee D-C, Lee S-G and Kim H-S (1996b) Production of D-*p*-hydroxyphenylglycine from D,L-5-(4-hydroxyphenyl)hydantoin using immobilized thermostable D-hydantoinase from *Bacillus stearothermopilus* SD-1. Enzyme Microb. Technol. **18**:35-40

Lee S-G, Lee D-C, Hong S-P, Sung M-H and Kim H-S (1995) Thermostable D-hydantoinase from thermophilic *Bacillus stearothermophilus* SD-1: characteristics of purified enzyme. Appl. Microbiol. Biotechnol. **43**:270-276

Lee S-G, Lee D-C and Kim H-S (1997a) Purification and characterisation of thermostable D-hydantoinase from thermophilic *Bacillus stearothermophilus* SD1. Appl. Biochem. Biotechnol. **62**:251-266

Lee S-G, Lee D-C, Sung M-H and Kim H-S (1994) Isolation of thermostable D-hydantoinase-producing thermophilic *Bacillus sp* SD-1. Biotechnol. Lett. **16(5)**:461-466

Louwrier A and Knowles CJ (1996) The purification and characterisation of a novel D(-)-specific carbamoylase enzyme from an *Agrobacterium* sp. Enzyme Microb. Technol. **19**:562-571

Louwrier A and Knowles CJ (1997) The aim of industrial enzymic amoxycillin production: characterization of a novel carbamoylase enzyme in the form of a crude, cell-free extract. Biotechnol. Appl. Biochem. **25**:143-149

Luksa V, Starkuviene V, Starkuviene B and Dagys R (1997) Purification and characterization of the D-hydantoinase from *Bacillus circulans*. Appl. Biochem. Biotechnol. **62**:219-231

May O, Habenicht A, Mattes R, Syldatk C and Siemann M (1998e) Molecular evolution of hydantoinases. Biol. Chem. **379**:743-747

May O, Nguyen PT and Arnold FH (2000) Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine. Nature Biotechnol. **18**:317-320

May O, Siemann M, Pietzsch M, Kiess M, Mattes R and Syldatk C (1998d) Substrate-dependent enantioselectivity of a novel hydantoinase from *Arthrobacter aurescens* DSM 3745: Purification and characterisation as new member of cyclic amidases. J. Biotechnol. **61**:1-13

May O, Siemann M, Siemann MG and Syldatk C (1998a) The hydantoin amidohydrolase from *Arthrobacter aurescens* DSM 3745 is a zinc metalloenzyme. J. Mol. Catal. B **5**:367-370

May O, Siemann M, Siemann MG and Syldatk C (1998b) Catalytic and structural function of zinc for the hydantoinase from *Arthrobacter aurescens* DSM 3745. J. Mol. Catal. B **4**:211-218

May O, Siemann M and Syldatk C (1998c) A new method for the detection of hydantoinases with respect to their enantioselectivitiy on acrylamide gels based on enzyme activity stain. Biotechnol. Techniques **12(4)**:309-312

May O, Siemann M, Syldatk C, Niefind K and Schomburg D (1996) Crystallization and preliminary X-ray analysis of a hydantoinase from *Arthrobacter aurescens* DSM 3745. Acta Cryst. D **52**:1209-1210

Meyer P and Runser S (1993) Efficient production of the industrial biocatalysts hydantoinase and *N*-carbamyl amino acid amidohydrolase: Novel non-metabolizable inducers. FEMS Microbiol. Lett. **109**:67-74

Möller A, Syldatk C, Schulze M and Wagner F (1988) Stereo- and substrate-specificity of a D-hydantoinase and a D-*N*-carbamyl amino acid amidohydrolase of *Arthrobacter crystallopoietes* AM 2. Enzyme Microb. Technol. **10**:618-625

Morin A (1993) Use of D-hydantoinase extracted from legumes to produce *N*-carbamyl D-amino acids. Enzyme Microb. Technol. **15**:208-214

Morin A, Hummel W and Kula M-R (1987) Enrichment and selection of hydantoinase-producing micro-organisms. J. Gen. Microbiol. **133**:1201-1207

Morin A, Hummel W, Schütte H and Kula M-R (1986) Characterisation of hydantoinase from *Pseudomonas*

flourescens strain DSM 84. Biotechnol. Appl. Biochem. **8**:564-574

Morin A and Lafond A (1992) Continuous production of *N*-carbamyl-D-alanine by *Peptococcus anaerobius* adsorbed on activated charcoal. Biotechnol. Lett. **14(2)**:117-118

