CHARACTERIZATION OF AMIDE BOND HYDROLYSIS IN NOVEL HYDANTOINASE-PRODUCING BACTERIA

THESIS

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ABSTRACT

This thesis describes a series of investigations into the amide bond-hydrolyzing activity of bacterial strains RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR, which were previously isolated for their ability to hydrolyze hydantoins to amino acids. The main aim of the study was to develop biotransformations with potential application in the production of enantiomerically pure amino acids and related compounds. Several compounds may be used as substrates by biocatalysts for the production of amino acids, such as hydantoins, amino nitriles, and amides. These compounds are not only important for amino acid production, but they may be used for production of other industrially important compounds, such as 2-arylpropionic acids, which are non-steroidal anti-inflammatory drugs. Thus, the ability of the above-mentioned strains to hydrolyze these substrates was investigated, with the view to utilizing the maximum potential of these biocatalysts.

The compounds used as substrates in the investigation are all essentially amides. Thus, the ability of the strains to hydrolyze imides, hydantoins, and amides, was investigated. In particular, imides have a structure which is very similar to that of hydantoins, and thus it was an objective of the study to determine whether these strains could hydrolyze imides. Imide-hydrolyzing activity has only recently been discovered in microorganisms.

Hydantoin conversion involves a two-step hydrolysis reaction which yields, initially, an *N*-carbamylamino acid intermediate, and subsequently, an α-amino acid. The hydantoin-hydrolyzing enzymes of a *Pseudomonas putida* strain, RU-KM3$_S$, were characterized in a crude extract preparation and reaction conditions for its biocatalytic application were optimized. The optimum conditions for conversion of 5-methylhydantoin were found to be 3 hours at 40°C, with conversion yields greater than 50% achieved. The enzymes of RU-KM3$_S$

demonstrated considerable stability, retaining 80% of their activity after incubation at 40°C for 3 hours. The activities of the enzymes were increased by the addition of a detergent to the extraction medium, suggesting that the enzymes might be membrane-bound. The results of the determination of the metal-dependence of the hydantoinase and *N*-carbamylase of RU-KM3$_S$ suggested that these enzymes required metal ions for activity, with metal ions such as Mg^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} resulting in activation of the enzymes. However, Cu^{2+} and Fe^{2+} caused inactivation of these enzymes. The stereoselectivity of the enzymes was investigated, and the results suggested that the hydantoinase was non-selective, whereas the *N*-carbamylase was L-selective.

The hydantoin substrate selectivity of RU-KM3$_S$ was compared to that of three other hydantoinase-producing bacteria, RU-KM1, RU-KM3$_L$, and RU-OR. The four strains were able to hydrolyze all of the seven substrates tested. However, there was a difference in activity levels between crude extract preparations and whole cells, with crude extracts generally showing higher activity than whole cells, except in the case of RU-KM1. Some difference was also observed in the order of preference of substrates between whole cells and crude extracts. The preferred substrate for RU-KM1 whole cells was isopropylhydantoin, whereas the crude extract preparation preferentially hydrolyzed *p*-hydroxyphenylhydantoin. RU-KM3$_L$ whole cells achieved a higher conversion yield with isobutylhydantoin, whereas the crude extract achieved a higher yield with 5-*t*-butylhydantoin. RU-KM3$_S$ whole cells and crude extract preferentially hydrolyzed 5-*n*-butylhydantoin, although the yield was greater with the crude extract. The highest conversion yields were observed with RU-KM3$_S$ crude extract, with conversion yields of 71.6% and 100% for *n*-butylhydantoin and *p*-hydroxyphenylhydantoin, respectively.

The ability of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ to hydrolyze nitriles, initially to amides and subsequently to carboxylic acids, was investigated. These strains were demonstrated to be unable to utilize acrylonitrile, propionitrile and benzonitrile as nitrogen sources, but were able to hydrolyze acrylonitrile, propionitrile and acetonitrile, in resting cell reactions. Nitrile hydrolysis was demonstrated to be inducible in all three strains, and the enzyme system responsible for nitrile hydrolysis was proposed to be a nitrile hydratase-amidase system.

Amidase activity in the four bacterial strains was investigated. The ability of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR to utilize amides as a nitrogen source was investigated, and the results showed that propionamide was a good nitrogen source for all four of the strains. Amide-hydrolyzing activity, by resting cells, was shown to be inducible by propionamide in all four strains. RU-KM3$_S$ demonstrated superior amide-hydrolyzing ability in that it hydrolyzed propionamide, acetamide, and acrylamide to a greater extent than the other strains.

Resting cells of RU-KM1 and RU-OR were demonstrated to have the ability to hydrolyze the imide substrate, succinimide, and this imidase activity was found to be inducible. These strains were also able to utilize this imide as the sole source of nitrogen for growth, which is a novel finding, as to date, bacteria have only be reported to utilize imides as a carbon source. The identity of the enzyme system responsible for succinimide hydrolysis is not yet clear.

In conclusion, the hydantoin-hydrolyzing enzymes of RU-KM3$_S$ have been shown to be possibly membrane associated, which is a novel finding that has also been proposed in three other hydantoinase-producing strains in our laboratory. This study has shown that the *N*-carbamylase of RU-KM3$_S$ is L-stereoselective, which, to our knowledge, is the first report of an L-stereospecific *N*-carbamylase in a *Pseudomonas putida*. Publication of these findings is already in progress. This is the first report on the study of imide hydrolysis in either an *Agrobacterium tumefaciens* or a *Pseudomonas* sp., and publications reporting these results are in preparation.

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ABBREVIATIONS

NCA – *N*-carbamylalanine

NCG – *N*-carbamylglycine

EDTA – Ethylene Diamine Tetra Acetic Acid

p-HPH – *p*-Hydroxyphenylhydantoin

HMM – Hydantoin minimal medium

p-HPG – *p*-Hydroxyphenylglycine

ACE – Acetylcholine esterase

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CHAPTER 1

Literature review

1.1 INTRODUCTION

This thesis describes an investigation into the amide-bond hydrolyzing activity of bacterial strains RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR, which are hydantoinase-producing bacteria. The main aim of the study was to develop biocatalytic routes to produce enantiomerically pure amino acids and related compounds.

Amino acids have attained a wide variety of applications in industry, and increasing interest has been directed towards the large scale synthesis of optically pure D- or L-"-amino acids (Boesten *et al.*, 1986; Syldatk *et al.*, 1990; Hermes *et al.*, 1993). Applications of amino acids include their utilization as additives in food and feed (Syldatk *et al.*, 1990; Ryu *et al.*, 1996; Wagner *et al.*, 1996), as intermediates in the synthesis of semisynthetic antibiotics (Syldatk *et al.*, 1990 and 1998; Kim and Kim, 1993; Lee *et al.*, 1994; Burton *et al.*, 1997; Hartley *et al.*, 1998; Ikenaka *et al.*, 1998b, Ogawa and Shimizu, 1999), as starting materials for the synthesis of peptides and peptide hormones (Kim and Kim, 1993; Gokhale *et al.*, 1996; Grifantini *et al.*, 1996; Drauz, 1997; Lee and Kim, 1998), as building blocks in the synthesis pesticides (Kim *et al.*, 1995; Grifantini *et al.*, 1996; Ikenaka *et al.*, 1998a; Sudge *et al.*, 1998; May *et al.*, 1998), and as building blocks for the synthesis of acetylcholine esterase (ACE) inhibitors, such as enalapril (Keil *et al.*, 1995; Chien and Hsu, 1996; Cannarsa, 1996; Tsuji *et al.*, 1997). Examples are D-phenylglycine and D-*p*-hydroxyphenylglycine, which are important building blocks for semisynthetic antibiotics (Hermes *et al.*, 1993 and 1994; van den Tweel, 1993). Other examples are L-valine, which is used in the production of Cyclosporin A (Hermes *et al.*, 1993), an immunosupressant (van den Tweel *et al.*, 1993), isovaline, which is a natural substituent of several antibiotics (Hermes *et al.*, 1994), and D-valine which is used as an intermediate in the production of Fluvalinate, an insecticide (Hermes *et al.*, 1993; van den Tweel, 1993). "-Alkyl-"-amino acids are also becoming very valuable pharmaceutical compounds or intermediates (van den Tweel, 1993). An example is L-"-methyl-3,4-dihydroxyphenylalanine, which is an important drug for hypertension.

Several methods have been reported in the literature for the production of optically active amino acids, both chemical and enzymatic in nature, as a result of the commercial demand for them in the pharmaceutical industry (Volkel and Wagner, 1995; Louwrier and Knowles, 1996). Four processes are commonly used for the commercial production of amino acids:

a) extraction from plant or animal hydrolysates
b) chemical synthesis using aminonitriles as intermediates
c) conversion cheap substrates such as sugars and molasses
d) enzymatic catalysis using stereoselective enzymes (biocatalysts) to convert amino acid amides (Morin *et al.*, 1990).

Biocatalysts possess a number of properties which make them advantageous to use, in place of conventional chemical catalysts (Polastro, 1989). They are capable of working under mild conditions, i.e. pH and temperature, they also exhibit high levels of regio- and enantio-selectivity or specificity, and they also raise fewer safety and pollution issues. Microbial enzymes are also advantageous to use since they are easily produced from fermentations, in high yields. A wide variety of enzymes may be obtained from one fermentation, i.e. from one bacterial culture, and a wide range of micro-environments give enzymes with a wide variety of stability and conditions for activity.

Our laboratory, whose main research activities involve enzymatic production of amino acids, had isolated four hydantoinase-producing bacteria, namely RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR, on the basis of their ability to hydrolyze hydantoin to glycine. A decision was taken to develop a process for the production of amino acids, using these strains.

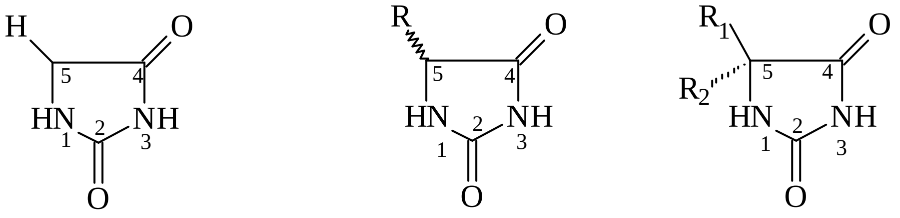
Production of amino acids from hydantoins essentially involves amide bond hydrolysis, and there are other enzyme systems which utilize other amide bond-containing substrates to produce amino acids, including nitrile-hydrolyzing enzymes, and amide-hydrolyzing enzymes. Imides are also amide bond-containing compounds, which have a basic structure similar to that of hydantoins. The common feature of these substrates is that their hydrolysis products are all carboxylic acids. Thus, a decision was taken to investigate the presence of these other enzyme systems in the four hydantoinase-producing strains, so as to develop biocatalysts which may be applied for the production of amino acids as well as a variety of other commercially important products. Below is a summary of the literature which has been published to date on the utilization of these enzyme systems as biocatalysts.

1.2 HYDANTOIN HYDROLYSIS

5-Substituted hydantoins are recognized as classical precursors of amino acids (Yamashiro *et al.*, 1988; Ishikawa *et al.*, 1993 and 1994; Ogawa *et al.*, 1995), and this is partly due to the fact that they easily undergo spontaneous racemization under mild conditions (Nishida *et al.*, 1987), and can be easily synthesized via a number of processes.

1.2.1 Synthesis of 5-monosubstituted hydantoins

The hydantoin ring system has been intensively studied, since its discovery by Baeyer in 1861 (Finkbeiner, 1965; Drauz *et al.*, 1995). The systematic terms for hydantoin are imidazolidine-2,4-dione or 2,4-diketotetrahydroimidazole (Drauz *et al.*, 1995). Much of the interest in hydantoin chemistry has been centred in two areas: the natural occurrence of hydantoins, and synthesis of a variety of compounds from them for use as pharmaceuticals (Finkbeiner, 1965). A wide spectrum of different 5-mono- and 5,5-disubstituted hydantoin derivatives of industrial and pharmacological interest has been described in the literature. 5-Monosubstituted hydantoins may be regarded as cyclic ureides of "-amino acids and are important precursors of "-amino acids (Yokozeki *et al.*, 1986; Syldatk *et al.*, 1990; Drauz *et al.*, 1995). 5,5-Disubstituted hydantoin derivatives, on the other hand, have been used for production of drugs used for the treatment of Parkinson's disease (Drauz *et al.*, 1995).



(1) **(2)** **(3)**

Figure 1.2.1 Structures of hydantoin (1), 5-monosubstituted hydantoin (2), and 5,5-disubstituted hydantoin

A number of methods used for the chemical synthesis of hydantoins have been reported in the literature (Drauz *et al.*, 1995; Syldatk *et al.*, 1990). The condensation of aldehydes to form 5-arylkylidine hydantoins and their subsequent reduction was first reported by Wheeler and Hoffman in 1911 (Finkbeiner, 1965). This reaction has since been used in the preparation of a large number of different amino acids.

The two main methods currently utilized for the synthesis of 5-substituted hydantoins are the Bucherer-Bergs synthesis from carbonyl compounds (Bucherer and Steiner, 1934) and the condensation of aldehydes with hydantoin (Syldatk *et al.*, 1990). Figure 1.2.1 illustrates the

Bucherer-Bergs method for hydantoin synthesis. The applicability of each method depends on the nature of the C-5 residue required and on the availability of precursors to allow the appropriate and cost-effective introduction of the 5-substituent (Syldatk *et al.*, 1990). During the Bucherer-Bergs synthesis, aldehydes are treated with potassium cyanide (KCN) and ammonium carbonate ($(NH_4)_2CO_3$) under mild conditions.

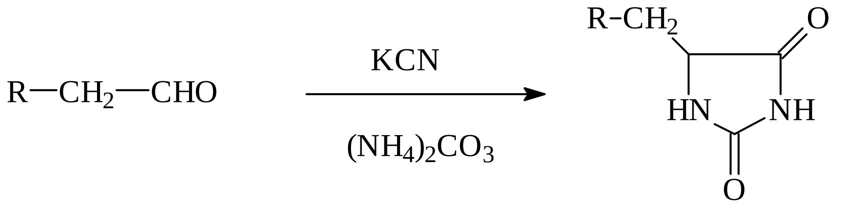


Figure 1.2.2 Bucherer-Bergs synthesis of C-5 substituted hydantoins (Adapted from Syldatk *et al.*, 1990)

These methods have also been modified for the synthesis of other substrates. 5-(4-hydroxyphenyl)-hydantoin (*p*-HPH) is synthesized *via* the amidoalkylation of phenol with glyoxylic acid and urea under acid conditions (Ohashi *et al.*, 1981). The advantage of this method is the exclusion of sodium cyanide and 4-hydroxybenzaldehyde, which are toxic reactants, and which also undergo product-contaminating side reactions. This method was optimized for the exclusion of *o*-HPH, which is co-produced with the *p*-HPH.

1.2.2 Chemical synthesis of amino acids

Alkaline chemical hydrolysis may be used to convert substituted hydantoins to "-amino acids (Syldatk *et al.*, 1990a and 1990b; Watabe *et al.*, 1992a and 1992b; Sudge *et al.*, 1998). However, this process yields racemic mixtures of amino acids, which require resolution to the optically active amino acid (Syldatk *et al.*, 1990b; Sudge *et al.*, 1998). Resolution is necessary because the synthesis of biologically active compounds requires only enantiomerically pure amino acids as building blocks (Runser *et al.*, 1990). The enantiomers have identical chemical and physical properties in a symmetrical environment. However, in a chiral environment, such as the human body, enantiomers may act as two different molecules with drastically different biological activities (Martens and Bhushan, 1989). For example, L-DOPA (3,4-dihydroxyphenylalanine) is an important drug for treatment of Parkinson's disease; D-penicillamine is an antibiotic, whereas the L-isomer is toxic (Martens and Bhushan, 1989). Another disadvantage of the chemical hydrolysis of hydantoins is that it also requires a large amount of energy (Yamashiro *et al.*, 1988).

A chemo-enzymatic method has been reported for the synthesis of amino acids from 5-substituted hydantoins (Takahashi *et al.*, 1979; Yamada *et al.*, 1990). In this process, a D,L-5-substituted hydantoin is asymmetrically hydrolyzed to the *N*-carbamoyl-D-amino acid using D-specific hydantoinase (dihydropyrimidinase, EC 3.5.2.2), and the intermediate is further chemically converted to the corresponding D-amino acid (Runser and Meyer, 1993; Lee *et al.*, 1995; Luksa *et al.*, 1997) using either nitrite (Takahashi *et al.*, 1979) or hydrochloric acid (Takahashi *et al.*, 1979; Syldatk *et al.*, 1990; Kim and Kim, 1993; Lee *et al.*, 1994 and 1995). There are several disadvantages associated with the chemical hydrolysis of *N*-carbamyl derivatives. Among these are high reaction temperature, low yields and a long reaction time (Olivieri *et al.*, 1978; Grifantini *et al.*, 1996). Another disdvantage of the chemical hydrolysis of *N*-carbamylderivatives is the generation of large amounts of waste (Kim and Kim, 1994; Grifantini *et al.*, 1996), which necessitates the consideration of waste treatment in the process development (Kim and Kim, 1994).

1.2.3 Enzymatic production of amino acids from hydantoins

A fully enzymatic, enantioselective method for the production of amino acids from 5-substituted hydantoins has been reported. This process involves the hydrolysis of the hydantoin derivative by the hydantoinase enzyme, and the hydrolysis of the *N*-carbamyl intermediate, catalyzed by *N*-carbamylamino acid amidohydrolase (*N*-carbamylase) (Olivieri *et al.*, 1979; Moller *et al.*, 1988; Kim and Kim, 1993; Ogawa *et al.*, 1994; Ikenaka *et al.*, 1998). A number of microorganisms, from various genera, have been reported to produce both an hydantoinase and an *N*-carbamylase. Examples include *Flavobacterium* (Nishida *et al.*, 1987), *Pseudomonas* (Chevalier *et al.*, 1989; Ishikawa *et al.*, 1993), *Arthrobacter* (Syldatk *et al.*, 1987), *Bacillus* (Yamashiro *et al.*, 1988), and *Agrobacterium* species (Hartley *et al.*, 1998). Figure 1.2.2 is an illustration of the enzymatic hydrolysis of hydantoins.

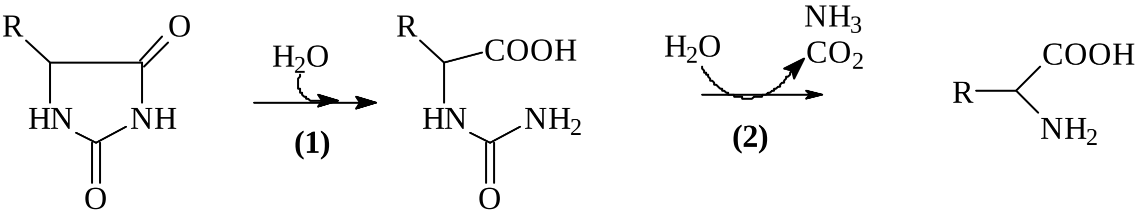


Figure 1.2.3 Enzymatic hydrolysis of 5-monosubtituted hydantoins

(Key: (1) = Hydantoinase; (2) = *N*-Carbamylase)

Due to the numerous disadvantages associated with the chemical hydrolysis of hydantoins, enzymatic hydrolysis is thought to be a feasible option as it offers a wide range of advantages. From a catalytic point of view, enzymes are regarded to be superior to chemical catalysts because they possess specificity (Tramper, 1985). Owing to their high specificity, enzymes have the ability to produce a very pure product, provided that the starting reaction mixture is free of contaminants. Unlike chemical catalysts, many enzymes possess stereospecificity, which enables enzymes to distinguish between enantiomers (Tramper, 1985). Thus, the use of enzymes does not require a chemical racemization step or a resolution step since it does not result in the production of racemic mixtures. Another advantage of the enzymatic hydrolysis of hydantoins is that the reaction occurs under mild reaction conditions, which can result in a cheap industrial process (Polastro, 1989; Hartley *et al.*, 1998).

Enzymatic production of amino acids from 5-substituted hydantoins is initiated by the ring-opening of the hydantoin, which results in the formation of the corresponding *N*-carbamylamino acid (Chevalier *et al.*, 1989; Chien *et al.*, 1996). This enzyme is widely distributed in microorganisms, plants, and animals (Bernheim and Bernheim, 1946; Yamada *et al.*, 1978; Syldatk *et al.*, 1990; Chien *et al.*, 1998). It is an inducible catabolic enzyme which is involved in the degradation of pyrimidine nucleotides (Syldatk *et al.*, 1990; Siemann *et al.*, 1999). The natural substrates of this enzyme have been suggested to be 5,6-dihydrouracil and 5,6-dihydrothymine (Siemann *et al.*, 1999). Hydantoinases are classified as D-, L-, or non-selective, according to their stereospecificity (Siemann *et al.*, 1999), and thus *N*-carbamyl-D- and/or L-amino acids (NCAA) may be produced, depending on the stereospecificity of the microorganism or enzyme used. The most widely distributed group of hydantoinases is the D-specific group, which have been isolated from several microbial, plant and animal sources (Syldatk *et al.*, 1990).

In most microorganisms hydantoinase is regarded as identical to dihydropyrimidinase. However, it has been shown in some microorganisms that hydantoin-hydrolyzing activity was distinct from pyrimidine hydrolyzing activity. For example, Runser and Ohleyer (1990) reported that dihydrouracil was poorly hydrolyzed by the D-hydantoinase of *Agrobacterium* sp. Runser and Meyer (1993) also reported that when dihydrouracil was used as a substrate for resting cells of *Agrobacterium* IP I-671, dihydropyrimidinase activity was lost upon heating, whereas hydantoinase activity was retained. This led to the suggestion that the hydantoin-hydrolyzing and dihydropyrimidine-hydrolyzing enzymes of this bacterium were different molecular entities.

The second step in the production of amino acids from hydantoins is the hydrolysis of the NCAA to produce the corresponding amino acid, catalyzed by *N*-carbamylase (Sano *et al.*, 1977; Olivieri *et al.*, 1981; Chien *et al.*, 1996; Ikenaka *et al.*, 1998). This enzyme is found in some microorganisms in co-existence with an hydantoinase, but it is also found in certain microorganisms that do not produce hydantoinase, such as *Comamonas* sp. E222c (Ogawa *et al.*, 1992). It is regarded to be identical to β-ureidopropionase (EC 3.5.1.6) (Moller *et al.*, 1988; Ogawa *et al.*, 1994; May *et al.*, 1998).

1.2.4 Induction of hydantoin-hydrolyzing enzymes

Durham and Weber (1995) investigated the production of hydantoinase by an *Agrobacterium tumefaciens* strain in the presence and absence of inducers, and they found that the enzyme was produced in low quantities unless an inducer was incorporated into the medium. Highest titers of enzyme were obtained with the inclusion of D,L-hydantoin or D,L-5-methylhydantoin in the growth medium. 2,4-Dithiouracil had no effect on hydantoinase production by this strain. However, hydantoinase production by another *Agrobacterium* strain was greatly improved by the addition of 2,4-dithiouracil to the growth medium (Runser and Meyer, 1993). The hydantoinase of this bacterium was also induced to a greater extent by *p*-hydroxyphenylhydantoin than by hydantoin, which suggested that the saturation of the chiral centre may be of importance for the efficiency of the inducer.

Moller *et al*. (1998) investigated the effect of the addition of various hydantoin derivatives to the culture medium on the formation of hydantoinase by *Arthrobacter crystallopoietes*, and found that maximum D,L-5-methylhydantoin transformation was obtained when hydantoin was used as an inducer.

1.2.5 Substrate selectivity of hydantoin-hydrolyzing enzymes

Many hydantoin derivatives are accepted as substrates for enzymatic reactions, and it has been known since the 1940s that some microorganisms are able to grow on media with D,L-5-monosubstituted hydantoins as the sole C- and/or N-source in mineral salt medium (Drauz *et al.*, 1995).

Several hydantoinases from different sources have been reported to have relatively wide substrate selectivity. However, there is also a wide variation in the stereoselectivity characteristics of individual enzymes. Olivieri *et al.* (1981) investigated the substrate selectivity of intact cells of *Agrobacterium radiobacter*, and the results showed that the hydantoinase and *N*-carbamylase of this bacterium hydrolyzed a wide range of 5-substituted hydantoins, including aliphatic and aromatic substrates. Syldatk *et al.* (1987) also reported a wide substrate selectivity for the hydantoinase from *Arthrobacter* sp. BH20, which showed no stereoselectivity in that it hydrolyzed D-, and -L-substrates. However, an hydantoinase from another *Arthrobacter, A. crystallopoietes* AM2, hydrolyzed a wide range of hydantoins, but was reported to be strictly D-specific (Moller *et al.*, 1988). Another D-specific hydantoinase was partially purified from *Bacillus stearothermophilus* NS1122A (Ishikawa *et al.,* 1994). This enzyme hydrolyzed D-hydantoin derivatives several-fold more efficiently than their L-forms. Lee *et al.* (1995) also reported a D-specific hydantoinase which preferentially hydrolyzed hydantoin and showed no activity toward 2-thiouracil and 2,4-

thiouracil. Hydantoin was hydrolyzed twice as efficiently than dihydrouracil by this enzyme. In contrast, dihydrouracil was the best substrate for the D-hydantoinase of an *Agrobacterium tumefaciens* strain (Durham and Weber, 1995). The hydantoinase of *Bacillus brevis* No. 102 was reported to possess relaxed substrate selectivity, resulting in the production of a wide variety of L-amino acids (Yamashiro *et al.*, 1988).

The hydantoinase extracted from green hulls of the pea plant, *Pisum sativum* showed high activity towards hydantoin (95% conversion), and low activity was observed towards 5-isopropylhydantoin (53%) (Morin *et al.*, 1995). This enzyme was also reported to efficiently hydrolyze 5-methylmercaptoethylhydantoin (74%) and 5-methylhydantoin (93%).

The *N*-carbamylase of an *Agrobacterium* species was reported to have a relatively wide substrate selectivity, as it hydrolyzed a number of *N*-carbamylamino acids, both aliphatic and aromatic (Louwrier and Knowles, 1996). The *N*-carbamylase of this bacterium showed strict D-stereospecificity, and no L-*N*-carbamylamino acids were hydrolyzed. Another D-specific *N*-carbamylase was reported for an *Agrobacterium* sp. KNK712, which also hydrolyzed a wide variety of substrates (Ikenaka *et al.*, 1999). The D-hydantoinase of *Bacillus circulans* also exhibited wide substrate selectivity, with preference for D,L-5-substituted hydantoins with longer aliphatic side chains (LukÑa *et al.*, 1998). However, the most readily hydrolyzed substrates were hydantoin and 5-phenylhydantoin. In contrast, the *N*-carbamylase enzyme from *Pseudomonas* sp. strain NS671 showed strict L-stereospecificity, and hydrolyzed a wide range of aliphatic substrates, as well as *N*-carbamylphenylalanine, which is an aromatic substrate (Ishikawa *et al.*, 1996). An L-specific *N*-carbamylase was purified from *Alcaligens xylosoxidans*, and, although it hydrolyzed a number of substrates, their relative rates of hydrolysis were low, with *N*-carbamylalanine hydrolyzed with the highest yield. However, the V_{max}/ K_m values indicated that *N*-carbamyl-L-valine was the best substrate for this enzyme (Ogawa *et al.*, 1995).

1.3 NITRILE HYDROLYSIS

Nitriles are cyanide-substituted carboxylic acids that occur naturally and synthetically (Babu *et al.*, 1995; Linardi *et al.*, 1996). Organonitriles are versatile intermediates in organic synthesis, and this can be attributed to the ease with which they can be prepared and subsequently transformed (Beard *et al.*, 1993; O'Grady and Pembroke, 1994; Linardi *et al.*, 1996). Synthetic nitrile compounds are used industrially in benzonitrile herbicides (Babu *et al.*, 1995; Linardi *et al.*, 1996), and as precursors for the synthesis of polyacrylonitrile plastics (Henahan and Idol, 1971, Babu *et al.*, 1995). Nitriles are also used as organic solvents (Linardi *et al.*, 1996), extractants and recrystallizing agents in a number of industrial processes (Henahan and Idol, 1971).

Much research on nitriles has been directed towards their transformation into a number of commercially important products (Beard *et al.*, 1993). The chemical bond of interest in nitriles is essentially a C-N bond, (a carbon atom bonded to a nitrogen atom with a triple bond). Hydantoins, which were discussed in the previous section, also contain a C-N bond. Hydrolysis of both types of compounds involves breaking this C-N bond. The products of hydrolysis of hydantoins are amino acids, which are carboxylic acids, and similarly, nitrile hydrolysis also results in the production of carboxylic acids. One of the major applications of nitrile hydrolysis is the production of 2-arylpropionic acids, which are important non-steroidal anti-inflammatory drugs, e.g., *S*-naproxen from racemic naproxen nitrile (Layh *et al.*, 1994). Hydrolysis of nitriles may be accomplished by the use of chemical processes or enzymatic methods.

1.3.1 Chemical hydrolysis of nitriles

Nitriles may be chemically transformed into amines, amides, carboxylic acids, carbonyl and other compounds (Kakeya *et al.*, 1991). The hydrolysis products of nitriles, namely amides and/or carboxylic acids, are used extensively in industry. The simplest functional group change that a nitrile can undergo, i.e. chemical hydrolysis to an amide or carboxylic acid is often difficult to achieve due to the harshness of the conditions required (either strongly acidic or strongly alkaline) (Bhalla *et al.*, 1992; Eyal and Charles, 1990). An example is the hydrolysis of "-aminonitriles into racemic amino acids (Bhalla *et al.*, 1992).

Another example of the use of nitriles for the production of useful compounds is the industrial production of 2-arylpropionic acids. This involves the resolution of isomers by physical means, which is a very costly process (Gilligan *et al.*, 1993). Acrylamide has been produced industrially via the catalytical hydrolysis of acrylonitrile by the copper salts (Jallageas *et al.*, 1980; Nagasawa *et al.*, 1989). However, the disadvantages of this process are the occurrence of side reactions and the difficulty of the regeneration of the catalyst. Another disadvantage of the chemical hydrolysis of nitriles is that racemic products are obtained (Bhalla *et al.*, 1992). It has also been reported that during the chemical hydrolysis of nitriles, especially in larger scale transformations, large quantities of inorganic salts are always produced as byproducts, which results in unfavourable ecological consequences (Honicke-Schmidt and Schneider, 1990; Eyal and Charles, 1990).

1.3.2 Enzymatic hydrolysis of nitriles

The ability of enzymes to hydrolyze nitriles is well known (Cohen *et al.*, 1990), and the enzymic hydrolysis of nitriles to amides and/or carboxylic acids represents a potentially useful synthetic method owing to the mildness under which the reactions can be carried out (Eyal and Charles, 1990; Cohen *et al.*, 1992).

The use of bacterial enzymes for the large-scale bioconversion of nitriles into useful amides and acids has been established in recent years (Nagasawa and Yamada, 1989 and 1990; Gilligan *et al.*, 1993). Gilligan *et al.* (1993) classify the use of resting cells for the bioconversion of nitriles into three categories. The first category involves the use of a nitrile hydratase enzyme, which converts nitriles to their corresponding amides. Nagasawa and Yamada (1990) reported the use of a nitrile hydratase for the production of acrylamide. The second category involves the use of a nitrilase, which directly converts nitriles to their corresponding carboxylic acids. Yamamoto *et al.* (1990) reported the use of a nitrilase for the production of ibuprofen from 2-phenylpropionitrile. The third category involves the use of two enzymes, nitrile hydratase and amidase, for the conversion of nitriles to acids via amide intermediates. The nitrile hydratase first converts the nitrile to the amide, which is subsequently converted to the corresponding carboxylic acid by the amidase (See section 1.3.3 for chemical reactions).

1.3.3 Distribution of nitrile-hydrolyzing activity

Nitrile-degrading enzyme systems are widely distributed through two of the three domains, namely plants, fungi, and bacteria (Cowan *et al.*, 1998). Nitrilase activity was reported in only 3 out of 21 plant families (Thimann and Mahadevan, 1964a cited in Cowan *et al.*, 1998), and in a limited number of fungal genera, which gave an indication that this activity was relatively rare (Cowan *et al.*, 1998). Nitrile-degrading activity appears to be more common in bacteria (Cowan *et al.*, 1998). The physiological role of nitrile-degrading enzymes in plants has not yet been elucidated (Cowan *et al.*, 1998). However, in plants, nitrile-degrading enzymes are involved in nutrient mobilization, particularly in the glucosinolates (Bestwick *et al.*, 1993 cited in Cowan *et al.*, 1998). It has also been suggested that these enzymes play a role in complex pathways controlling both the production and degradation of cyanogenic glycosides and related compounds and where aldoximes are key intermediates (Cowan *et al.*, 1998). Nitrilase is also involved in the biosynthesis of the plant hormone, indole-3-acetic acid, from indole-3-acetonitrile (Kobayashi *et al.*, 1998). The nitrile-degrading enzymes have been reported to be endoenzymes or cell-associated enzymes (Ingvorsen *et al.*, 1988).

The hydration of nitriles in plants and microorganisms is catalyzed by two distinct enzymatic processes (Jallageas *et al.*, 1980; Amarant *et al.*, 1989; Cohen *et al.*, 1990; Beard *et al.*, 1993; Bauer *et al.*, 1998; Stolz *et al.*, 1998; Almatawah *et al.*, 1999). The first pathway is the hydration of a nitrile by two molecules of water to form the corresponding carboxylic acid and ammonia is catalyzed by the enzyme nitrilase and proceeds without the release of the amide intermediate (Fig. 1.3.1) (Amarant *et al.*, 1989; Cohen *et al.*, 1990; Kobayashi *et al.*, 1993; Cramp and Cowan, 1999). However, there are a relatively limited number of examples of this type of reaction (Jallageas *et al.*, 1980), and the nitrilase frequently shows a strong preference for aromatic substrates (Cowan *et al.*, 1998).

Figure 1.3.1 Hydrolysis of nitriles by nitrilase

The more common reaction is the second pathway (Jallageas *et al.*, 1980), which involves a nitrile hydratase in combination with an amidase. Nitriles are firstly hydrated to the corresponding amides by the nitrile hydratase, and this is followed by the hydrolysis of the amides to the corresponding carboxylic acids and ammonia by amidase (Fig. 1.3.2) (Macadam and Knowles, 1985; Nagasawa and Yamada, 1990; Pogorelova *et al.*, 1996; Layh *et al.*, 1997; Payne *et al.*, 1997; Kobayashi *et al.*, 1993 and 1998; Pereira *et al.*, 1998). However, Kobayashi *et al.* (1998) have also reported a nitrilase which is able to catalyze amide hydrolysis.

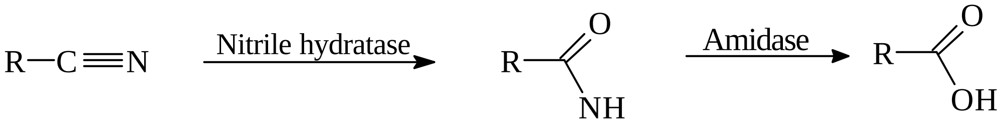


Figure 1.3.2 Hydrolysis of nitriles by nitrile hydratase and amidase

1.3.4 Induction of nitrile-hydrolyzing enzymes

Nitrilases and nitrile hydratases have been reported to be inducible proteins, and the analysis of a limited number of nitrilase and nitrile hydratase operons suggests that both upstream and downstream open reading frames are involved in positive transcriptional regulation (Cowan *et al.*, 1998).

The nitrilase activity of *Nocardia rhodochrous* was investigated after growth of the bacterium on either acetonitrile or acetate as the carbon source, and the results showed that no activity was observed after growth on succinate, which suggested that acetate did not induce enzyme activity (Collins and Knowles, 1983). The "-aminonitrile-hydrolyzing nitrilase of *Rhodococcus rhodochrous* PA34, was reported to be induced by isobutyronitrile, whereas acetonitrile was a good source of nitrogen for the bacterium, but did not induce nitrile-degrading activity (Bhalla *et al.*, 1990). Unlike most reported inducible nitrile-degrading enzymes which require nitriles for induction, the nitrile hydratase of *Rhodococcus equi*

TG328 was reported to prefer an amide, crotonamide, as an inducer (Gilligan *et al.*, 1993). Another *Rhodococcus* species, *R. rhodochrous* J1 was reported to be induced by several amide compounds as well as urea (Nagasawa *et al.*, 1991 cited in Mizunashi *et al.*, 1998), whereas that from a transformant of *R. rhodochrous* was induced by methacrylamide and crotonamide (Mizunashi *et al.*, 1998). The nitrile hydratase of *Pseudomonas chlororaphis* was also induced by methacrylamide (Nagasawa *et al.*, 1989 and 1990). Babu *et al.* (1995) reported that the nitrile hydratase from *Pseudomonas* marginalis was an inducible enzyme after it failed to catalyze the hydrolysis of nitriles after being cultured on glucose instead of acetonitrile (carbon source). The nitrile hydratase of *Agrobacterium tumefaciens* strain d3 was also shown to require a nitrile compound, phenylacetonitrile, in the growth medium in order to obtain maximal nitrile hydratase activity (Bauer *et al.* (1998).

A number of inducer-independent nitrile-hydrolyzing enzymes have been reported. Examples are enzymes from *Brevibacterium* species, *Brevibacterium* R-312 (Eyal and Charles, 1990), and *Brevibacterium imperiale* (Battistel *et al.*, 1997), a *Pseudomonas putida* (Fallon *et al.*, 1997), and *Bacillus* sp. (Pereira *et al.*, 1998). Jallegeas *et al.* (1980) also reported the hydrolysis of nitriles by several inducer-independent bacterial strains belonging to the genera *Micrococcus* and *Brevibacterium*.

1.3.5 Substrate selectivity of nitrile-hydrolyzing enzymes

The substrate range of nitrile-hydrolyzing enzymes depends mainly on the source of the enzyme, nitrile hydratases and nitrilases from different sources showing preference for certain substrates. Nitrilases are generally considered to have preference for aromatic substrates, whereas nitrile hydratases generally prefer aliphatic substrates (Collins and Knowles, 1983; Cowan *et al.*, 1998; Almatawah *et al.*, 1999). However, there have been some reports of enzyme systems which do not conform to this rule, i.e. nitrile hydratases which are able to hydrolyze both aliphatic and aromatic substrates.

The nitrile hydratase of *Rhodococcus* sp. CH5 was reported to have a wide substrate selectivity, because it hydrolyzed a wide range of nitriles, both aliphatic and aromatic (Ingvorsen *et al.*, 1988). O'Grady and Pembroke (1994) reported the isolation of an *Agrobacterium* sp. capable of degrading a wide range of nitrile substrates, including aliphatic and aromatic nitriles. This bacterium hydrolyzed nitriles via the two-step pathway, i.e. nitrile hydratase and amidase, and it was reported to hydrolyze some nitriles which did not support growth. Layh *et al.* (1997) isolated several microorganisms which were able to grow on various nitriles, including some *Pseudomonas* species, *Rhodococcus* species, and *Alcaligens denitrificans*. These isolates converted a fairly broad range of substrates, but highest yields were observed with the nitrile used during the enrichment process. The nitrile hydratase of a *Bacillus* sp. was reported to hydrolyze a wide range of aliphatic nitriles, but did not hydrolyze aromatic substrates such as benzonitrile and benzylcyanide (Pereira *et al.*, 1998).

Kakeya *et al.* (1991) reported the hydrolysis of nitriles by *Rhodococcus butanica*, utilizing a nitrile hydratase in combination with an amidase, as well as a nitrilase. The nitrilase of this bacterium hydrolyzed a wide variety of aromatic substrates, including substituted benzonitriles and arylnitriles (Kakeya *et al.*, 1991). Blakey *et al.* (1995) reported a nitrile hydratase from *Rhodococcus* AJ270, which was able to hydrolyze aliphatic and aromatic nitriles. Another *Rhodococcus* sp. was reported to have the nitrile hydratase and amidase enzymes, but no nitrilase, and an immobilized enzyme preparation from this bacterium was used to hydrolyze a wide variety of aromatic dinitriles (Cohen *et al.*, 1990 and 1992).