Morin A, Leblanc D, Lingnau A and LaPointe G (1997) Cyclic amide amidohydrolase activities in *Pisum sativum* hulls. Biores. Technol. **61(1)**:91-97

Morin A, LeBlanc D, Paleczek A, Hummel W and Kula M-R (1990) Comparison of seven microbial D-hydantoinases. J. Biotechnol. **16**:37-48

Morin A , Poitras E, Moresoli C and Brion F (1995a) Extraction of cyclic amide amidohydrolase from green hulls of *Pisum sativum* and its use as biocatalyst for *N*-carbamyl amino acids. Biores. Technol. **53**:31-37

Morin A, Tran Trung NH, LaPointe G and Dubeau H (1995b) Conditions used with a continuous cultivation system to screen for D-hydantoinase-producing microorganisms. Appl. Microbiol. Biotechnol. **43**:259-266

Mukohara Y, Ishikawa T, Watabe K and Nakamura H (1993) Molecular cloning and sequencing of the gene for a thermostable *N*-carbamyl-L-amino acid amidohydrolase from *Bacillus strearothermophilus* strain NS1122A. Biosci. Biotech. Biochem. **57(11)**:1935-1937

Mukohara Y, Ishikawa T, Watabe K and Nakamura H (1994) A thermostable hydantoinase of *Bacillus stearothermophilus* NS1122A: cloning, sequencing, and high expression of the enzyme gene, and some properties of the expressed enzyme. Biosci. Biotech. Biochem. **58(9)**:1621-1626

Nakai T, Hasegawa T, Yamashita E, Yamamoto M, Kumasaka T, Ueki T, Nanba H, Ikenaka Y, Takahashi S, Sato M and Tsukihara T (2000) Crystal structure of *N*-carbamyl-D-amino acid amidohydrolase with a novel catalytic framework common to amidohydrolases. Structure **8**:729-737

Nanba H, Ikenaka Y, Yamada Y, Yajima K, Takano M, Ohkubo K, Hiraishi Y, Yamada K and Takahashi S (1998b) Immobilization of *N*-carbamyl-D-amino acid amidohyrolase. Biosci. Biotechnol. Biochem. **62(10)**:1839-1844

Nanba H, Ikenaka Y, Yamada Y, Yajima K, Takano M, Ohkubo K, Hiraishi Y, Yamada K and Takahashi S (1999) Immobilisation of thermotolerant *N*-carbamyl-D-amino acid amidohydrolase. J. Mol. Catal. B **6**:257-263

Nanba H, Ikenaka Y, Yamada Y, Yajima K, Takano M and Takahashi S (1998a) Isolation of *Agrobacterium sp*. Strain KNK712 that produces *N*-carbamyl-D-amino acid amidohydrolase, cloning of the gene for this enzyme, and properties of the enzyme. Biosci. Biotechnol. Biochem. **62(5)**:875-881

Neal RJ, Griffin AM and Gorham HC (1999) D-*N*-carbamoyl-amino acid amidohydrolase and hydantoinase. US Patent 5 858 759, SmithKline Beecham p.l.c.

Neugebauer J (1988) A guide to the properties and uses of detergents in biology and biochemistry. Calbiochem-Novabiochem International.

Nishida Y, Nakamichi K, Nabe K and Tosa T (1987) Enzymatic production of L-tryptophan from DL-5-indolylmethylhydantoin by *Flavobacterium* sp. Enzyme Microb. Technol. **9**:721-725

O'Fagain C, Sheehan H, O'Kennedy R and Kilty C (1988) Maintenance of enzyme structure-possible methods for enhancing stability. Process Biochem. **23**:166-171

Ogawa J, Chung MC-M., Hida S, Yamada H and Shimizu S (1994b) Thermostable *N*-carbamoyl-D-amino acid amidohydrolase: screening, purification and characterization. J. Biotechnol. **38**:11-19

Ogawa J, Kaimura T, Yamada H and Shimizu S (1994a) Evaluation of pyrimidine- and hydantoin-degrading enzyme activities in aerobic bacteria. FEMS Microbiol. Lett. **122**:55-60