1.4 AMIDE HYDROLYSIS

Aliphatic amides, such as acrylamide and methylacrylamide are used extensively as grouting agents in the manufacture of acrylic and methacrylic resins and fibres and as solvents in the petrochemical industry (Nawaz *et al.*, 1994). Also, a number of amides are either active ingredients of herbicides and pesticides or intermediates of pesticide degradation. The extensive usage and indiscriminate discharge of acrylamide has led to the contamination of terrestrial, aquatic and plant ecosystems (Nishikawa *et al.*, 1978, cited in Nawaz *et al.*, 1998). Biodegradation of these compounds is of great importance because of their detrimental health effects (Hayashi *et al.*, 1989, cited in Nawaz *et al.*, 1994) such as carcinogenecity, teratogenecity and neurotoxic properties (Nawaz *et al.*, 1998).

Several microorganisms have been shown to degrade various aliphatic and aromatic amides (Kagayama and Ohe, 1990; Nawaz *et al.*, 1998; Wyborn *et al.*, 1994). Microorganisms catalyze this activity via the use of an amidase (acylamide amidohydrolase; EC 3.5.1.4) (Wyborn *et al.*, 1994). Amidases are cytoplasmic enzymes (Skouloubris *et al.*, 1997) which hydrolyze amides to produce ammonia and the corresponding carboxylic acid (Skouloubris *et al.*, 1997; Nawaz *et al.*, 1994; Wyborn *et al.*, 1994; Amarant *et al.*, 1989). Figure 1.4.1 shows the hydrolysis of amides catalyzed by an amidase. Amidases have been found in both procaryotic and eucaryotic organisms, catalyzing deamidation of various endogenous and foreign substrates (Ettinger and DeLuca, 1995).

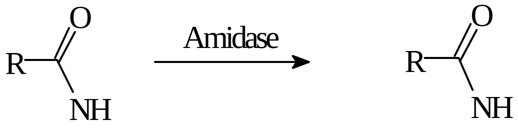


Figure 1.4.1 Enzymatic hydrolysis of amides

1.4.1 Biocatalytic applications of amidases

Bioconversion of amides is important for the enzymatic production of some commercially important products. Such products include 2-arylpropionic acids (Yamamoto *et al.*, 1996; Hayashi *et al.*, 1997), amino acids (Hermes *et al.*, 1993), acrylic acid (Brennan *et al.*, 1995), and "-alkyl substituted amino acids (Hermes *et al.*, 1994). Optically active 2-arylpropionic acids are useful pharmaceutically active substances, the most important of these compounds being the nonsteroidal anti-inflammatory drugs known as 'profens' (Yamamoto *et al.*, 1996; Hayashi *et al.*, 1997). The enantiomers of most profens have different biological activities, with the exception of naproxen. Despite their different activities, these drugs are administered as racemic mixtures, because there are few industrial processes for producing the enantiomers (Yamamoto *et al.*, 1996). However, there is an increasing demand for the separate enantiomers of the drugs as they are more effective in therapy (Hayashi *et al.*, 1997). Some microorganisms have been reported to have the ability to produce enantiomerically pure 2-arylpropionic acids from 2-arylpropionamides via the action of enantioselective amidases (Beard *et al.*, 1993; Hirrlinger *et al.*, 1996; Hayashi *et al.*, 1997).

Acrylic acid and its salts are important commodity chemicals annually produced on a large scale (Brennan *et al.*, 1995). These chemicals are used for the manufacture of a variety of polymers including flocculants, adhesives, thickeners and superabsorbents. A wide range of microorgansims produce acrylic acid from acrylamide, utilizing an amidase (Asano *et al.*, 1982; Ciskanik *et al.*, 1994; Hayashi *et al.*, 1997; Nawaz et. al, 1994; Skouloubris *et al.*, 1997), or from acrylonitrile utilizing amidase in combination with nitrile hydratase (Bui *et al.*, 1984; Collins and Knowles, 1983; Asano *et al.*, 1982; Ingvorsen et. al, 1988). Acrylic acid is used for the production of esters which are then used as feedstock for polymerizations which give coating, lacquer and resins products (Hughes *et al.*, 1998). Other uses of acrylic acid include production of its homo- and co-polymers, which are used as print thickeners, dispersants in pigment suspensions like ink and paints, and as flocculants in alkaline mineral solutions (Hughes *et al.*, 1998).

1.4.2 Distribution and induction of amidase activity

Several microorganisms that have been reported, in the literature, to have the ability to hydrolyze amides, are also reported to require inducers for efficient production of amidases. Collins and Knowles (1983) reported an inducible amidase from *Norcadia rhodochrous* LL100-21, induced by acetamide and benzamide. However, the ability of the enzyme to hydrolyze the substrate was inducer-dependent, in that acetamide-induced cells were unable to hydrolyze benzamide, and benzamide-induced cells were unable to hydrolyze acetamide. The amidase of *Pseudomonas putida* Sc2 was reported to be induced by *N*-acetyl arylalkylamine, since no amidase activity was detected in the absence of this substrate in the growth medium (Shimizu *et al.*, 1992). The amidase of *Ochrobactrum anthropi* NCIMB 40321 was reported to be significantly reduced when the bacterium cultured in media containing ammonium chloride, ammonium sulfate, or urea, whereas amidase activity was induced by addition of "-substituted amino acid amides (van den Tweel, 1993). *O. anthropi* was unable to grow in defined media with acetamide or benzylamide as nitrogen source. An L-carnitine amidase reported by Joeres and Kula (1994) was also an inducible enzyme, with L-carnitine amide being the best inducer of enzyme activity. Acetamide did not induce enzyme activity, and acrylamide did not support growth of the L-carnitine amidase-producing bacterium. Moreau *et al.* (1994) reported an amidase from a *Brevibacterium* sp. which was induced by acetamide and by *N*-methylacetamide, an amide analogue. The amidase of *Pseudomonas marginalis* was reported to be induced by acetonitrile, and the bacterium was able to grow on a variety of amides, including acetamide (Babu *et al.*, 1995). Several amides induced production of amidase in *Pseudomonas chlororaphis* B23, including isobutyramide, propionamide, and acrylamide (Ciskanik *et al.*, 1995), but was not induced by acetamide. The naproxen amide-hydrolyzing amidase of *R. erythropolis* MP50 was induced after growth of the strain with naproxen nitrile or naproxen amide (Layh *et al.*, 1995; Hirrlinger *et al.*, 1996), but this enzyme was not induced by urea, acetamide, and nitriles such as propionitrile, isovaleronitrile or phenylacetonitrile (Hirrlinger *et al.*, 1996). The amidase activity of *Comamonas acidovorans* KPO-2771-4 was reported to be enhanced by the presence of isobutyronitrile in the growth medium, although it was constitutively expressed (Yamamoto *et al.*, 1996).

An inducer-independent amidase has also been reported; the amidase of a *Brevibacterium* sp. was found to have the same amidase activity level whatever the carbon or nitrogen source used (Moreau *et al.*, 1994).

A number of authors have investigated the growth phase at which amidase activity is most pronounced. The amidase activity of *O. anthropi* (van den Tweel, 1993), *P. marginalis* (Babu *et al.*, 1995), and *Corynebacterium nitrophilus* (Brennan *et al.*, 1995) was reported to be maximal during the exponential growth phase. However, the amidase activity of *P. putida* Sc2 (Shimizu *et al.*, 1992), *Brevibacterium* sp. (Moreau *et al.*, 1994), and *R. erythropolis* (Hirrlinger *et al.*, 1996) was reported to be maximal at the end of exponential growth phase.

1.4.3 Substrate selectivity of amidases

The ability of amidases from different sources to hydrolyze a range of amides has been reported in the literature. Asano *et al.* (1982) reported an amidase from *Arthrobacter* sp. J-1 which was able to efficiently hydrolyze acetamide, acrylamide and propionamide. The relative activities for these substrates were 100% for acetamide, 330% for acrylamide, and 216% for propionamide. However, this bacterium was unable to hydrolyze a number of other amides such as formamide, *n*-valeramide, benzamide, phenylacetamide, glycinamide, succinamide, and malonamide. These results showed that the amidase of *Arthrobacter* sp. J-1 had a narrow substrate selectivity (Asano *et al.*, 1982). Collins and Knowles (1983) also reported a narrow substrate-specificity amidase from *Norcadia rhodochrous*, where hydrolysis of amides was strictly selective for the amide used to induce activity. Another narrow spectrum amidase was reported by Kagayama and Ohe (1990). This amidase was purified from *Pseudomonas* sp. GDI 211, and it only hydrolyzed a few aromatic amides, and no aliphatic amides. Hermes *et al.* (1994) also reported a narrow spectrum amidase from *Mycobacterium neoaurum*, where activity was reported to be restricted to "-amino acid amides with an H atom at the C"- position (e.g. D,L-valinamide) or "-methyl-substituted molecules (e.g. DL-"-methyl-valinamide).

A wide spectrum amino acid amidase was reported by Boesten *et al.* (1986). This amidase hydrolyzed a variety of amino acid amides, including phenylalnine amide and phenylglycine amide. Van den Tweel *et al.* (1993) investigated the hydrolysis of various amides by *O. anthropi*, and reported the amidase to have a relatively wide substrate spectrum, as it hydrolyzed a wide range of "-H-, "-alkyl, *N*-hydroxy-"-amino acid amides and "-hydroxy acid amides.

Another wide spectrum amidase was reported by Kobayashi *et al.* (1993). This enzyme was purified from recombinant *E. coli*, and it was shown to efficiently hydrolyze a wide range of amides including aromatic, aliphatic, and amino acid amides. However, this enzyme was unable to hydrolyze amino acids with an amide group (Kobayashi *et al.*, (1993). Joeres and

Kula (1994) reported an amidase which hydrolyzed L-carnitine amide, isobutyramide, acrylamide and other amino acid amides. However, this amidase was unable to hydrolyze the aliphatic amide acetamide, even though the organism was able to grow on this amide. Hayashi *et al.* (1997) reported a wide substrate spectrum amidase from *Comamonas acidovorans*, which was able to hydrolyze aromatic amides and aliphatic amides with long carbon chains. The peptide amidase of *Stenotrophomonas maltophilia* was shown to hydrolyze a wide range of amino acid amides (Stelkes-Ritter *et al.*, 1995). However, unlike the amidase reported by Kobayashi *et al.* (1993), it was unable to hydrolyze amino acid amides with free amino groups, and only hydrolyzed amino acid amides with protected amino groups. *P. marginalis* was reported to hydrolyze a wide spectrum of amides, including aromatic and aliphatic amides (Babu *et al.*, 1995). Among the amides tested as substrates, acetamide showed the highest activity as a substrate, followed by propionamide. Acrylamide was not hydrolyzed by this bacterium (Babu *et al.*, 1995).

Another wide spectrum amidase was purified from *P. chlororaphis* B23, which showed efficient activity toward a variety of amides, including aliphatic amides, with the highest activity observed with isobutyramide, propionamide, and aromatic amides such as D,L-phenylalanine amide and nicotinamide (Ciskanik *et al.*, 1995). No activity was observed with urea, *N*-methylbenzamide, and *N*-methylacetamide. The purified amidase of *R. erythropolis* MP50 was reported to hydrolyze a broad spectrum of amides (Hirrlinger *et al.*, 1996), including a number of aliphatic amides and aromatic amides. Among the substrates tested, urea showed the least activity, and acetamide, isovaleramide and acrylamide were also poorly hydrolyzed (Hirrlinger *et al.*, 1996).

1.5 IMIDE HYDROLYSIS

1.5.1 Distribution of cyclic imide-transforming activity

Cyclic imides have structures similar to those of cyclic ureides and are known to be hydrolyzed by mammalian dihydropyrimidinase, which functions in pyrimidine metabolism (Dudley *et al.*, 1976; Ogawa *et al.*, 1996). Figure 1.5.1 illustrates the structures of cyclic imides and cyclic ureides.

Cyclic imide **Cyclic ureide**

Figure 1.5.1 Diagram illustrating the structures of cyclic imides and cyclic ureides

Imides are not natural metabolites, but they are encountered as xenobiotics (Yang *et al.*, 1993). Examples of imides are the succinimide drugs "-phenylsuccinimide, and *N*-methyl-"-methyl-"-phenylsuccinimide (antiepileptic agents) (Dudley *et al.*, 1976). A number of mammalian enzymes which are capable of hydrolyzing imides have been reported in the literature. Examples are dihydropyrimidinase from the dog (Dudley *et al.*, 1976), and rat liver imidase (Yang *et al.*, 1993). The first report of microbial hydrolysis of imides was published by Ogawa *et al.* (1994), and the microorganism involved was *Blastobacter* sp. strain A17p-4. A D-hydantoinase was reported to catalyze imide hydrolysis in *Blastobacter* sp. strain A17p-4 (Ogawa *et al.*, 1999). The above-mentioned enzymes are involved in detoxification, and they are characterized by broad specificity for compounds bearing a particular functional group rather than for an individual compound or a type of carbon skeleton (Yang *et al.*, 1993). This broad specificity is elucidated in reports of several microbial species which utilize the first step of pyrimidine degradation (dihydropyrimidinase) to catalyze the ring-opening step of hydantoin hydrolysis (Takahashi *et al.*, 1978; Ogawa *et al.*, 1994). Runser and Ohleyer (1990) have also reported that hydantoin and pyrimidine hydrolysis in an *Agrobacterium* sp. are catalyzed by the same enzyme.

Soong *et al.* (1998) studied the distribution of cyclic imide-transforming activity in microorganisms, and reported that this activity was widely distributed in microorganisms, with more activity observed in bacteria than in yeasts and molds. A variety of microorganisms were observed to have the ability to assimilate succinimide. These authors found that there was no correlation among succinimide-, dihydrouracil- and D,L-5-methylhydantoin-hydrolyzing activities in bacteria, which suggests that cyclic imide and cyclic ureide transformation involve different enzyme systems. However, they found that cyclic imide transformation and cyclic ureide transformation was similar in yeasts and in molds. They therefore suggested that cyclic imide- and cyclic ureide-hydrolyzing activities are catalyzed by an identical enzyme system in eukaryotes.

1.5.2 Imidase

Imidase is an enzyme which specifically hydrolyzes the simple cyclic imides succinimide, glutarimide, and sulfur-containing cyclic imides (Ogawa *et al.*, 1997 and 1999). This enzyme catalyzes the first step of cyclic imide metabolism (Ogawa *et al.*, 1999), resulting in the production of monoamidated dicarboxylates (Ogawa *et al.*, 1997). These monoamidated dicarboxylates are deamidated by an amidase enzyme to yield dicarboxylates (Fig. 1.5.2) (Ogawa *et al.*, 1996).

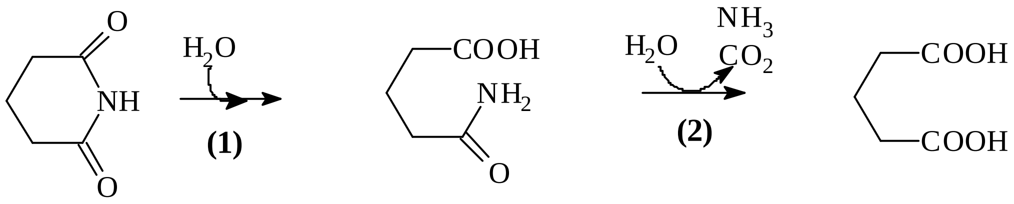
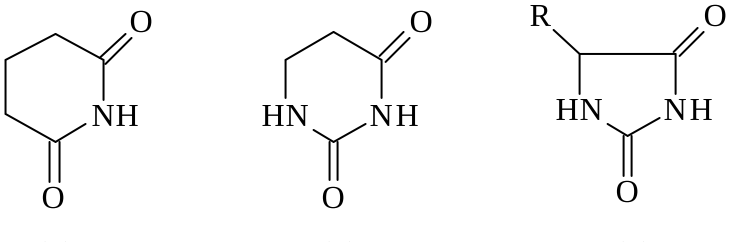


Figure 1.5.2 Enzymatic hydrolysis of cyclic imides

(Key: (1) = imidase; (2) = amidase)

The deamidation of monoamidated dicarboxylates is followed by a transformation pathway similar to that in the tricarboxylic acid cycle (TCA) (Ogawa *et al.*, 1996; Soong *et al.*, 1998). Imidase is different from cyclic ureide-transforming enzymes (Ogawa *et al.*, 1996; Soong *et al.*, 1998) in that it only hydrolyzes simple cyclic imides, whereas hydantoinase acts on complex cyclic imides (Soong *et al.*, 1998). The microbial imidase has a broad substrate spectrum as it hydrolyzes different imides, dihydropyrimidines and hydantoin, but in contrast to dihydropyrimidinase, it does not hydrolyze 5-monosubstituted hydantoins (Ogawa *et al.*, 1997; Syldatk *et al.*, 1999). Figure 1.5.3 shows the structures of cyclic imides, dihydropyrimidines, and 5-monosubstituted hydantoins.



(1) **(2)** **(3)**

Figure 1.5.3 Structures of dihydropyrimidine (1), cyclic imide (2), and 5-monosubstituted hydantoin

1.5.3 Induction of imide-transforming enzymes

The enzymes involved in imide hydrolysis have been reported to be inducible in *Blastobacter* sp. A17p-4, in which succinimide- and succinamic acid-hydrolyzing activity was higher in succinimide-grown cells than in sucrose-grown cells. (Ogawa *et al.*, 1996). This suggests that both enzymes involved in imide metabolism were induced by succinimide, and it was concluded that the induction of successive enzymes contributes to the utilization of cyclic imides for cell growth (Ogawa *et al.*, 1996).

1.5.4 Substrate selectivity of imide-transforming enzymes

The substrate specificity of *Blastobacter* sp. A17p-4 was investigated by Ogawa *et al.* (1997). The imidase from this bacterium showed slightly different substrate specificity from the imidase reported by Yang *et al.* (1993) in that diacetamide and dihydrothymine, which were hydrolyzed by the rat liver imidase, were not hydrolyzed by the imidase from *Blastobacter* sp. A17p-4 (Ogawa *et al.*, 1997). However, like the rat liver imidase, it did not hydrolyze *N*-substituted cyclic imides, nor did it hydrolyze cyclic imides with bulky substituents on the ring structure. The imidase of *Blastobacter* sp. A17p-4, although it hydrolyzed cyclic ureides, showed higher affinity and activity toward cyclic imides than toward cyclic ureides in the ring-opening reaction. 5-Substituted hydantoins were not hydrolyzed by the imidase of this bacterium, whereas cyclic imides with a sulfur atom at the N_1-position of hydantoin were substrates for this enzyme (Ogawa *et al.*, 1997).

1.5.5 Reversal of cyclic imide hydrolysis

The imidase from *Blastobacter* sp. A17p-4 differed from the eucaryotic enzymes in that it catalyzed the cyclization of monoamidated dicarboxylates as well as *N*-carbamylamino acids to cyclic imides and ureides, respectively (Ogawa *et al.*, 1997). However, this reverse reaction was reported to proceed much slower than the ring-opening reaction. The D-hydantoinase enzyme of *Blastobacter* sp. strain A17p-4 was also reported to catalyze the cyclization of phthalamidic acid (monoamidated amide) to phthalimide (cyclic amide) under acidic conditions (Ogawa *et al.*, 1999). The D-hydantoinase of this bacterium, however, did not catalyze the cyclization of succinamic acid to succinimide, which is a substrate for imidase.

1.6 RESEARCH OBJECTIVES

The major objective of this study was the development of a biocatalytic process for the production of amino acids. Our laboratory had previously isolated four bacterial strains, namely RU-KM1, RU-KM3_L, RU-KM3_S, and RU-OR, which are able to hydrolyze hydantoin and hydantoin derivatives to their corresponding amino acids, utilizing an hydantoinase in combination with an *N*-carbamylase (Burton *et al.*, 1998). The activity of the hydantoin-hydrolyzing enzymes of these strains had been characterized, to a certain extent, in whole cells. Enzyme activity has also been partially characterized in crude extracts of RU-KM1, RU-KM3_L, and RU-OR, to determine the effects of disruption of cell membranes on enzyme activity. No work had been done, thus far, on crude extracts of RU-KM3_S, which is the strain which showed highest activity among the four isolates. Thus the first objective of this study was the characterization of enzyme activity in crude extracts of RU-KM3_S. This was necessary because a comparison of enzyme activity in whole cells and crude extracts would be useful in that one would then be able to select the best biocatalytic system of the two.

In developing a biocatalytic process for amino acid production from hydantoins by microorganisms, it would be valuable to have a microorganism which is able to hydrolyze a broad range of hydantoin substrates, and thus be able to produce a wide variety of amino acids. Information on which hydantoin substrate is best hydrolyzed by which strain would also be valuable in that it would allow for utilization of that microorganism for bulk production of a specific amino acid. Thus, the second objective of this study was to determine the hydrolysis of different hydantoin substrates by the four strains so as to:

a) compare their activities with that of RU-KM3_S;

b) determine the substrate range hydrolyzed by each strain; and

c) determine the best substrate for each strain, and thus elucidate which strain, among the four, is most suitable for production of which amino acid.

In addition to hydantoin and its derivatives, a number of other compounds may be used as substrates for biocatalytic production of amino acids, as discussed in the preceeding literature review. These include nitriles, and amino acid amides. The biocatalytic reactions by which these compounds and hydantoins are hydrolyzed all involve amide bond hydrolysis. Thus it was postulated that the enzymatic amide bond-hydrolyzing activity possessed by these microorganisms, i.e., hydantoinase activity, could be applied in a number of industrial processes. Hydrolysis of the substrates of these enzymes, and related compounds, could result in the production of a wide range of commercially important products.

In addition to nitriles and amides, imides are also enzymatically hydrolyzed *via* an amide bond, and they have a basic structure which is very similar to that of hydantoins. Therefore, a decision was taken to investigate hydrolysis of nitriles, amides, and imides by the four hydantoin-hydrolyzing microorganisms, with the view of developing biocatalyts with more diverse applications, and thus to fully exploit the biocatalytic capacity of the microorganisms.

CHAPTER 2

Characterization of the hydantoin-hydrolyzing enzymes in RU-KM3$_S$ crude extract

2.1 INTRODUCTION

This chapter deals with the characterization of the hydantoin-hydrolyzing enzymes of RU-KM3$_S$, a mesophilic, hydantoinase-producing *Pseudomonas putida* strain. The aim of this investigation was to compare the activity of this strain in a crude extract preparation with that observed with whole cells, so as to select the best biocatalyst for production of amino acids.

In this study, the ability of this strain to hydrolyze a range of hydantoin derivatives was to be investigated. Preliminary research had already been done on RU-KM3$_S$, mostly using resting cell reactions (Buchanan, K., 1996). The optimum pH and temperature for conversion of hydantoin to glycine were found to be pH 8.0 and 40°C, respectively. The reaction time required in order to obtain maximum product formation by RU-KM3$_S$ resting cells was reported to be 3 hours. Whole cells of RU-KM3$_S$ were reported to be able to catalyze four successive hydantoin biotransformations over a 12-hour period, with only a small decrease in enzyme activity (Buchanan, K., 1996).

The decision was taken to characterize the activity of RU-KM3$_S$ in crude extract preparations, since the disruption of cell membranes might lead to hydrolysis of a wider substrate range, due to the increased access of the substrate to the enzymes. This would also allow the comparison of the activity of the hydantoin-hydrolyzing enzymes of RU-KM3$_S$, *viz.*, hydantoinase and *N*-carbamylase, in whole cells and crude extract form. The enzymes might behave differently in the two biocatalytic forms, due to the alteration of the cell environment in which the enzymes have demonstrated high activity. However, disruption of cell membranes may lead to unavailability of cofactors, which may be required for hydantoin hydrolysis. Also, the two enzymes involved in the reaction in the intact cells may be situated in positions which would allow the second enzyme to gain access to the intermediate of the hydantoin hydrolysis reaction, namely, the *N*-carbamyl-intermediate.

The hydrolysis of 5-methylhydantoin by the crude extract of RU-KM3$_S$ under various conditions was investigated. 5-Methylhydantoin was chosen as a substrate due to the availability of its pure enantiomers, i.e. D- and L-5-methylhydantoin, which would allow determination of the stereoselectivity of the RU-KM3$_S$ enzymes. In addition to this, this substrate is a 5-monosubstituted hydantoin with a very short side chain (CH_3-), and was therefore considered to be a representative substrate, which could be used for the investigation of 5-monosubstituted hydantoins by RU-KM3$_S$. Enzymatic hydrolysis of 5-methylhydantoin results in the production of *N*-carbamylalanine and alanine.

The effect of various additives (metal ions, detergent, and stabilizing agents) on the activity of the hydantoin-hydrolyzing enzymes of RU-KM3$_S$ was investigated, using 5-methylhydantoin as a substrate.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

5-Methylhydantoin was chemically synthesized by the method of Bucherer-Bergs (Bucherer and Steiner, 1934). *N*-carbamylalanine and alanine were purchased from Sigma-Aldrich Chemicals. All other reagents were of analytical grade.

2.2.2 Culture of microorganism

RU-KM3$_S$ from 3 day-old hydantoin minimal medium (HMM) agar plates (Appendix 1.1) was inoculated into 50mL HMM broth supplemented with 1% hydantoin as sole nitrogen source and 1% glucose as the carbon source. This was incubated at 28°C on an orbital shaker at 200 rpm until stationary phase (3 - 4 days). This was termed the starter culture.

The starter culture was then inoculated into nutrient broth (Biolab Diagnostics, S.A.) supplemented with 0.1% hydantoin (growth medium), to give an OD$_{600}$ of 0.02. This was subsequently incubated at 28°C on an orbital shaker at 200 rpm, until stationary phase (" 18 hours) was reached.

2.2.3 Preparation of the crude extract of RU-KM3$_S$

The cells were harvested by centrifugation at 7000 rpm for 10 minutes at 4°C. The cell pellet was then washed with cold 0.1M potassium phosphate buffer, pH 8.0, followed by centrifugation at 8000g for 10 minutes at 4°C. The cell pellet obtained after washing the cells was resuspended in 0.1M potassium phosphate buffer to give a final concentration of 0.1g cells/mL, which was calculated as follows:

$$\text{Wet cell mass} = (\text{Mass of centrifuge bottle} + \text{Pellet}) - \text{Mass of centrifuge bottle}$$

$$\text{Volume buffer} = \frac{\text{Wet cell mass}}{0.1}$$

The cells were disrupted by 1 pass through a Yeda-press (LINCA Lamon Instrumentations Co., Ltd. Tel-Aviv) at 15 MPa, 4°C, at a flow rate of 1 drop per second. The extract thus obtained was frozen in liquid nitrogen, and dried on a vacuum dryer at – 40°C to obtain a crude extract powder, which was stored at –20°C.

2.2.4 Quantification of the products of the hydantoin hydrolysis reaction

2.2.4.1 Ehrlich’s assay

This is a standard colorimetric assay which was used for the quantification of *N*-carbamylamino acids produced. It was based on that reported by Yamada *et al.*, (1978).

The protocol was as follows:

< the reaction mixtures and controls were microfuged in 1.5mL eppendorf tubes at 13 000g for 5 minutes
< 1mL of the supernatant was transferred to test tubes containing 0.5mL of 12% trichloroacetic acid
< this was mixed on a vortex mixer
< 3mL of deionised water was added to the mixture
< 0.5mL of Ehrlich s reagent was added
< the tubes were allowed to stand at room temperature for 20 minutes
< the absorbance of the samples was read at 420nm on a UV-Visible spectrophotometer
< the concentration of the *N*-carbamylamino acid produced was calculated using a standard curve prepared with *N*-carbamylglycine (NCG) (See Appendix 3.1 for standard curve).

2.2.4.2 Ninhydrin assay (Plummer, 1987)

This colorimetric assay was used for the quantification of amino acids.

The protocol was as follows:

< 0.980mL aliquots of 0.1M potassium phosphate buffer, pH 8.0, were added to test tubes

- 20FL of each supernatant was added to the test tubes
- 1mL of ninhydrin reagent was added to the test tubes
- the test tubes were boiled for 15 minutes
- they were then allowed to cool to room temperature ("10 minutes)
- 3mL of 50% ethanol was added to the samples
- these were allowed to stand at room temperature for 10 minutes
- the absorbance was read at 570nm

The concentration of the amino acid produced was calculated using a standard curve prepared with alanine (See Appendix 3.2 for standard curve)

2.2.5 Determination of the optimum reaction time for *N*-carbamylalanine and alanine production by the RU-KM3$_S$ crude extract

A solution containing 0.1g/mL freeze-dried extract powder, which had been stored frozen, in 0.1M potassium phosphate buffer, pH 8.0, was prepared. Aliquots (1mL) of this solution were mixed with 1mL aliquots of a 100mM 5-methylhydantoin solution (giving a starting concentration of 50mM) in Bijou bottles. All reaction mixtures were prepared in triplicate. Appropriate controls, *viz.* extract solution without substrate, substrate without extract solution, plus a potassium phosphate buffer blank, were prepared. The reaction mixtures and controls were then incubated at 40°C in an incubator with an orbital shaker set at 200rpm Samples were taken out hourly, from 1 hour up to 6 hours, and microfuged. The production of *N*-carbamylalanine (to indicate hydantoinase activity) and alanine (to indicate *N*-carbamylase activity) were measured from the supernatant using a modification of the Erhlich's method of Yamada *et al.* (1978), for the hydantoinase activity, and the ninhydrin assay for amino acid production.

2.2.6 Determination of the stability of the hydantoin-hydrolyzing enzymes of RU-KM3$_S$

A solution containing 0.1g/mL crude extract was prepared as before. 1mL aliquots were transferred to reaction bottles, and these were incubated at 40°C, shaking at 200 rpm. At the start of the experiment, 1.0mL of 50mM methylhydantoin was added to three reaction bottles. After 1 hour, this set was taken out and microfuged, after which hydantoinase and *N*-carbamylase activities were analyzed by measuring the product concentrations in the supernatant as described above. Identical sets of 3 triplicate samples were incubated for 1, 2, and 3 hours, respectively, before addition of the substrate. The biocatalytic reactions were carried out for 1 hour, after which the activities of the two hydantoin-hydrolyzing enzymes were assayed by analyzing the product concentrations in the supernatants.

2.2.7 The effect of detergent W-1 on the hydantoin-hydrolyzing enzymes in RU-KM3$_S$ crude extract

An extract solution with a concentration of 0.1g/mL powder was prepared. The solution was aliquoted into reaction bottles (1mL/reaction). A stock solution of W-1 (4%) was added to each of the bottles to give final W-1 concentrations of 0.1, 0.5, and 1%. A 0% control was also prepared. All reactions and controls were prepared in triplicate. The reaction bottles were then incubated on ice for 10 minutes, after which the substrate (1mL of 50mM 5-methylhydantoin) was added. The reaction bottles were then incubated at 40°C for 1 hour, shaking at 200 rpm. The samples were then microfuged, and concentrations of *N*-carbamylalanine and alanine in the supernatant were analyzed.

2.2.8 The effect of addition of metal ions on the hydantoinase and *N*-carbamylase of RU-KM3$_S$ extract

A crude extract solution was prepared as before, and 1mL aliquots were transferred to reaction bottles. Stock solutions of different metal sulphates were added to the reaction bottles containing extract solutions to give final metal ion concentrations of 2.5mM. The metal ions used were Mg^{2+}, Cu^{2+}, Co^{2+}, and Mn^{2+}. Each reaction was carried out in triplicate. The substrate, 1mL of 50mM methylhydantoin, was added to each and the reaction bottles were incubated at 40°C for 1 hour, shaking at 200rpm. Controls containing the crude extract solution and metal sulphate solutions without the substrate were also prepared. The samples were microfuged after incubation, and product (*N*-carbamyalanine and alanine) concentrations in the supernatants were determined.

2.2.9 The effect of metal ions on the hydantoin-hydrolyzing enzymes of RU-KM3$_S$ crude extract: Removal and back-addition of metal ions

RU-KM3$_S$ was cultured in HMM and nutrient broth supplemented with 0.1% hydantoin until early stationary phase and harvested as before. Cells were then disrupted by French - pressing, and the extract obtained was dialyzed against 0.1M potassium phosphate buffer, pH 8.0, containing 2.5mM EDTA for 1.5 hours, at 4°C, with buffer changes every 30 minutes. The extract was then aliquoted to reaction bottles (1mL/reaction), and 1mL of substrate (50mM hydantoin solution) was added. Metal sulphate solutions (Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+} and Co^{2+}) were added to a final concentration of 2.5mM, in triplicate. The following controls were also prepared: whole cells, French-pressed extract, French-pressed extract dialyzed against plain buffer, whole cells left standing in buffer in cold room for 1.5 hours. The biocatalytic reactions were carried out for 3 hours at 40°C. Product concentrations in the supernatants were assayed.

2.2.10 The effect of organic solvents on the hydantoin-hydrolyzing enzymes in RU-KM3$_S$ crude extract

The effect of the addition of different solvents to the reaction mixture was determined using two solvent concentrations, *viz.*, 5 and 10%. The solvents used were ethanol, acetone, and acetonitrile. Stock solutions of the solvents were added to reaction bottles containing 1mL of a 0.1g/mL extract solution to give the desired solvent concentration. The bottles were left on ice for 10min, after which the substrate (1mL of 50mM methylhydantoin) was added. A control to which no solvent was added was also prepared. The biocatalytic reactions were carried out at 40°C for 1 hour. The concentrations of *N*-carbamyalanine and alanine in the supernatants were determined.

2.2.11 The effect of varying substrate concentration on the hydantoinase and *N*-carbamylase in RU-KM3$_S$ crude extract

A solution containing 0.1g/mL crude extract was prepared in potassium phosphate buffer (0.1M; pH 8.0). 1mL aliquots of this solution were transferred to reaction bottles. A stock solution of 5-methylhydantoin (100mM) was aliquoted into each of the bottles to give final substrate concentrations of 10, 20, 30, 40, and 50mM, in a total reaction volume of 2mL. The biocatalytic reactions were carried out at 40°C for 1 hour, shaking at 200rpm. The concentrations of *N*-carbamyalanine and alanine in the supernatants were analyzed after the samples were microfuged at 13 000g for 5 minutes.

2.2.12 Investigation of the stereoselectivity of the hydantoin-hydrolyzing enzymes in RU-KM3$_S$ crude extract

A solution containing 0.1g extract/mL was prepared as before. This was aliquoted into reaction bottles, 1mL per bottle. Optically pure substrate solutions were prepared (50mM each), using D-5-methylhydantoin, and L-5-methylhydantoin. A racemic solution was also prepared using equal amounts of D- and L-methylhydantoin, with the final concentrations of each being 25mM, thus achieving a total D,L-5-methylhydantoin concentration of 50mM. A 1mL aliquot of each substrate solution was added to reaction bottles containing the crude extract solution, each reaction mixture prepared in triplicate. The biocatalytic reactions were carried out for 1 hour at 40°C, with shaking at 200rpm on an orbital shaker. The samples were then microfuged at 13000g for 5 minutes. The concentrations of *N*-carbamyalanine and alanine in the supernatant were analyzed.

2.3 RESULTS AND DISCUSSION

Enzymatic hydrolysis of 5-methylhydantoin is achieved by two enzymes, an hydantoinase and an *N*-carbamylase enzyme (Figure 2.3.1)

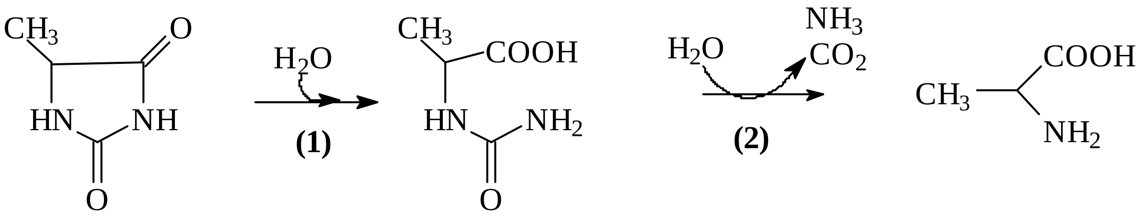


Figure 2.3.1 Enzymatic hydrolysis of 5-methylhydantoin.

(**Key**: (1) = Hydantoinase; (2) = *N*-carbamylase)

The effects of various conditions and additives on the hydrolysis of 5-methylhydantoin by RU-KM3$_S$ crude extract were tested. Since this is a two-enzyme system, the effects observed will be a result of the combination of the effects of that particular reaction condition on the enzymes, individually. The figures in this section show the results observed during the course of this investigation. Most of the results are represented in the form of bar graphs, with the first bar series representing the concentration (mM) of *N*-carbamylalanine measured, and the second bar series representing the concentration of alanine measured. The third bar series represents total 5-methylhydantoin conversion (% or mM, as indicated in Key below figure), i.e. *N*-carbamylalanine plus alanine, and thus represents total hydantoinase activity. Total hydantoinase activity includes the amount of amino acid produced, since the *N*-carbamylase enzyme converts *N*-carbamylalanine, which has been produced by the hydantoinase enzyme, to alanine. Where bar charts are too complex, results are shown as tables.

Conversion of 5-methylhydantoin was achieved at yields greater than 50%, and this is due to the non-stereoselectivity of the hydantoinase enzyme of RU-KM3$_S$ (This is discussed in a later Section 2.3.8). The yields did vary between experiments, but a comparison was made between samples in the same experiment.

2.3.1 Determination of the optimum reaction time for 5-methylhydantoin hydrolysis by RU-KM3$_S$ crude extract

The aim of this experiment was to determine the length of reaction time required by both hydantoin-hydrolyzing enzymes of RU-KM3$_S$ to yield the maximum amount of product, and thus to optimize the reaction time. The results are illustrated in Figure 2.3.2.

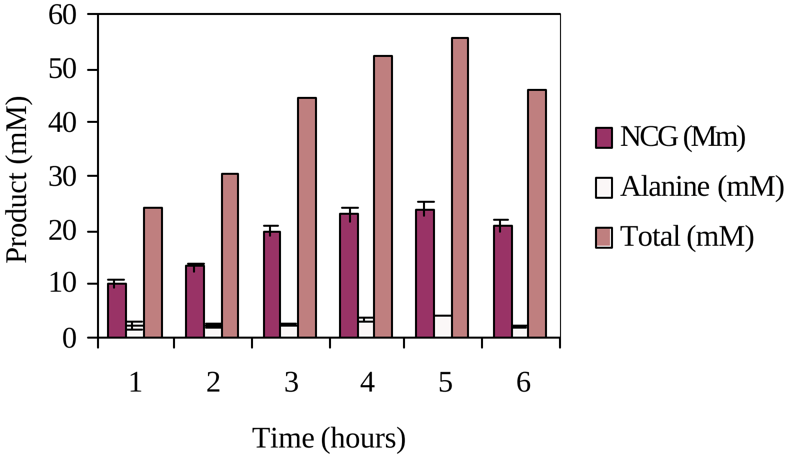


Figure 2.3.2 The optimum reaction time for maximum product formation by the hydantoinase and *N*-carbamylase in RU-KM3$_S$ crude extract

(**Key:** NCA = *N*-carbamyalanine; Total (%) = Total conversion of 5-methylhydantoin)

The length of the reaction time required for maximum *N*-carbamylalanine and alanine formation by the enzymes involved in hydantoin hydrolysis in RU-KM3$_S$ was found to be between 3 and 6 hours. The total hydantoinase yield after 3, 4, and 5 hours was 44.66%, 52.46%, and 55.56%, respectively. There was a definite increase in the amount of *N*-carbamyalanine produced after 1 hour of incubation as compared to 3 hours (9.97mM was produced after 1 hours, compared to 19.4mM after 3 hours). Beyond 3 hours of incubation there was only a relatively small further increase in the amount of *N*-carbamyalanine produced, and thus 3 hours would be sufficient to run the biocatalytic reaction, to observe high *N*-carbamyalanine production by the hydantoinase enzyme of RU-KM3$_S$.