Ogawa J, Min Kim J, Mirdnoy W, Amano Y, Yamada H and Shimizu S (1995a) Purification and characterisation of an ATP-dependent amidohydrolase, *N*-methylhydantoin amidohydrolase, from *Pseudomonas putida* 77. Eur. J. Biochem. **229**:284-290

Ogawa J, Miyake H and Shimizu S (1995b) Purification and characterization of *N*-carbamoyl-L-amino acid amidohydrolase with broad substrate specificity from *Alcaligenes xylosoxidans*. Appl. Microbiol. Biotechnol. **43**:1039-1043

Ogawa J and Shimizu S (1997) Diversity and versatility of microbial hydantoin transforming enzymes. J. Mol. Catal. B. **2**:163-176

Ogawa J, Shimizu S and Yamada H (1993) *N*-Carbamoyl-D-amino acid amidohydrolase from *Comamonas* sp. E222c. Purification and characterisation. Eur. J. Biochem. **212**:684-691

Oh K-H, Nam S-H and Kim H-S (2002) Directed evolution of *N*-carbamyl-D-amino acid amidohydrolase for simultaneous improvement of oxidative and thermal stability. Biotechnol. Prog. **18(3)**:413-417

Olivieri R, Fascetti E, Angelini L and Degen L (1979) Enzymatic conversion of *N*-carbamoyl-D-amino acids to D-amino acids. Enzyme Microb. Technol. **1**:201-204

Olivieri R, Fascetti E, Angelini L and Degen L (1981) Microbial transformation of racemic hydantoins to D-amino acids. Biotechnol. Bioeng. **23**:2173-2183

Park J-H, Kim G-J and Kim H-S (2000) Production of D-amino acid using whole cells of recombinant *Escherichia coli* with separately and co-expressed D-hydantoinase and *N*-carbamoylase. Biotechnol. Prog. **16(4)**:564-570

Park J-H, Oh KH, Lee DC and Kim HS (2002) Modeling and kinetic analysis of the reaction system using whole cells with separately and co-expressed D-hydantoinase and *N*-carbamoylase. Biotechnol. Bioeng. **78(7)**:779-793

Park JK and Chang HN (2000) Microencapsulation of microbial cells. Biotechnol. Adv. **18**:303-319

Park J, Kim G, Lee S and Kim H (1998) Biochemical properties of thermostable D-hydantoinase from *Bacillus thermocatenulatus* GH-2. Ann. N. Y. Acad. Sci. **864**:337-340

Pasquinelli RS, Shepherd RE, Koepsel RR, Zhao A and Ataai MM (2000) Design of affinity tags for one-step protein purification from immobilized zinc columns. Biotechnol. Prog. **16**:86-91

Pietzsch M, Wiese A, Ragnitz K, Wilms B, Altenbuchner J, Mattes R and Syldatk C (2000) Purification of recombinant hydantoinase and L-*N*-carbamoylase from *Arthrobacter aurescens* expressed in *Escherichia coli*: comparison of wild-type and genetically modified proteins. J. Chromatography B **737**:179-186

Plummer DT (1987) The quantitative estimation of amino acids using the ninhydrin reaction. In: Practical Biochemistry, Plummer DT (editor), 3rd edition. Wiley-Interscience, New York, p.158

Polastro ET (1989) Enzymes in the fine chemicals industry: dreams and realities. Bio/Technol. **7**:1283-1241

Ragnitz K, Pietzsch M and Syldatk C (2001a) Immobilization of the hydantoin cleaving enzymes from *Arthrobacter aurescens* DSM 3747. J. Biotechnol. **92**:179-186

Ragnitz K, Syldatk C and Pietzsch M (2001b) Optimization of the immobilization parameters and operational stability of hydantoinase and L-*N*-carbamoylase from *Arthrobacter aurescens* for the production of optically pure L-amino acids. Enzyme Microb. Technol. **28**:713-720

Rai R and Taneja V (1998) Production of D-amino acids using immobilized D-hydantoinase from lentil,

Lens esculenta, seeds. Appl. Microbiol. Biotechnol. **50**:658-662

Runser S, Chinski N and Ohleyer E (1990) D-*p*-Hydroxyphenylglycine production from DL-5-*p*-hydroxyphenylhydantoin by *Agrobacterium* sp. Appl. Microbiol. Biotechnol. **33**:382-388

Runser SM and Meyer PC (1993) Purification and biochemical characterization of the hydantoin hydrolyzing enzyme from *Agrobacterium* species. A hydantoin with no 5,6-dihydropyrimidine amidohydrolase activity. Eur. J. Biochem. **213**:1315-1324

Runser S and Ohleyer E (1990) Properties of the hydantoinase from *Agrobacterium* sp. IP I-671. Biotechnol. Lett. **12(4)**:259-264

Russell IM (1998) The development of an immobilised-enzyme bioprobe for the detection of phenolic pollutants in water. MSc thesis. Rhodes University

Ryu OH, Ju JY and Shin CS (1997) Continuous L-cysteine production using immobilised cell reactors and product extractors. Process Biochem. **32**:201-209

Sareen D, Sharma R, Nandanwar HS and Vohra RM (2001) Two-step purification of D(-)-specific carbamoylase from *Agrobacterium tumefaciens* AM 10. Protein Expression and Purification **21**:170-175

Schulze B and Wubbolts MG (1999) Biocatalysis for industrial production of fine chemicals. Curr. Opin. Biotechnol. **10**:609-615

Scopes RK (1987) Protein purification: principles and practice. Springer-Verlag, New York

Scouten WH, Luong JHT and Brown RS (1995) Enzyme or protein immobilization techniques for applications in biosensor design. TIBTECH **13**:178-184

Sharma R and Vohra RM (1997) A thermostable D-hydantoinase isolated from a mesophilic *Bacillus sp*. AR9. Biochem. Biophys. Res. Commun. **234**:485-488

Shimizu S, Ogawa J, Kataoka M, Kobayashi M (1997) Screening of novel microbial organisms for the production of biologically and chemically useful compounds. Adv. Biochem. Eng. Biotechnol. **58**:45-56

Shimizu S, Shimada H, Takahashi O, Ohashi T, Tani Y and Yamada H (1980) Synthesis of *N*-carbamyl-D-2-thienyl-glycine and D-2-thienlyglycine by microbial hydantoinase. Agric. Biol. Chem. **44(9)**:2233-2234

Siemann M, Alvarado-Marin A, Pietzsch M and Syldatk C (1999) A D-specific hydantoin amidohydrolase: properties of the metalloenzyme purified from *Arthrobacter crystallopoietes*. J. Mol. Catal. B **6**:387-397

Siemann M, Syldatk C and Wagner F (1993) Detection and comparison of strains with selective L-hydantoin cleaving activity using polyclonal antibodies. Biotechnol. Techniques **7(5)**:361-366

Siemann M, Syldatk C and Wagner F (1994) Enhanced stability of an L-hydantoinase mediated by its corresponding polyclonal antibody. Biotechnol. Lett. **16(4)**:349-354

Smith RJ, Pietzsch M, Waniek T, Syldatk C and Bienz S (2001) Enzymatic synthesis of enantiomerically enriched D- and L-3-silylated alanines by deracemization of D,L-5-silylmethylated hydantoins. Tetrahedron: Asymmetry **12**:157-165

Soong C-L, Ogawa J, Honda M and Shimizu S (1999) Cyclic-imide-hydrolysing activity of D-hydantoinase from *Blastobacter* sp. Strain A17p-4. Appl. Environ. Microbiol. **65(4)**:1459-1462

Soong C-L, Ogawa J and Shimizu S (2001) Cyclic ureide and imide metabolism in microorganisms producing a D-hydantoinase useful for D-amino acid production. J. Mol. Catal. B **12**:61-70

Sudge SS, Bastawde KB, Gokhale DV, Kalkote UR and Ravindranathan T (1998) Production of D-hydantoinase by halophilic *Pseudomonas* sp. NCIM 5109. Appl. Microbiol. Biotechnol. **49**:594-599

Syldatk C, Cotoras D, Dombach G, Gross C, Kallwass H and Wagner F (1987) Substrate- and stereospecificity, induction and metallo-dependence of a microbial hydantoinase. Biotechnol. Lett. **9(1)**:25-30

Syldatk C, Läufer A, Müller R and Höke H (1990a) Production of optically pure D- and L-" -amino acids by bioconversion of D,L-5-monosubstituted hydantoin derivatives. Adv. Biochem. Eng./Biotechnol. **41**:29-75

Syldatk C, Mackowiak V, Hoke H, Gross C, Dombach G and Wagner F (1990b) Cell growth and enzyme synthesis of a mutant of *Arthrobacter* sp. (DSM 3747) used for the production of L-amino acids from D,L-5-monosubstituted hydantoins. J. Biotechnol. **14**:345-362