The amount of alanine produced after 1 hour and 3 hours of reaction was similar, 2.15mM and 2.39mM, respectively. There was only a slight increase in the amount of alanine produced after 4 and 5 hours of incubation, 3.26mM and 4.04mM. These results correspond well with those obtained with whole cells of RU-KM3$_S$ (Buchanan, K., 1996), where maximum product formation occurred after 3 hours. The same was observed with another *Pseudomonas putida* strain, RU-KM3$_L$ (Phehane, V., 1998). This finding makes RU-KM3$_S$ a good candidate for biocatalytic conversion of hydantoins to amino acids, as the reaction time required is relatively short, and thus is less likely to be expensive.

A decrease in total 5-methylhdyantoin conversion was observed after 6 hours of reaction. The total hydantoinase yield decreased from 55.56% after 5 hours, to 45.82%. The reason for this decrease in the amount of product detected could possibly be due to utilization of the *N*-carbamyl-intermediate by other enzymes in the crude extract. This needs to be investigated using purified enzymes.

Because appreciable levels of activity were observed for both enzymes after 1 hour, for practical reasons, further investigations were carried out for 1 hour. The purpose of this investigation was mainly to optimize biocatalytic reaction conditions under the most convenient analytical conditions, and to compare different biocatalytic reactions, rather than to optimise for process conditions at this stage.

2.3.2 Determination of the stability of the hydantoin-hydrolyzing enzymes of RU-KM3$_S$ in the crude extract

Thermostable biocatalysts are of great importance in industrial applications. For this reason, the stability of the hydantoinase and *N*-carbamylase of RU-KM3$_S$, in crude extract form, was investigated. The investigation was carried out under the normal assay temperature, i.e., at 40°C, to determine whether the enzymes lost any activity with prolonged incubation time. The results are shown in Figure 2.3.3.

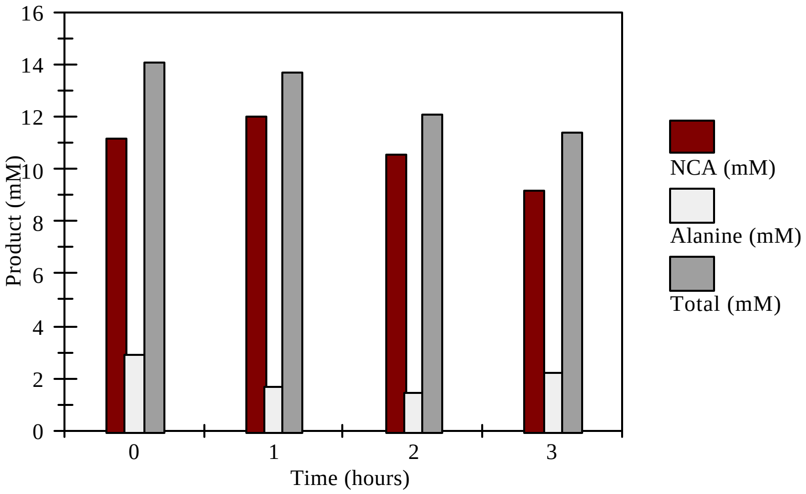


Figure. 2.3.3 The stability of the hydantoin-hydrolyzing enzymes in RU-KM3$_S$ crude extract at 40°C

(NCA = *N*-carbamylalanine, Total = Total hydantoinase yield. The results represent a mean"SEM of triplicate determinations, with standard errors #0.03)

The results indicated that the enzymes had retained most of their biocatalytic activity after 3 hours of incubation (Fig. 2.3.3). The total hydantoinase product yield after 1, 2, and 3 hours was 13.67mM, 12.04mM, and 11.39mM respectively.

The activity of the *N*-carbamylase enzyme apparently decreased after 1 and 2 hours of incubation, compared to that observed without pre-incubation of the extract. The amount of alanine formed when no pre-incubation was performed was 2.92mM, and after 1 and 2 hours it was 1.65mM and 1.47mM, respectively. However, the activity of this enzyme seemed to increase after 3 hours of incubation, although not to a great extent (2.21mM). These results are all very similar, and suggest that the enzyme was not greatly affected by incubation at 40°C. This further confirms that the hydantoinase enzyme, which which hydrolyzes 5-methylhydantoin to *N*-carbamylalanine, and thus provides the substrate for the *N*-carbamylase, was not greatly affected by incubation at 40°C for 3 hours.

These results have positive implications for the industrial application of RU-KM3_S in the production of amino acids from 5-monosubstituted hydantoins, because they show that the enzymes can retain most of their activity under the reaction conditions required for high levels of hydantoin hydrolysis, which involve incubation of the biocatalyst with the substrate at 40°C for 3 hours.

2.3.3 The effect of polyethylene ether W-1 on the hydantoinase and *N*-carbamylase in RU-KM3_S crude extract

Polyethylene ether W-1 is a detergent which has the ability to disrupt cell membranes. It was used to investigate the possibility of enhancing the activity of the hydantoin-hydrolyzing enzymes of RU-KM3_S, by releasing the enzymes from the cell debris, and thus making the enzymes more accessible to the substrate. A range of concentrations of the detergent was used, as high concentrations may be detrimental to enzyme activity, due to denaturation.The results of this investigation are shown in Figure 2.3.4.

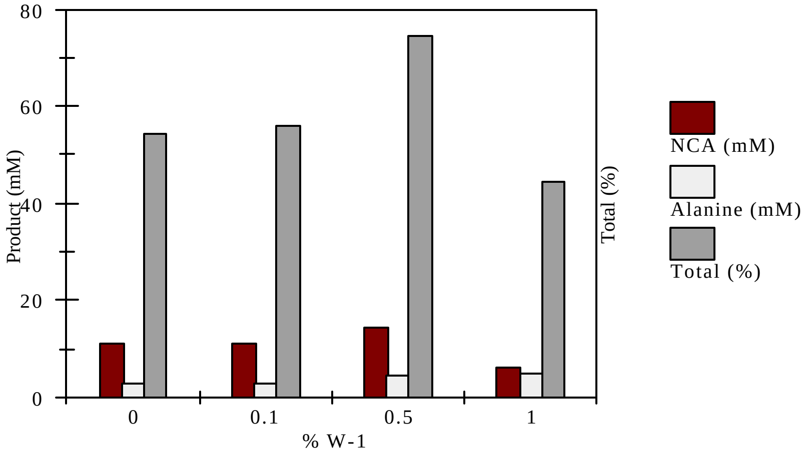


Figure 2.3.4 The effect of W-1 on 5-methylhydantoin hydrolysis by RU-KM3$_S$ crude extract

(NCA = *N*-carbamylalanine, Total = Total hydantoinase yield. The results represent a mean"SEM of triplicate determinations, with standard errors #0.045)

Addition of detergent W-1 at a concentration of 0.1% resulted in a slight increase in the total hydantoinase activity of RU-KM3$_S$ crude extract (Figure 2.3.4) The total conversions of 5-methylhydantoin with and without addition of 0.1% detergent were 54.36% and 55.76%, respectively. Addition of 0.5% W-1 also resulted in an increase in the activity of the hydantoinase enzyme, with total hydantoinase conversion being 74.48%.

An increase was observed in the activity of the *N*-carbamylase with the addition of 0.5% and 1% W-1. The amount of alanine produced by the control where no detergent was added was

2.77mM, whereas addition of 1% detergent resulted in the production of 5.02mM. However, total hydantoinase conversion was decreased by the addition of 1% detergent.

The total hydantoinase conversion observed with the control was 54.36%, whereas 1% detergent resulted in total hydantoinase conversion of 44.6%. These results are similar to the results obtained in our laboratories with another *Pseudomonas putida* strain and an *Agrobacterium tumefaciens*, where the hydantoinase activity was increased by the addition of 0.1% and 0.5% W-1, respectively (Burton *et al.*, 1998). This finding suggests that the hydantoin-hydrolyzing enzymes of RU-KM3$_S$ may be membrane-associated. W-1 disrupts cell membranes and this could result in the increased availability of enzymes to the substrate. This is a novel finding, as thus far there has been no report in the literature on membrane-bound hydantoinases. The location of these enzymes in cells is currently under further investigation in our laboratories.

2.3.4 Metal dependence of the hydantoinase and *N*-carbamylase of RU-KM3$_S$

A number of hydantoin-hydrolyzing enzymes have been reported, in the literature, to be metal-dependent. Thus, it was necessary to determine whether this was the case with the enzymes of RU-KM3$_S$. The first experiment was carried out by the direct addition of metal ions (in the form of metal sulphates) to the reaction mixture, at a concentration of 2.5mM. The results of this experiment are illustrated in Figure 2.3.5.

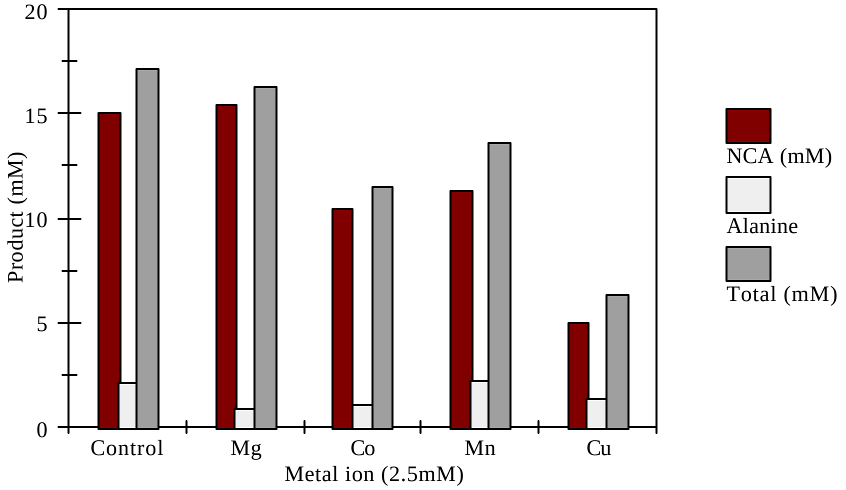


Figure 2.3.5 The effect of metal ions on 5-methylhydantoin hydrolysis by RU-KM3$_S$ crude extract

(NCA = *N*-carbamylalanine, Total = Total hydantoinase yield. The results represent a mean"SEM of triplicate determinations, with standard errors #0.036)

Direct addition of Mg^{2+} to the reaction mixture resulted in a slight increase in the amount of *N*-carbamylalanine produced by RU-KM3$_S$ crude extract (15.44mM), compared to the control (14.99mM). However, the total conversion of 5-methylhydantoin was slightly decreased by the addition of this metal ion. The total hydantoinase conversion in the control was 68.56%, whereas addition of Mg^{2+} resulted in 65.16% substrate conversion. Addition of Mn^{2+} and Co^{2+} both caused an 18% decrease in the total conversion shown by the hydantoinase enzyme. However, Cu^{2+} caused a more significant decrease in the total hydantoinase activity, giving 44.36% conversion.

Addition of Co^{2+} caused a slight increase in the production of alanine, since the amount of alanine formed in the control was 2.15mM, and that formed after the addition of this metal ion was 2.23mM. However, addition of Mn^{2+} caused an apparent decrease in the activity of this enzyme, with 1.34mM alanine being formed. The decrease in the amount of alanine formed with the addition of Mn^{2+} and Cu^{2+} could be due to one of two things: a) inactivation of the *N*-carbamylase enzyme by these metal ions, and b) a decrease in the amount of *N*-carbamylalanine available for hydrolysis, resulting from inactivation of the hydantoinase enzyme.

The effect of Cu^{2+} on the activity of the *N*-carbamylase enzyme is similar to that reported by Louwrier and Knowles (1997), who reported that this metal ion inactivated the *N*-carbamylase enzyme. Ishikawa *et al.* (1994) also reported that Co^{2+} activated the *N*-carbamylase enzyme, which is similar to the results observed in this study. Activation of the hydantoinase enzyme by Mg^{2+} and inactivation by Cu^{2+} are similar to results reported in the literature (Ogawa *et al.*, 1995; Sharma and Vohra, 1997).

2.3.5 The effect of removal and back-addition of metal ions on the hydantoinase and *N*-carbamylase of RU-KM3$_S$

The aim of this experiment was to further investigate the metal-dependency of the hydantoin-hydrolyzing enzymes of RU-KM3$_S$. This was done by first dialyzing a crude extract of RU-KM3$_S$ against potassium phosphate buffer containing EDTA. EDTA is a metal-chelating agent, and thus it was used to remove any metal ions that may have already been bound to the enzymes. The dialyzed crude extract was then used in reaction mixtures containing different metal ions, at a concentration of 2.5mM. A control where no metal ion was added was prepared, to determine whether there was any loss of activity due to dialysis against the buffer containing EDTA. Another control, where the extract was dialyzed against potassium phosphate buffer, was also prepared to investigate whether the dialysis process itself had any effect on the activity of RU-KM3$_S$. Some cells were left standing at 4°C to determine the effect this would have on the activity of the enzymes. The last control was a normal resting cell reaction, so as to compare the activity under normal conditions with that of the other controls as well as the experimental reactions. The results of this experiment are given in Tables 2.3.1 and 2.3.2.

Table 2.3.1 Hydantoin hydrolysis by different controls used in determination of metal ion effects on hydantoin hydrolysis by RU-KM3$_S$

Control	NCG (mM)	Glycine (mM)	Total converted (mM)
Whole cells	21.68"0.02	9.65"0.09	31.33
French-pressed extract	18.65"0.02	6.91"0.12	25.55
Dialyzed extract	16.83"0.01	5.34"0.03	22.18
Dialyzed + EDTA	14.47"0.03	4.81"0.10	19.27
Standing	18.49"0.01	6.14"0.03	24.64

Table 2.3.2 Effect of back-addition of metal ions to dialyzed extract of RU-KM3$_S$

Metal ion Added	NCG (mM)	Glycine (mM)	Total converted (mM)
Mg^{2+}	17.26"0.00	4.00"0.09	21.27
Mn^{2+}	18.13"0.04	8.02"0.01	26.15
Zn^{2+}	18.63"0.02	3.58"0.02	22.21
Co^{2+}	17.7"0.01	9.15"0.05	26.84
Cu^{2+}	5.47"0.01	1.35"0.09	6.83
Fe^{2+}	11.39"0.02	2.83"0.01	14.22
Co^{2+} + Mg^{2+}	18.88"0.01	9.35"0.02	28.23

The results of this experiment showed that French pressing, French pressing and dialyzing in potassium phosphate buffer with and without EDTA, and leaving cells standing at 4°C for the same amount of time as the duration of dialysis, all resulted in some decrease in the activity both enzymes. Dialysis in buffer containing EDTA resulted in the greatest decrease in activity. The total amount of 5-methylhydantoin converted was 25.55mM, 22.18mM, 19.27mM, and 24.64mM, respectively, compared to 31.33mM converted by whole cells (Table 2.3.1). The large decrease caused by dialyzing the crude extract in buffer containing EDTA suggests that the hydantoin-hydrolyzing enzymes had native metal ions bound to them, and these are required for optimal activity. The decrease in hydantoin conversion caused by French-pressing may be due to destabilization of the enzyme conformation, resulting from membrane disruption, or separation of enzymes from cofactors required for activity, or the change in the biocatalytic environment.

Addition of Mg^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} restored the activity of the hydantoinase enzyme in the extract dialyzed in potassium phosphate buffer with EDTA (Table 2.3.2) to that of the French-pressed extract, prior to dialysis (Table 2.3.1). The total hydantoin conversions were 21.27mM, 26.15mM, 22.21mM, and 26.84mM, respectively, compared to 19.27mM converted by the extract dialyzed in potassium phosphate buffer containing EDTA. However, Cu^{2+} and Fe^{2+} both resulted in a decrease in the activity of the hydantoinase enzymes, compared to the French-pressed extract dialyzed with EDTA control (19.27mM, Table 2.3.1). In the presence of Cu^{2+} only 6.83mM hydantoin was converted, whereas in the presence of Fe^{2+}, 14.22mM hydantoin was converted. Addition of Mg^{2+} and Co^{2+} together restored the activity of the hydantoinase enzyme to slightly higher than that observed with the non-dialyzed French-pressed extract control. The latter converted 25.55mM hydantoin, whereas addition of the two metal ions resulted in conversion of 28.23mM hydantoin.

The activation of the hydantoinase enzyme by Zn^{2+} has been reported in the literature (Lee *et al.*, 1995; Sharma and Vohra, 1997; Siemann *et al.*, 1998). The effect of Fe^{2+} observed here is contrary to that reported in another *Pseudomonas putida*, where the D-hydantoinase was activated, rather than inactivated, by Fe^{2+} ions (Takahashi *et al.*, 1978).

The activity of the *N*-carbamylase was lowered by addition of Mg^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+}. These metal ions resulted in conversion of 4.0mM, 3 .58mM, 2.83mM, 1.35mM *N*-carbamylglycine, respectively, compared to conversion of 6.91mM *N*-carbamylglycine achieved by the non-dialyzed French-pressed extract. The effect of zinc observed in this investigation is similar to that reported for an *N*-carbamylase from an *Agrobacterium* species (Louwrier and Knowles, 1997).

Addition of Co^{2+} alone and in combination with Mg^{2+} restored the activity of the *N*-carbamylase to that of intact cells. Addition of Co^{2+} alone resulted in conversion of 9.15mM *N*-carbamyglycine, whereas addition of Co^{2+} in combination with Mg^{2+} resulted in conversion of 9.35mM *N*-carbamyglycine. This was higher than the amount converted by the non-dialyzed French-pressed extract (6.91mM), which suggests that French-pressing might disrupt metal binding in these cells. Addition of Mn^{2+} also caused an increase in the activity of the *N*-carbamylase, resulting in conversion of 8.02mM *N*-carbamylglycine, compared to 6.91mM, which was achieved by the non-dialyzed French-pressed extract.

In conclusion, the hydantoinase and *N*-carbamylase of RU-$KM3_S$ show some degree of metal-dependence, since addition of EDTA, a metal chelating agent, resulted in a decrease in enzyme activity, which was then restored by the addition of some metal ions. The highest activation of the enzymes was achieved by Mn^{2+}, Mg^{2+}, Co^{2+}, and a combination of Mg^{2+} and Co^{2+}. Thus it may be suggested that the hydantoinase and *N*-carbamylase of RU-$KM3_S$ are metallo-enzymes. However, further investigations, such as determination of the effect of these metal ions on purified enzymes, are required to confirm this.

2.3.6 The effects of organic solvents on the hydantoin-hydrolyzing enzymes in RU-KM3$_S$ crude extract

The effect of the addition of ethanol, acetone, and acetonitrile to the reaction mixture on the hydrolysis of 5-methylhydantoin was investigated in an attempt to characterize hydantoinase and *N*-carbamylase activities in these solvents. The main objective here was to determine whether these solvents could be used for hydrolysis of poorly soluble substrates, e.g. *p*-HPH. Table 2.3.3 shows the results obtained from this experiment.

Table 2.3.3 The effect of organic solvent on 5-methylhydantoin hydrolysis by RU-KM3$_S$ crude extract

Solvent	**% Solvent**	**NCA (mM)**	**Alanine (mM)**	**Total (mM)**
Ethanol	5	2.18	0.02	2.20
	10	1.65	0.05	1.70
Acetone	5	2.68	0.24	2.92
	10	0.89	0.04	0.93
Acetonitrile	5	0.00	4.62	4.62
	10	0.03	0.00	0.03
Control	0	12.21	1.68	18.88

(The results represent a mean"SEM of triplicate determinations, with standard errors #0.005)

The results showed that the solvents significantly inactivated the hydantoinase enzyme at both concentrations used. The total 5-methylhydantoin conversion yields were 2.20mM and 1.70mM, for 5 and 10% ethanol, respectively, whereas with 5 and 10% acetone they were 2.92mM and 0.93mM, respectively. This result is similar to that observed in our laboratories with another *P. putida* strain, where the activity was decreased by these solvents at the same concentrations.

Addition of acetonitrile resulted in an increase in the activity of the *N*-carbamylase enzyme, resulting in formation of 4.62mM alanine, compared to 1.68mM formed by the control containing no organic solvent. Ethanol and acetone, on the other hand, caused a decrease in the activity of the *N*-carbamylase enzyme. When these solvents were present at a concentration of 5%, ethanol and acetone resulted in formation of 0.02mM and 0.24mM alanine, respectively, whereas at 10% concentration 0.05mM and 0.04mM alanine was formed. However, it is not clear whether this decrease in alanine production is caused by inactivation of the enzyme by these solvents, or by the decrease in the amount of *N*-carbamylalanine available for conversion, due to inactivation of the hydantoinase enzyme.

Since these solvents had detrimental effect on the hydantoin hydrolyzing enzymes of RU-KM3_S, no further investigations using these solvents were considered, since addition of organic solvents at concentrations less than 5% is unlikely to have any significant effect on substrate solubility.

2.3.7 The effect of substrate concentration on hydantoin hydrolysis by RU-KM3_S extract

The effect of substrate concentration on the hydrolysis of hydantoins by the RU-KM3_S extract was investigated, using 5-methylhydantoin as a substrate. The concentrations used were 10, 20, 30, 40, and 50mM. The results are illustrated in Figure 2.3.6.

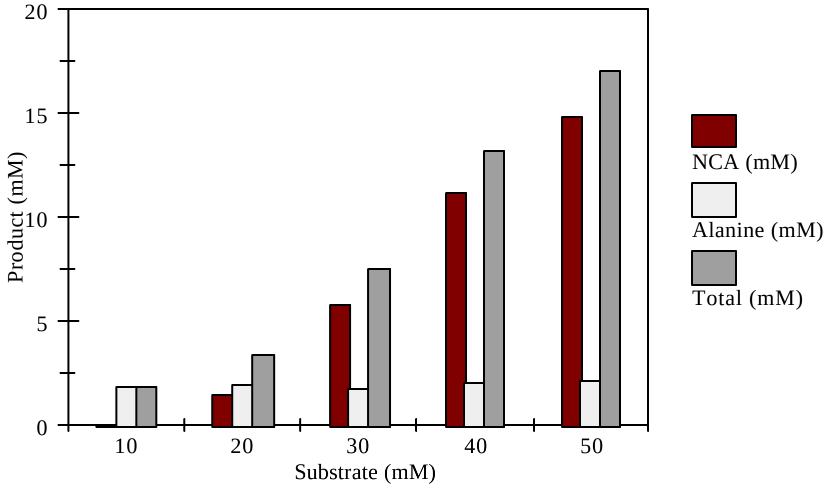


Figure 2.3.6 Hydrolysis of increasing concentrations of 5-methylhydantoin by RU-KM3$_{S}$ crude extract

(NCA = *N*-carbamylalanine; Total = Total 5-methylhydantoin converted. The results represent a mean"SEM of triplicate determinations, with standard errors #0.034)

The results showed that total hydantoinase activity increased as the initial substrate concentration increased (Figure 2.3.6). In this experiment, the total concentrations of hydantoin converted were 1.83mM, 3.39mM, 7.48mM, 13.15mM, and 16.98mM, at starting substrate concentrations of 10, 20, 30, 40, and 50mM, respectively.

Amino acid production followed a different pattern. There was little difference in the amount of alanine produced with the different substrate concentrations. The amount of alanine formed from 10, 20, 30, 40, and 50mM 5-methylhydantoin 1.83, 1.89, 1.73, 2.00, and 2.14mM, respectively. Thus, alanine production did not increase greatly with increased *N*-carbamylalanine availability.

This could possibly be a limiting factor in the application of this biocatalyst to amino acid production, and thus further optimization to improve the activity of the *N*-carbamylase enzyme is required.

2.3.8 The stereoselectivity of the hydantoinase and *N*-carbamylase in RU-KM3$_S$ extract

The stereoselectivities of the hydantoin-hydrolyzing enzymes of RU-KM3$_S$ were investigated using 25mM each of optically pure, and racemic, 5-methylhydantoin. The results are shown in Table 2.3.4 and Figure 2.3.7.

Table 2.3.4 Hydrolysis of D-, L-, and D,L-methylhydantoin by the RU-KM3$_S$ extract

Substrate	NCA (mM)	Alanine (mM)	Total (mM)
L-Methylhydantoin	3.91	4.10	8.01
D-methylhydantoin	7.51	2.39	9.9
D,L-Methylhydantoin	5.41	3.47	8.88

(The results represent a mean"SEM of triplicate determinations, with standard errors #0.05)

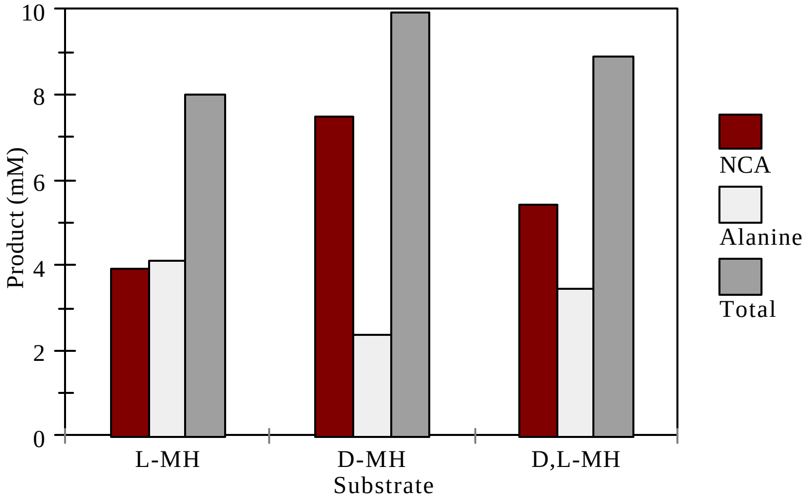


Figure 2.3.7 Hydrolysis of D-, L- and D,L-methylhydantoin by RU-KM3$_S$ crude extract

(L-MH = L-methylhydantoin; D-MH = D-methylhydantoin; D,L-MH = D,L-methylhydantoin. The results represent a mean"SEM of triplicate determinations, with standard errors #0.05)

The use of enzymes is considered to be advantageous over chemical methods for the production of industrially important intermediates and products, and this is partially due to their stereoselectivity. Thus, the stereoselectivity of RU-KM3$_S$ was investigated, using D-, L-, and D,L-methylhydantoin as substrates. This method was based on the assumption that an L-selective enzyme, for example, would not be efficient in hydrolyzing D-substrates, and *vice versa*. As illustrated in Table 2.3.4 and Figure 2.3.7, the total conversions of L-, D,- and D,L-5-methylhydantoin, were fairly similar (8.01mM, 9.90mM, and 8.88mM, respectively). However, the amounts of *N*-carbamylalanine and alanine produced from the different substrates differed. When D-methylhydantoin was used as a substrate, the amount of alanine produced was less than that produced from L-methylhydantoin (2.39mM and 4.1mM, respectively). The amount of alanine produced from D,L-methylhydantoin was also less than that produced from L-alanine (3.47mM and 4.1mM, respectively).

These results suggest that the *N*-carbamylase enzyme of RU-KM3_S is L-stereoselective, whereas the hydantoinase enzyme appears to be non-selective. This is indicated by the fact that although the total substrate conversion was the same, irrespective of the stereoselectivity of the substrate, more alanine was produced when the L-substrate was used. It is also indicated by the fact that the hydantoinase enzyme hydrolyzed approximately equal amounts of substrates, but of the *N*-carbamylalanine products formed, the *N*-carbamylase of RU-KM3_S preferentially hydrolyzed the L-form. In some previous experiments, product yields greater than 50% were achieved, which further confirms that the hydantoinase of RU-KM3_S is non-selective, since a stereoselective enzyme would only be able to catalyze the hydrolysis of only one isomer.

L-stereoselective *N*-carbamylases have been reported in the literature (Sano *et al.*, 1977; Watabe *et. al.*, 1992; Wagner *et al.*, 1996). However, to our knowledge, this is the first indication of an L-selective *N*-carbamylase in a *Pseudomonas putida* strain (Burton *et al.*, 1999, in press).

The hydantoinase enzyme of RU-KM3_S is different from those reported in the literature for other *P. putida* strains (Chien *et al.*, 1997; LaPointe *et al.*, 1994), which were D-selective, in that it appears to be non-selective, i.e. it can hydrolyze both D-, and L-substrates efficiently. A similar enzyme system to that observed in this study, i.e. non-selective hydrolysis of 5-monosubstituted hydantoins in combination with L-selective hydrolysis of *N*-carbamylamino acids was also reported by Wagner *et al.* (1996).

2.4 CONCLUSIONS

Characterization of the hydantoin-hydrolyzing biocatalyst, RU-KM3$_S$, in the form of a crude extract demonstrated that, although French-pressing resulted in some loss of the activity of the hydantoinase and *N*-carbamylase enzymes of RU-KM3$_S$, the use of the crude extract for hydantoin-hydrolysis is feasible because significant levels of activity were still retained. The optimum reaction time required for maximum substrate conversion by the RU-KM3$_S$ extract is similar to that reported for whole cells of this strain, *viz.*, 3 hours. This short reaction time would be advantageous if RU-KM3$_S$ were to be considered for industrial production of amino acids, because time consuming processes are generally expensive. The hydantoin-hydrolyzing enzymes of RU-KM3$_S$ retained most of their activity after incubation for 3 hours at 40°C, which means that the hydantoin hydrolyzing enzymes of RU-KM3$_S$ are relatively stable under the optimal reaction conditions, which are pH 8.0, and 3 hours at 40°C. These characteristics make this strain a suitable candidate as a biocatalyst for industrial production of amino acids.

The hydantoin-hydrolyzing enzymes of RU-KM3$_S$ apparently require metal ions for optimum activity. However, further investigations are required to confirm this. It is not yet clear which metal ions affect which enzyme, since effects on one enzyme inevitably result in some effect on the other. This problem would be overcome by purification of the enzymes, since this would enable one to study the effects of various metal ions on the individual enzymes. *N*-carbamylamino acids would have to be used as substrates for the purified *N*-carbamylase enzyme.

The hydantoinase of RU-KM3$_S$ was found to be non-stereoselective, whereas the *N*-carbamylase is apparently L-selective. Further investigations to confirm this would require the analysis of the reaction products to determine whether they are L- or D-isomers, possibly by chiral HPLC, which was not available at the time of this study. The L-selectivity of RU-KM3$_S$ makes it a potential biocatalyst for production of L-amino acids, for which there are very few industrial processes.

It has been shown in this investigation that RU-KM3$_S$ may be used as a biocatalyst for production of amino acids, in both resting cells and crude extract form. This, together with previous information that RU-KM1, RU-KM3$_L$, and RU-OR may also be used as biocatalysts in these forms, led to the investigation of the range of substrates that may be hydrolyzed by these strains, using both biocatalytic forms. This investigation is dealt with in the next chapter.

CHAPTER 3

Substrate selectivity of the hydantoin-hydrolyzing enzymes of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

3.1 INTRODUCTION

Hydantoinases are commercially valuable enzymes which can be used for the production of optically pure amino acids (Achary *et al.*, 1997; Syldatk *et al.*, 1997). The structure of the amino acid produced depends on the side chain of the hydantoin derivative used, i.e., the 5-substituent, and hydantoinases show varied substrate selectivity and stereoselectivity, depending on the source of the enzyme (Syldatk *et. al.*, 1990).

This chapter deals with the investigation of the substrate specificity of four previously isolated mesophilic hydantoinase-producing bacteria, *viz.*, RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR. This investigation was carried out on all four strains to compare their activity with that of RU-KM3$_S$, which had demonstrated superior hydantoinase activity, among the isolates, when hydantoin was used as a substrate (pers. comm., C. Hartley). The motivation behind this was that the other strains might have better activity with hydantoin derivatives with different side chains. Also, the hydantoinase activity of RU-KM3$_S$ with different substituted hydantoin substrates could be elucidated.

The investigation of the ability of these strains to hydrolyze various hydantoin substrates would elucidate which substrate was best converted by which strain, and thus which strain would be the most suitable candidate for production of a specific amino acid, depending on the preferred substrate. Thus, various 5-monosubstituted hydantoin substrates were synthesized for use as substrates.

The conditions used for the biocatalytic reactions of the four strains with the different substrates were based on the results discussed in Chapter 2 (for RU-KM3$_S$), and work done by others in our laboratory.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Hydantoin was purchased from Sigma-Aldrich. Acetaldehyde was purchased from Fluka Chemika, whereas the other aldehydes were purchased from Sigma-Aldrich. Ammonium carbonate was purchased from Merck N.T. Laboratory Supplies (Pty) Ltd. (S.A.) and potassium cyanide (KCN) was obtained from Saarchem (S.A.). For the synthesis of *p*-hydroxyphenylhydantoin glyoxylic acid was obtained from Sigma-Aldrich, and urea and phenol were purchased from Merck Laboratory Supplies (Pty) Ltd.

3.2.2 Synthesis of aliphatic 5-monosubstituted hydantoin substrates

The method employed was that of Bucherer-Bergs (Bucherer and Steiner, 1934). 90 mmoles of the appropriate aldehyde was dissolved in 100mL of 50% ethanol. This was followed by the addition of 18.2g (0.19 moles) of ammonium carbonate and 5.2g (79 mmoles) of KCN. The reaction mixture was then stirred under reflux at 60°C for 2 hours. The mixture was concentrated to two thirds volume, using a rotary evaporator (Bћchi Rotavapor, Labotech) at 60°C. The liquid was then cooled on ice, after which it was acidified to pH 2.0 with 50% HCl. The mixture was then frozen in liquid nitrogen and dried on a freeze drier.

The powder obtained after freeze-drying was dissolved in 100mL of absolute ethanol. The residue obtained was removed by filtration using a Buchner funnel and vacuum pump. Ethanol was removed from the filtrate by evaporation on a rotary evaporator at 60°C. The thick oil resulting from this step was left to crystallize at 4°C. The crystals were washed with diethyl ether in a Buchner funnel, and left to dry in a vacuum dessicator. Products were analyzed by ^{1H}NMR, using DMSO as a solvent. Spectra were recorded on a Bruker AMX spectrometer at 30°C.

3.2.3 Synthesis of *p*-hydroxyphenylhydantoin

The starting materials used for *p*-hydroxyphenylhydantoin (*p*-HPH) synthesis were water (16.88g, 0.94 mol), 32% hydrochloric acid (15.36g, 0.42 mol), phenol (4.29g, 46 mmol), and urea (5.55g, 92 mmol). These were mixed together in a round bottom flask and heated to 90°C, with constant stirring. Aqueous glyoxylic acid (6.01g, 81 mmol) was added drop-wise over 10 hours, while the temperature was maintained at 90°C. The reaction was carried out under reflux conditions. During the first few hours of the reaction, an off-white precipitate was formed in the reaction mixture. After about four hours, a dense white precipitate was observed. After the reaction time had lapsed, the reaction was left to cool to 4°C overnight. The precipitate (product) was filtered, washed with water, and left to dry in a vacuum dessicator. The product was analyzed by ^{1H}NMR, using DMSO as a solvent.

3.2.4 Culture conditions for RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

Seed cultures were prepared by inoculating the four bacterial strains (RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR) from minimal medium agar plates containing hydantoin as the sole nitrogen source into 100mL liquid minimal medium (Appendix 1.1) and incubating these at 28°C for 3- 4 days, in 500mL conical flasks. Aeration of the bacteria was achieved by placing the flasks on an orbital shaker at 200rpm. Bacterial growth was monitored by measuring the change in optical density (600nm), on a Shimadzu UV-Visible spectrophotometer every 24 hours. The seed cultures were used to inoculate nutrient broth supplemented with 0.1% hydantoin to give an initial cell optical density (600nm) of 0.02. These were incubated at 28°C, with shaking at 200rpm, until stationary phase was reached.

3.2.5 Resting cell reactions with hydantoin and 5-monosubstituted hydantoin substrates

Cells were harvested by centrifugation at 7000g for 10 minutes at 4°C, washed with 0.1M phosphate buffer (pH 7.0), and the cell pellet recovered by centrifugation at 8000g. The cell pellet was resuspended in the same buffer at a concentration of 0.1g cells/mL. Biocatalytic reaction mixtures were prepared with the hydantoin substrates at a final concentration of 25mM and 1mL of the cell suspension in a total volume of 2 .0mL. Due to the poor solubility of *p*-HPH, this substrate was prepared at a concentration of 30mM, and thus reaction mixtures contained 15mM of this substrate at the start of the reaction. Reactions were carried out for 3 hours with all bacterial strains except RU-OR, where the reaction time was 6 hours (pers. comm. C. Hartley), at 40°C, with shaking at 200rpm. Hydrolysis of the different substrates by the bacteria was monitored by analysis of the supernatant, using Ehrlich's reagent for *N*-carbamylamino acid detection, and ninhydrin reagent for amino acid detection (Chapter 2).

3.2.6. Hydrolysis of hydantoin and its derivatives by crude extracts of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

Cells were harvested as described above and a 0.1g/mL cell suspension was prepared. The crude extract was prepared by passing cells through a French-press at 15 MPa, at a rate of one drop/sec. This procedure was carried out at 4°C, to minimize inactivation of the enzymes. Biocatalytic reactions were conducted using 1.0mL of the respective crude extract preparations with substrates, at a final concentration of 25mM for the aliphatic substrates and 15mM for *p*-HPH, in a total volume of 2.0mL. Reaction conditions and product detection were as described above (Section 3.2.5).

3.3 RESULTS AND DISCUSSION

3.3.1 Synthesis of 5-monosubstituted hydantoin substrates

A selection of 5-monosubstituted hydantoins were synthesized by the Bucherer-Bergs method, for use as substrates in biocatalytic reactions. Table 3.3.1 shows the structures of the synthesized hydantoin derivatives and the amino acids resulting from their hydrolysis by the hydantoinase and *N*-carbamylase enzymes. After synthesis, the 5-monosubstituted hydantoin substrates were analyzed by ^{1H}NMR to confirm their structures. The results of this analysis are reported in Appendix 4.

Table 3.3.1 5-monosubstituted hydantoin substrates synthesized, and the amino acid products of hydrolysis by hydantoinase and *N*-carbamylase enzymes

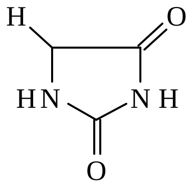
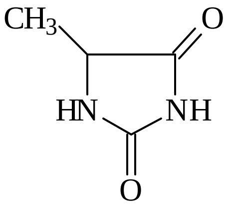
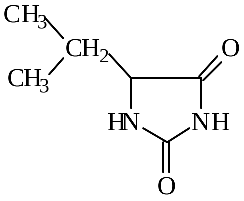
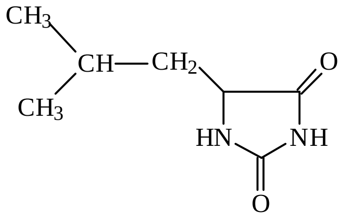
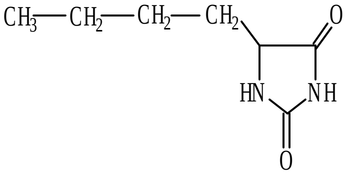
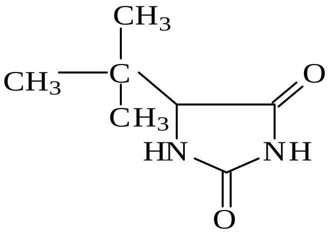
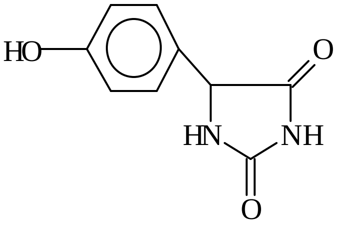
Chemical structure	Name	Yield (%)	Amino acid
	Hydantoin	Commercial	Glycine
	5-Methylhydantoin	21.5	Alanine
	5-Isopropylhydantoin	19.4	Valine

Table 3.3.1 (contd.) List of 5-monosubstituted hydantoin substrates and their amino acid products

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

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

3.3.2 Hydrolysis of hydantoin subtrates by resting cells and crude extracts of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

In order to investigate the substrate selectivity of the four strains, hydrolysis of various hydantoin substrates by these bacteria was investigated using 0.1g/mL solutions of either whole cells or crude extracts, with starting concentrations of 25mM for all substrates except *p*-HPH, for which the starting concentration was 15mM. 0.1g/mL suspensions were used as a means of comparison between cells and extracts with regard to biocatalytic efficiency. This, however, does not suggest that the specific enzyme activities are identical in cells and crude extracts.