Syldatk C, May O, Altenbuchner J, Mattes R and Siemann M (1999) Microbial hydantoinases - industrial enzymes from the origin of life? Appl. Microbiol. Biotechnol. **51**:293-309

Syldatk C and Pietzsch M (1995) Hydrolysis and formation of hydantoins. In: Enzyme catalysis in organic synthesis, Drauz K and Waldmann H, editors. Weinheim, Germany, VCH-Verlag, pp.409-431

Takahashi S, Kii Y, Kumagai H and Yamada H (1978) Purification, crystallization and properties of

hydantoinase from *Pseudomonas striata*. J. Ferment. Technol. **56(5)**:492-498

Takahashi S, Ohashi T, Kii Y, Kumagai H and Yamada H (1979). Microbial transformation of hydantoins to *N*-carbamyl-D-amino acids. J. Ferment. Technol. **57(4)**:328-332

Tramper J (1985) Immobilising biocatalysts for use in organic syntheses. Trends Biotechnol. **3(2)**:45-50

Tripathi CKM, Bihari V and Tyagi RD (2000) Microbial production of D-amino acids. Process Biochem. **35**:1247-1251

Wagner T, Hantke B and Wagner F (1996) Production of L-methionine from D,L-5-(2-methylthioethyl)hydantoin by resting cells of a new mutant strain of *Arthrobacter* species DSM 7330. J. Biotechnol. **46**:63-68

Watabe K, Ishikawa T, Mukohara Y and Nakamura H (1992) Purification and characterisation of the hydantoin racemase of *Pseudomonas* sp. strain NS671 expressed in *Escherichia coli*. J. Bacteriol. **174**:7989-7995

Weber JE and Durham DR (1997) Hydantoinase from *Agrobacterium tumefaciens* and its use for the preparation of optically pure *N*-carbamyl-D-amino acids from racemic hydantoins. Biofutur **1997**:44-45

Wiese A, Pietzsch M, Syldatk C, Mattes R and Altenbuchner J (2000) Hydantoin racemase from *Arthrobacter aurescens* DSM 3747: heterologous expression, purification and characterization. J. Biotechnol. **80**:217-230

Wilms B, Wiese A, Syldatk C, Mattes R and Altenbuchner J (2001) Development of an *Escherichia coli* whole cell biocatalyst for the production of L-amino acids. J. Biotechnol. **86**:19-30

Xu G and West TP (1994) Characterisation of dihydropyrimidinase from *Pseudomonas stutzeri*. Arch. Microbiol. **161**:70-74

Yamada H (1981) Enzymatic processes for the synthesis of optically active amino acids. In: Chibata I, Fukui S and Wingard, LB (eds.) Enzyme Engineering **6**:97-106

Yamada H, Shimizu S, Shimada H, Tani Y, Takahashi S and Ohashi T (1980) Production of D-phenylglycine-related amino acids by immobilized microbial cells. Biochimie **62**:395-399

Yin B-D, Chen Y-C, Lin S-C and Hsu W-H (2000) Production of D-amino acid precursors with permeabilized recombinant *Escherichia coli* with D-hydantoinase activity. Process Biochem. **35**:915-921

Yokozeki K, Nakamori S, Eguchi C, Yamada K and Mitsugi K (1987b) Screening of microorganisms producing D-*p*-hydroxyphenyl-glycine from D,L-5-(*p*-hydroxyphenyl)hydantoin. Agric. Biol. Chem. **51(2)**:355-362

Yokozeki K, Nakamori S, Yamanaka S, Eguchi C, Mitsugi K and Yoshinaga F (1987c) Optimal conditions for the enzymatic production of D-amino acids from the corresponding 5-substituted hydantoins. Agric. Biol. Chem. **51(3)**:715-719

Yokoseki K, Sano K, Eguchi C, Yamada K and Mitsugi K (1987a) Enzymatic production of L-tryptophan from D,L-5-indolmethylhydantoin by mutants of *Flavobacterium* sp. T-523. Agric. Biol. Chem. **51(2)**:363-36

Zhang WC, Yuan HJ, Hao SF, Peng QZ, Wang SJ, Zhu HC and Huang LY (2001) Studies on bioconversion conditions and stereoselectivity of *Arthrobacter* K1108 hydantoinase. Chinese J. Biotechnol. **17**:635-638