3.3.2.1 Hydrolysis of hydantoin substrates by RU-KM1 whole cells and crude extracts

The hydrolysis of hydantoin and 5-monosubstituted hydantoin substrates by RU-KM1 whole cells and RU-KM1 crude extract was investigated. The results are shown in Table 3.3.2 and Table 3.3.3. The substrates are listed in order of decreasing total conversion (%).

Table 3.3.2 Hydrolysis of hydantoin substrates by RU-KM1 whole cells

Hydantoin substrate	**Amino acid**	**NCAA* (mM)**	**Amino acid (mM)**	**%NCAA converted***	**%Total conversion***
5-Isopropyl-	Valine	10.93	3.33	23.35	57.04
5-*n*-Butyl-	Norleucine	9.51	1.51	13.70	44.08
5-Methyl-	Alanine	7.64	0.63	7.62	33.08
5-*t*-Butyl-	*tert*-leucine	4.08	3.55	46.53	30.52
Hydantoin	Glycine	4.71	2.12	31.13	27.32
p-HPH	*p*-HPG*	2.51	0.92	26.82	22.87
5-Isobutyl-	Leucine	2.30	0.41	15.13	10.84

(The data represent the mean ("SEM) of triplicate determinations, standard errors #0.05)

Table 3.3.3 Hydrolysis of hydantoin substrates by RU-KM1 crude extract

Hydantoin substrate	**Amino acid**	**NCAA* (mM)**	**Amino acid (mM)**	**% NCAA converted***	**%Total conversion***
p-HPH	*p*-HPG*	6.71	1.17	14.8	52.53
5-Isopropyl-	Valine	10.39	1.33	11.35	45.56
5-*n*-Butyl-	Norleucine	9.50	0.11	1.14	38.44
5-Methyl-	Alanine	7.88	0.66	7.73	34.16
5-*t*-Butyl-	*tert*-Leucine	5.39	1.04	16.17	25.72
Hydantoin-	Glycine	3.41	2.22	39.43	22.52
5-Isobutyl-	Leucine	3.06	1.11	26.62	16.68

(The data represent the mean ("SEM) of triplicate determinations, standard errors #0.048)

Key: NCAA* = *N*-carbamylamino acid
p*-HPG = *p*-Hydroxyphenylglycine
% NCAA converted* = percentage of NCAA produced by hydantoinase that was converted by the *N*-carbamylase
Total conversion* = Total hydantoinase yield

Table 3.3.4 Comparison of total hydantoin substrate conversion by RU-KM1 whole cells and crude extract

Hydantoin substrate	**Total conversion (%)** **By whole cells**	**Total conversion (%)** **by the crude extract**
Hydantoin	27.32	22.52
5-Methyl-	33.08	34.16
5-Isopropyl-	57.04	45.56
5-*n*-Butyl-	44.08	38.44
5-Isobutyl-	10.84	16.68
5-*t*-Butyl-	30.52	25.72
p-HPH	22.87	52.53

RU-KM1 whole cells and crude extract demonstrated different substrate selectivity patterns. The preferred substrate for whole cells was 5 -isopropylhydantoin, with a total hydantoin conversion of 57.04%, whereas the preferred substrate for the crude extract was *p*-HPH, with a total conversion of 52.53%. However, the least hydrolyzed substrate in both cases was 5-isobutylhydantoin, with whole cells and crude extract achieving 10.84% and 16.68% conversion of this hydantoin, respectively. The results also indicated that, in general whole cells showed higher hydantoin hydrolyzing activity than the crude extract, with hydantoin, isopropylhydantoin, *n*-butylhydantoin, and *t*-butylhydantoin (Table 3.3.4). Hydantoinase and *N*-carbamylase enzymes have been suggested, in the literature, to consist of multi-subunits, and investigations in our laboratory have indicated that these enzymes could be membrane-associated. Thus, French-pressing could result in the disturbance of the conformational stability of the enzymes in RU-KM1.

Hydrolysis of methylhydantoin was achieved to similar extents by both whole cells and crude extract (33.08% and 34.16%, respectively). Of the seven hydantoin substrates tested, only two were hydrolyzed to a greater extent by the crude extract than by whole cells, *viz.*, isobutylhydantoin and *p*-hydroxyphenylhydantoin. The reason for this could be related to the relatively low solubility of these substrates, or alternatively, because of some impedance to transporting these substrates into the cells. Disruption of cell membranes could possibly have resulted in easier access of these substrates to the enzymes, and hence the higher activity with the crude extract.

Higher NCAA conversion yields were also observed with whole cells than with the crude extract. This is possibly also related to the disturbance of the conformational stability of the *N*-carbamylase enzyme by French-pressing. Whole cells and the crude extract of RU-KM1 achieved 31.13% and 39.43% conversion of *N*-carbamylglycine, respectively. *N*-carbamyl-*tert*-leucine was also hydrolyzed at high yield by RU-KM1 whole cells (46.53% conversion).

These results suggest that RU-KM1 whole cells would be a feasible biocatalyst for the production of various amino acids from 5-monosubstituted hydantoin substrates. This biocatalyst is potentially most suitable for production of glycine (from hydantoin), *tert*-leucine (from *t*-butylhydantoin), and valine (from 5-isopropylhydantoin). Glycine is used in industrial synthesis of peptides, *tert*-leucine is used in the synthesis of a number of antiviral, antitumor, and anti-inflammatory agents, whereas valine is used in the production of Fluvalinate, an insecticide (Drauz *et al.*, 1997).

3.3.2.2 Hydrolysis of hydantoin substrates by RU-KM3$_L$ whole cells and crude extract

The results of the investigation of the substrate selectivity of RU-KM3$_L$ whole cells and crude extract are reported in the tables below. The hydantoin substrates are listed in the order of decreasing total conversion in Tables 3.3.5 and 3.3.6.

Table 3.3.5 Hydrolysis of hydantoin substrates by RU-KM3$_L$ whole cells

Hydantoin substrate	**Amino acid**	**NCAA* (mM)**	**Amino acid (mM)**	**%NCAA converted***	**% Total conversion***
5-Isobutyl-	Leucine	12.32	0.00	0.00	49.28
5-*t*-Butyl-	*tert*-leucine	6.95	1.73	19.9	34.72
Hydantoin	Glycine	2.28	2.49	52.2	19.08
p-HPH-	*p*-HPG*	1.07	1.60	59.9	17.8
5-Methyl-	Alanine	3.93	0.42	9.66	17.4
5-Isopropyl-	Valine	3.06	0.60	16.39	14.64
5-*n*-Butyl-	Norleucine	2.12	0.085	3.85	12.48

(The data represent the mean of triplicate determinations, standard errors #0.05)

Key: NCAA* = *N*-carbamylamino acid
p-HPG* = *p*-Hydroxyphenylglycine
% NCAA converted* = percentage of NCAA produced by hydantoinase that was converted by the *N*-carbamylase
Total conversion* = Total hydantoinase yield

Table 3.3.6 Hydrolysis of hydantoin substrates by RU-KM3$_L$ crude extracts

Hydantoin substrate	**Amino acid**	**NCAA* (mM)**	**Amino acid (mM)**	**%NCAA converted***	**% Total conversion***
5-*t*-Butyl-	*tert*-leucine	12.26	0.56	4.37	51.28
5-Methyl-	Alanine	9.37	0.24	2.50	38.44
Hydantoin	Glycine	5.87	0.98	14.31	27.4
5-Isopropyl-	Valine	3.58	1.68	31.94	21.04
5-Isobutyl-	Leucine	4.70	0.00	0.00	18.8
p-HPH-	*p*-HPG*	2.42	0.36	5.23	18.53
5-*n*-Butyl-	Norleucine	2.64	0.89	25.21	14.12

(The data represent the mean ("SEM) of triplicate determinations, standard errors #0.078)

Key: NCAA* = *N*-carbamylamino acid
p-HPG* = *p*-Hydroxyphenylglycine
% NCAA converted* = percentage of NCAA produced by hydantoinase that was converted by the *N*-carbamylase
Total conversion* = Total hydantoinase yield

Table 3.3.7 Comparison of total hydantoin substrate conversion by RU-KM3$_L$ whole cells and crude extract

Hydantoin substrate	**Total conversion (%) By whole cells**	**Total conversion (%) by the crude extract**
Hydantoin	19.08	27.4
5-Methyl-	17.4	27.4
5-Isopropyl-	14.64	38.44
5-*n*-Butyl-	12.48	14.42
5-Isobutyl-	49.28	18.8
5-*t*-Butyl-	34.72	51.28
p-HPH	17.8	18.53

RU-KM3_L whole cells and crude extract demonstrated different substrate selectivity patterns (Tables 3.3.5 and 3.3.6). Whole cells preferentially hydrolyzed 5-isobutylhydantoin (49.28% conversion) over the other hydantoin substrates tested, whereas the crude extract preferentially hydrolyzed 5-*t*-butylhydantoin (51.28% conversion). However, the least hydrolyzed substrate in both cases was 5-*n*-butylhydantoin, with whole cells achieving 12.48% conversion and the crude extract achieving 14.12% conversion of this hydantoin. The results showed that RU-KM3_L crude extract demonstrated higher total hydantoin-hydrolyzing activity than whole cells (Table 3.3.7). For six of the substrates tested, RU-KM3_L crude extract gave higher total conversion than whole cells. The exception was 5-isobutylhydantoin. This finding suggests that French-pressing, and thus cell membrane disruption, increased availability of the enzymes to the substrates, hence the higher activity. Thus it may be suggested that the lower activity observed with whole cells might be due to difficulties in transportation of the substrates into cells, and this was overcome by the disruption of cell membranes.

The *N*-carbamylase enzyme, on the other hand, demonstrated higher activity in intact cells than in the crude extract (% NCAA converted). In intact cells, the hydantoin-hydrolyzing enzymes are possibly situated such that the *N*-carbamylase enzyme can gain easy access to the *N*-carbamyl-intermediate, produced by the hydantoinase. The lower activity observed with the crude extract could thus possibly be due to separation of the enzyme from its substrate, caused by French-pressing. The highest NCAA conversion was observed with *N*-carbamylhydroxyphenylglycine (59.9%), with whole cells, whereas with the crude extract it was observed with *N*-carbamylvaline (31.94%).

Higher conversion yields were observed with 5-isobutylhydantoin, in the case of whole cells, and with 5-*t*-butylhydantoin, in the case of crude extracts, suggesting that RU-KM3$_L$ would be a feasible biocatalyst for the production of leucine and *tert*-leucine, which are used for the industrial production of an immunostimulant (Bestain) and antiviral and antitumor agents, respectively.

3.3.2.3 Hydrolysis of hydantoin substrates by RU-KM3$_S$ whole cells and crude extract

The results of the investigation of the substrate selectivity of RU-KM3$_S$ are illustrated in the tables below. The substrates are listed in the order of decreasing total conversion in Tables 3.3.8 and 3.3.9.

Table 3.3.8 Hydrolysis of hydantoin substrates by RU-KM3$_S$ whole cells

Hydantoin substrate	**Amino acid**	**NCAA* (mM)**	**Amino acid (mM)**	**% NCAA converted***	**% Total conversion***
5-*n*-Butyl-	Norleucine	13.22	1.10	7.68	57.28
5-Isobutyl-	Leucine	12.27	1.23	9.11	54.0
5-Isopropyl-	Valine	10.76	1.07	9.04	47.32
5-Methyl-	Alanine	7.59	1.19	13.55	35.12
Hydantoin	Glycine	3.84	2.62	40.56	25.84
p-HPH-	*p*-HPG*	3.00	0.40	11.76	22.67
5-*t*-Butyl-	*tert*-Leucine	2.08	0.20	8.77	9.12

(The data represent the mean of triplicate determinations, standard errors #0.046)

Key: NCAA* = *N*-carbamylamino acid
p*-HPG = *p*-Hydroxyphenylglycine
% NCAA converted* = percentage of NCAA produced by hydantoinase that was converted by the *N*-carbamylase
Total conversion* = Total hydantoinase yield

Table 3.3.9 Hydrolysis of hydantoin substrates by RU-KM3$_S$ crude extracts

Hydantoin substrate	**Amino acid**	**NCAA* (mM)**	**Amino acid (mM)**	**% NCAA converted***	**% Total conversion***
p-HPH-	*p*-HPG*	14.13	1.57	1.57	100
5-*n*-Butyl-	Norleucine	16.51	1.39	7.77	71.6
5-*t*-Butyl-	*tert*-Leucine	8.90	1.61	15.32	42.04
5-Methyl-	Alanine	8.97	0.54	5.68	38.04
Hydantoin	Glycine	6.39	1.42	18.18	31.24
5-Isopropyl-	Valine	4.54	1.43	23.95	23.88
5-Isobutyl-	Leucine	3.58	0.00	0.00	14.32

(The data represent the mean of triplicate determinations, standard errors #0.064)

Key: NCAA* = *N*-carbamylamino acid
p*-HPG = *p*-Hydroxyphenylglycine
% NCAA converted* = percentage of NCAA produced by hydantoinase that was converted by the *N*-carbamylase
Total conversion* = Total hydantoinase yield

Table 3.3.10 Comparison of total substrate conversion by RU-KM3$_S$ whole cells and crude extract

Hydantoin substrate	**Total conversion (%) By whole cells**	**Total conversion (%) By the crude extract**
Hydantoin	25.84	31.24
5-Methyl-	35.12	38.04
5-Isopropyl-	47.32	23.88
5-*n*-Butyl-	57.28	71.6
5-Isobutyl-	54.0	14.32
5-*t*-Butyl-	9.12	42.04
p-HPH	22.67	100.0

RU-KM3$_S$ whole cells showed a different substrate selectivity pattern from that observed with the crude extract. *n*-Butylhydantoin was the preferred substrate for whole cells, whereas for the crude extract, the preferred substrate was *p*-hydroxyphenylhydantoin. Whole cells and crude extract of RU-KM3$_S$ achieved 57.28% and 71.6% conversion of *n*-butylhydantoin, respectively, which was the most hydrolyzed aliphatic hydantoin in both cases. 5-Isobutylhydantoin was the substrate least hydrolyzed by the crude extract (14.32% conversion), whereas the second highest conversion of substrate (54%) was achieved with this substrate in the case of whole cells.

The RU-KM3$_S$ crude extract demonstrated higher activity than whole cells with 5 of the substrates tested, *viz.*, hydantoin, *n*-butyl-, *t*-butyl-, methyl-, and *p*-HPH (Table 3.3.10). These results suggest that the RU-KM3$_S$ crude extract would possibly be a better biocatalyst than whole cells for the production of glycine, norleucine, *tert*-leucine, alanine, and *p*-hydroxyphenylglycine, which are derived from these hydantoins. The highest activity of the *N*-carbamylase enzyme was demonstrated with *N*-carbamylglycine in whole cells (40.56% conversion), and with *N*-carbamylvaline in the crude extract (23.88%). Glycine finds industrial application in peptide synthesis, whereas *tert*-leucine is used in the synthesis of a number of antiviral, antitumor and anti-inflammatory agents (Drauz, 1997).

An interesting observation made in these studies was the difference in the substrate selectivity patterns of RU-KM3$_L$ and RU-KM3$_S$, which are both *Pseudomonas putida* strains. The preferred substrate for RU-KM3$_L$ whole cells was 5-isobutylhydantoin, whereas for RU-KM3$_S$ whole cells the preferred subsrate was *n*-butylhydantoin. RU-KM3$_L$ crude extract preferentially hydrolyzed *t*-butylhydantoin, whereas RU-KM3$_S$ crude extract preferentially hydrolyzed *p*-HPH and *n*-

butylhydantoin. However, the two showed similarity by achieving higher conversion rates in crude extract form than whole cells. These findings support earlier findings in our laboratory that these strains are different.

3.3.2.4 Hydrolysis of hydantoin substrates by RU-OR whole cells and crude extract

The results of the investigation of the substrate selectivity of RU-OR are illustrated in the following tables. The substrates are listed in the order of decreasing total conversion, in Tables 3.3.11 and 3.3.12.

Table 3.3.11 Hydrolysis of hydantoin substrates by RU-OR whole cells

Hydantoin substrate	**Amino acid**	**NCAA* (Mm)**	**Amino acid (mM)**	**% NCAA converted***	**% Total conversion***
5-Methyl-	Alanine	9.70	2.67	21.58	49.48
5-Isopropyl-	Valine	9.55	1.43	13.02	43.92
5-Isobutyl-	Leucine	7.30	1.46	15.98	35.04
5-*t*-Butyl-	*tert*-Leucine	6.76	0.50	6.89	29.04
5-*n*-Butyl-	Norleucine	4.47	0.22	4.69	18.76
p-HPH-	*p*-HPG*	1.46	0.79	35.11	15.00
Hydantoin	Glycine	0.81	1.69	67.6	10.00

(The data represent the mean of triplicate determinations, standard errors #0.030)

Key: NCAA* = *N*-carbamylamino acid
p-HPG* = *p*-Hydroxyphenylglycine
% NCAA converted* = percentage of NCAA produced by hydantoinase that was converted by the *N*-carbamylase
Total conversion* = Total hydantoinase yield

Table 3.3.12 Hydrolysis of hydantoin substrates by RU-OR crude extract

Hydantoin substrate	**Amino acid**	**NCAA* (mM)**	**Amino acid (mM)**	**% NCAA converted***	**% Total converted***
5-Isopropyl-	Valine	11.36	1.51	11.73	51.48
p-HPH-	*p*-HPG*	6.28	0.97	13.38	48.33
5-Isobutyl-	Leucine	7.60	3.02	28.44	42.48
5-Methyl-	Alanine	6.76	2.74	28.84	38.00
5-*t*-Butyl-	*tert*-Leucine	9.13	0.36	3.79	37.96
Hydantoin	Glycine	2.77	3.12	52.97	23.56
5-*n*-Butyl-	Norleucine	4.19	0.14	3.23	17.32

(The data represent the mean of triplicate determinations, standard errors #0.018)

Key: NCAA* = *N*-carbamylamino acid
p*-HPG = *p*-Hydroxyphenylglycine
% NCAA converted* = percentage of NCAA produced by hydantoinase that was converted by the *N*-carbamylase
Total conversion* = Total hydantoinase yield

Table 3.3.13 Comparison of total substrate conversion by RU-OR whole cells and crude extract

Hydantoin substrate	**Total conversion (%) By whole cells**	**Total conversion (%) By the crude extract**
Hydantoin	10.0	23.56
5-Methyl-	49.48	38.0
5-Isopropyl-	43.92	51.48
5-*n*-Butyl-	18.76	17.32
5-Isobutyl-	35.04	42.48
5-*t*-Butyl-	29.04	37.96
p-HPH	15.0	48.33

RU-OR whole cells showed a different substrate selectivity pattern from that observed with the crude extract (Tables 3.3.11 and 3.3.12). The preferred substrate for whole cells was 5-methylhydantoin, with 49.48% conversion achieved. However, the preferred substrate for the crude extract was 5-isopropylhydantoin, with 51.48% conversion. RU-OR crude extract hydrolyzed the aromatic 5-substituted hydantoin, *p*-HPH to a greater extent than whole cells. Conversion rates were 48.33% and 15.0%, respectively. This is indicative of the disruption of cell membranes resulting in the improvement of conversion rates of substrates. This is further confirmed by the observation that the crude extract achieved higher conversion rates than whole cells with 5 of the tested substrates, namely hydantoin, 5-methyl-, 5-isobutyl-, 5-*t*-butyl, and *p*-HPH (Table 3.3.13). 5-*n*-Butylhydantoin was hydrolyzed to similar extents by both whole cells and crude extract, with conversion rates of 18.76% and 17.32%, respectively. The lower activity observed with whole cells is possibly due to limited transportation of substrates into cells, which might have been overcome by disruption of cell membranes, and hence higher activity was observed with the crude extract.

Since appreciable levels of hydrolysis of 5-isopropyl-, *p*-hydroxyphenyl-, and 5-isobutylhydantoin were achieved by RU-OR crude extract, it may be suggested that this biocatalyst has great potential for application in the production of the amino acids produced from the enzymatic hydrolysis of these hydantoin substrates. These amino acids are valine (from isopropylhydantoin), *p*-hydroxyphenylglycine (from *p*- HPH), and leucine (from isobutylhydantoin). These amino acids have valuable industrial applications. Valine is used in the production of Fluvalinate, a pyrethoid insecticide, and Valaciclovir, which is a reverse transcriptase inhibitor. *p*-HPH is used in the synthesis of semi-synthetic antibiotics such as amoxycillin, whereas leucine is used in the synthesis of an immunostimulant, Bestain (Drauz, 1997). RU-OR whole cells, on the other hand, achieved high levels of methyl- and isopropylhydantoin conversion, and thus have potential application in the synthesis of alanine and valine. Alanine is used in the synthesis of enalapril, which is an ACE inhibitor.

3.4 CONCLUSIONS

5-Monosubstituted hydantoin substrates were synthesized successfully by the method of Bucherer-Bergs, and the resulting products were demonstrated to be sufficiently pure for the purpose of this study, i.e. hydrolysis by hydantoin-hydrolyzing enzymes. The synthesized substrates were subsequently hydrolyzed by the hydantoinase-producing bacteria investigated in this study, *viz.* RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR. All four strains hydrolyzed the seven substrates tested, thus demonstrating relaxed substrate selectivity. This is an advantageous feature to have in a biocatalyst, in that it means they may be applied to the synthesis of a number of commercially important amino acids. The highest conversion of substrates was observed with RU-KM3$_S$, confirming the superiority of its hydantoin-degrading enzymes over the other strains dealt with in this study.

The biocatalytic reaction conditions for amino acid production by these hydantoinase-producing bacteria could still be optimized further. One of the ways in which this could be attempted is by determining the effects of culturing the bacteria in minimal media containing the different hydantoin derivatives as the sole nitrogen source. Factors such as biomass production and induction of enzyme activity should be investigated. It would also be beneficial to determine whether induction with a specific hydantoin derivative resulted in higher conversion of that substrate in subsequent biocatalytic reactions. The reason for the difference in activity between whole cells and crude extracts also needs to be determined. It has been suggested in this study that French-pressing, in some cases, achieves an increase in enzyme activity by facilitating transport of substrates to the enzymes by disrupting cell membranes, but this needs further investigation. The effect of French-pressing on the enzyme conformation also needs to be elucidated. These investigations may be conducted by purifying the enzymes.

Our laboratory has subsequently been granted an Innovation Fund grant to develop the production of amino acids (or hydantoin-degrading enzymes) by these bacterial strains to commercialization, largely on the basis of the results of this investigation.

The possibility of using these bacteria for production of other commercially valuable compounds, in addition to amino acids, was also investigated, and this is dealt with in the next chapter.

CHAPTER 4

Investigation of the nitrile-hydrolyzing activity of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$

4.1 INTRODUCTION

The major objective of this study was the development of biocatalytic processes for production of amino acids. Previous chapters have dealt with the use of hydantoins as starting substrates for amino acid production. This chapter deals with the investigation of the possibility of using alternative starting substrates, namely nitriles.

Organonitriles are versatile intermediates in organic synthesis, as they can be prepared relatively easily, and they can be transformed into a variety of commercially useful compounds (Beard, *et. al.*, 1993). Amino nitriles may be chemically or enzymatically tranformed to amino acids. Hence, the ability of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$, which are microorganisms that have the ability to produce amino acids from hydantoins, to hydrolyze nitriles, was investigated. The aim of this investigation was to ascertain that the best enzyme system in these bacteria was utilized for amino acid production. The study of nitrile hydrolysis in these bacteria could provide a comparison as to whether utilization of the hydantoinase system was suitable for amino acid production.

The simplest functional group change that a nitrile can undergo, i.e. hydrolysis to an amide or carboxylic acid, is often difficult to achieve chemically due to the harshness of the conditions required (either strongly acidic or strongly alkaline). Another disadvantage of the chemical hydrolysis of nitriles is that racemic products are obtained. For example, the chemical hydrolysis of "-aminonitriles yields racemic amino acids (Bhalla *et. al.*, 1992). It has also been reported that during the chemical hydrolysis of nitriles, especially in larger scale transformations, large quantities of inorganic salts, which result in unfavourable ecological consequences, are always produced as byproducts (Hönicke - Schmidt and Schneider, 1990).

Enzymatic hydrolysis of nitriles has several advantages over chemical syntheses for some products (Eyal and Charles, 1990). The main advantage is that milder reaction conditions, *viz.*, lower temperature and neutral pH, are required for enzymatic hydration (Hönicke - Schmidt and Schneider, 1990). The ability of enzymes to catalyze nitrile hydrolysis is well documented in the literature, but considerably under-exploited (Hönicke - Schmidt and Schneider, 1990; Beard, *et. al.*, 1993). The hydration of nitriles in plants and microorganisms proceeds via two distinct enzymatic pathways (Amarant, *et. al.*, 1989; Nagasawa and Yamada, 1990; Almatawah *et. al.*, 1999). One pathway is the direct hydrolysis of a nitrile to a carboxylic acid, which is catalyzed by a nitrilase enzyme (Figure 4.1.1). The second pathway is a two step process in which the nitrile is first converted to an amide, by a nitrile hydratase, and subsequently to a carboxylic acid by an amidase (Figure 4.1.2).

Figure 4.1.1 Hydrolysis of nitriles to carboxylic acids by nitrilase

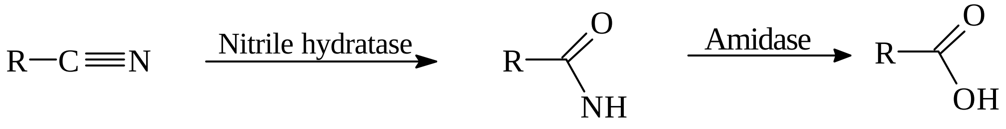


Figure 4.1.2 Hydrolysis of nitriles catalyzed by nitrile hydratase in combination with amidase

One of the major industrial applications of a nitrile hydrolyzing microorganism is the production of acrylamide from acrylonitrile (Nagasawa *et. al*., 1990). Enzymatic nitrile hydrolysis has also been applied to the production of 2-arylpropionic acids, which are important non-steroidal anti-inflammatory drugs, e.g., *S*-naproxen from racemic naproxen nitrile (Layh *et. al*., 1994).

4.2 MATERIALS AND METHODS

The bacterial strains used were RU-KM1, RU-KM3$_L$, and RU-KM3$_S$, which had all been previously selected for their ability to hydrolyze 5-monosubstituted hydantoins.

4.2.1 Chemicals

Acrylonitrile, acrylamide, acrylic acid, propionamide, propionic acid, and benzonitrile were all purchased from Sigma-Aldrich Chemical Co. Propionitrile was purchased from Fluka Chemika. Acetonitrile was purchased from BDH Laboratory Supplies (S.A.), and acetamide was purchased from Sigma-Aldrich Chemical Co. For product detection, sodium nitroprusside and potassium hydroxide were purchased from Saarchem (Pty) Ltd. (S.A.), and sodium hypochlorite was purchased from Associated Chemical Enterprises (S.A.). Phenol was obtained from NT Laboratory Supplies (S.A.).

4.2.2 Growth of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ in acrylonitrile minimal medium

The microorganisms, initially cultured in hydantoin minimal medium, were each inoculated into 100mL minimal medium containing acrylonitrile as the sole nitrogen source, with glucose as the carbon source. Acrylonitrile was added to a final concentration of 1% (v/v) (Appendix 1.2). The flasks were incubated at 28°C, with shaking at 200rpm. Growth was monitored by measuring the change in optical density at 600nm, every 24 hours.

4.2.3 Growth of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ on other nitriles

The ability of the four bacterial strains to utilize other nitriles as their sole source of nitrogen was also investigated. Minimal media were prepared as before (Appendix 1.2), except that different nitriles were added in place of acrylonitrile, at a final concentration of 10mM in each case. The strains were inoculated into 50mL of broth in 250mL conical flasks and incubated at 28°C, with shaking at 200rpm. Growth was monitored by observing the change in optical density at 600nm, every 24 hours.

4.2.4 Growth in nutrient broth supplemented with acrylonitrile

The bacteria were first cultured in hydantoin minimal medium until stationary phase was reached, giving seed cultures, which were subsequently used to inoculate 400mL nutrient broth (Biolab) supplemented with 0.1% (v/v) acrylonitrile (in 2L conical flasks) as an inducer of nitrile-hydrolyzing activity. The inoculum used was such that the starting OD_{600} in the nutrient broth was 0.02. The cultures were incubated at 28°C with shaking at 200rpm. This experiment was also carried out using nutrient broth supplemented with 0.01% acrylonitrile as an inducer.

4.2.5 Resting cell reactions with nitriles

Cells were cultured in 400mL nutrient broth supplemented with 0.01% acrylonitrile by centrifugation at 7000g for 10 minutes. The pellet thus obtained was resuspended in half the initial culture volume (200mL) of cold 0.1M potassium phosphate buffer, pH 7.0, and the suspension was centrifuged at 8000g for 10 minutes. The cell pellets were subsequently resuspended in 0.1M potassium phosphate buffer, pH 7.0, to give a 0.1g cells/mL cell suspension.

For the biocatalytic reactions, 1mL of the respective cell suspension was added to 1mL of a 50mM solution of the nitrile substrate. Thus the final cell concentration in each reaction vessel was 50mg/mL, and the final substrate concentration was 25mM. Each reaction was carried out in triplicate. The following controls were also prepared: 1mL cell suspension + 1mL buffer; and 1mL substrate solution + 1mL buffer. The reaction mixtures and controls were all maintained at 30°C for 1.5 hours, with shaking at 200rpm. After the reaction time had lapsed, the samples were transferred to 1.5mL eppendorf tubes and microfuged at 13 000g for 5 minutes. The supernatants were then transferred to clean eppendorf tubes, and the concentration of ammonia produced from nitrile hydrolysis was determined colorimetrically, using the phenol-hypochlorite method.

4.2.6 Detection of ammonia produced from nitrile hydrolysis by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$

A colorimetric assay was used to detect the release of ammonia during nitrile hydrolysis, *viz.*, the phenol-hypochlorite assay (Maestracci *et al.*, 1988; Pereira *et al*, 1998). The substrates used for resting cell reactions were acetonitrile, acrylonitrile, benzonitrile, and propionitrile. The cells were cultured in nutrient broth supplemented with 0.01% acetonitrile as an inducer, and harvested in the exponential phase of growth by centrifugation at 7000g for 10 minutes. The cells were then washed with 0.1M phosphate buffer, pH 7.0, after which they were centrifuged for another 10 minutes at 8000g. The pellets were resuspended in the same buffer to get a final cell concentration of 0.1g/ml. Biocatalytic reaction mixtures consisting of 1mL of the respective cell suspension plus 1mL of the respective substrate solution (50mM, giving 25mM concentration in the reaction) were prepared and incubated at 30°C for 3 hours, with shaking at 200rpm.

4.2.6.1 Phenol-hypochlorite protocol 1 (Maestracci *et al.*, 1988)

After the biocatalytic reaction time had lapsed, samples were microfuged at 13 000g for 5 minutes. A 1mL aliquot of each of the supernatants thus obtained was transferred to small test tubes. 0.5mL of solution 1 (Appendix 2.3) was added to the supernatants, which were then vortexed to mix the contents. 0.1mL of solution 2 (Appendix 2.3) was added to each of the test tubes, which were then left to stand for 5 minutes at room temperature, for colour development. The absorbance of the solutions was measured at 600nm on an UV-visible spectrophotometer. The concentration of ammonia produced from nitrile hydrolysis was determined from a standard curve prepared using ammonium sulfate.

4.2.6.2 Phenol-hypochlorite protocol 2 (Pereira *et al.*, 1998)

At the end of the biocatalytic reaction time, the samples were microfuged at 13 000g for 5 minutes. A 100µL aliquot of the supernatant was transferred into 1.5mL eppendorf tubes, and 400µL of Solution A (0.59M phenol and 1mM sodium nitroprusside) was added. After mixing, 400µL of Solution B (0.11M sodium hypochlorite and 2.0M sodium hydroxide) was added. The mixtures were left to stand for 30 minutes at room temperature, after which the absorbance was measured at 600nm. The concentration of the ammonia produced was determined from a standard curve prepared using ammonium sulfate (Appendix 3.3).

4.2.7 Determination of inducer dependence for nitrile hydrolysis by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$

The 4 strains were each inoculated into 200mL nutrient broth supplemented with 0.01% acetonitrile (inducer), and into nutrient broth without acetonitrile. The cultures were incubated at 28°C. Cells were harvested as before and resting cell reactions were conducted with 25mM acrylonitrile as the substrate, at 30°C for 3 hours. The reaction mixtures were microfuged at 13 000g for 5 minutes, and the amount of ammonia released into the supernatant was quantified using the phenol-hypochlorite method (Section 4.2.6.2).

4.2.8 Investigation of the substrate selectivity of the nitrile-hydrolyzing enzymes of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$

The cells were cultured in 400mL each of nutrient broth supplemented with 0.01% acetonitrile and incubated at 28°C until the log-phase of growth. They were then harvested as before and resting cell reactions consisting of 1mL of a 0.1g/mL cell suspension plus 1mL of a 50mM substrate solution (acetonitrile, acrylonitrile, propionitrile, and benzonitrile) were conducted at 30°C for 3 hours. The reaction mixtures were microfuged at 13 000g for 5 minutes, and the amount of ammonia produced in the supernatants, from hydrolysis of the different substrates, was determined using the phenol-hypochlorite method.

4.2.9 The effect of urea on nitrile hydrolysis by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$

The strains were cultured in nutrient broth supplemented with 0.01% acetonitrile (v/v) and harvested as before. One set of reaction mixtures consisted of 1mL of a 0.1g/mL cell suspension (of each strain) plus 1mL of 50mM acrylonitrile. The second set consisted of 1mL cell suspension prepared in 50mM urea, and 1mL of 50mM acrylonitrile. Thus, the total biocatalytic reaction volume was 2mL. The reactions were carried out at 30°C for 3 hours, with shaking at 200rpm. The reaction mixtures were microfuged at 13 000g for 5 minutes, and the amount of ammonia produced quantified in the supernatant using the phenol-hypochlorite method Section 4.2.6.2).

4.3 RESULTS AND DISCUSSION

4.3.1 Growth of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ in nitrile minimal media

The ability of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ to grow in defined media containing acrylonitrile as the sole nitrogen source was investigated. The results are illustrated in Figure 4.3.1. No growth was observed with RU-OR, and no activity was observed in preliminary experiments. Thus, no further investigations were conducted on this strain. The difference in nitrile-hydrolyzing activity between RU-OR and the other strains is expected, as RU-OR is an *Agrobacterium* species, whereas the other three strains are *Pseudomonas* species.

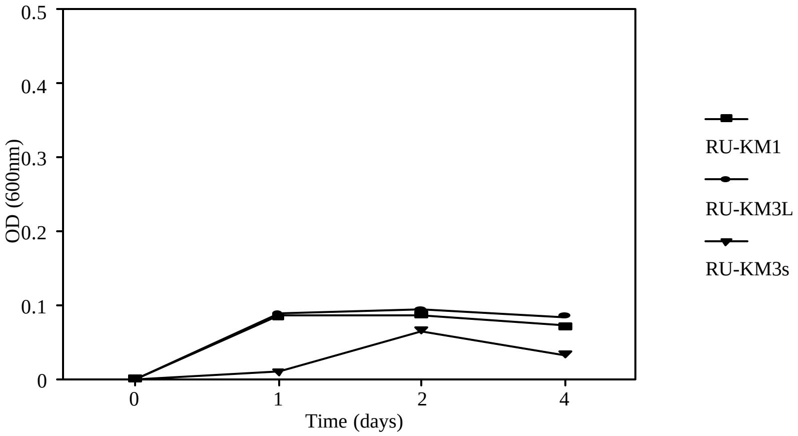


Figure 4.3.1 Growth of strains RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ in acrylonitrile minimal medium

The results of this experiment showed that none of the three strains were able to utilize acrylonitrile, at a concentration of 1% (v/v), as a sole nitrogen source, since none of them were able to reach optical densities greater than 0.1, even after 4 days of incubation. However, since there was an initial increase in the optical densities of the RU-KM1 and RU-KM3$_L$ cultures after 1 day of incubation, and after two days of incubation in the case of RU-KM3$_S$, it was suggested that these bacteria possibly did have nitrile-degrading ability. The hindrance to further growth might be the high concentration of acrylonitrile used, since nitriles are highly toxic compounds. Hence it was suggested that a lower nitrile concentration be tested, and acetonitrile was chosen due to its simple structure. The results of this investigation are illustrated below.

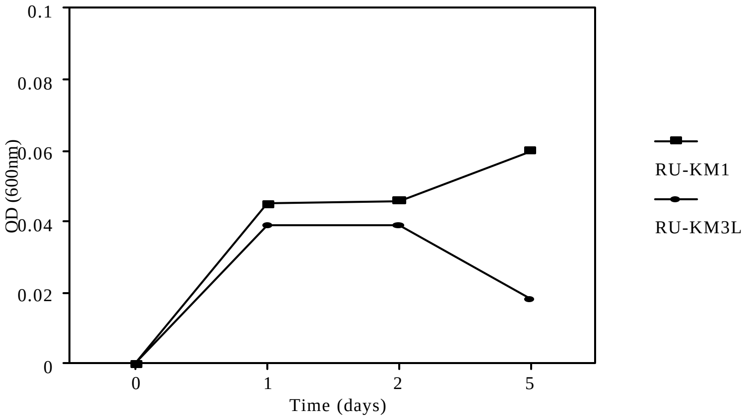


Figure 4.3.2 Growth of RU-KM1 and RU-KM3$_L$ in minimal medium with 10mM acetonitrile as the sole nitrogen source

The results showed poor utilization of acetonitrile as the sole source of nitrogen by the bacterial strains, since optical densities did not reach 0.1 even after 5 days of incubation. Propionitrile was tested as an alternative nitrogen source, and the results of this experiment are shown in Fig. 4.3.3.

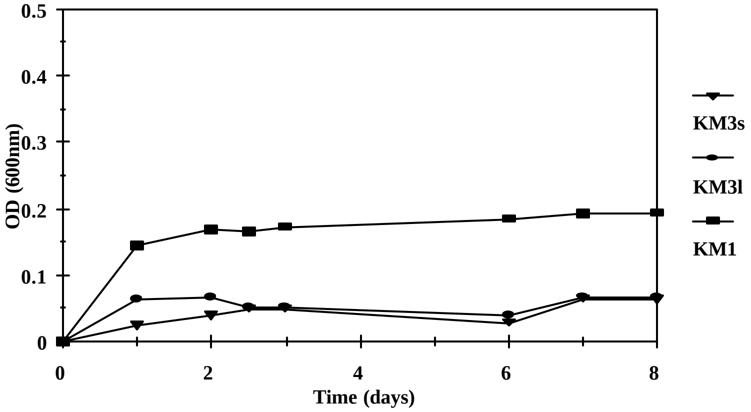


Figure 4.3.3 Growth of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ in propionitrile minimal medium

RU-KM1 was able to reach an optical density of 0.2 after 2 days of incubation, but no further increase was observed. RU-KM3$_L$ and RU-KM3$_S$, on the other hand, were unable to grow in this medium, since their respective optical densities did not even reach 0.1. Due to the poor utilization of the aliphatic nitriles tested by these strains, it was suggested that the utilization of an aromatic nitrile, benzonitrile, be tested for its ability to support growth of these strains. The results are shown below.

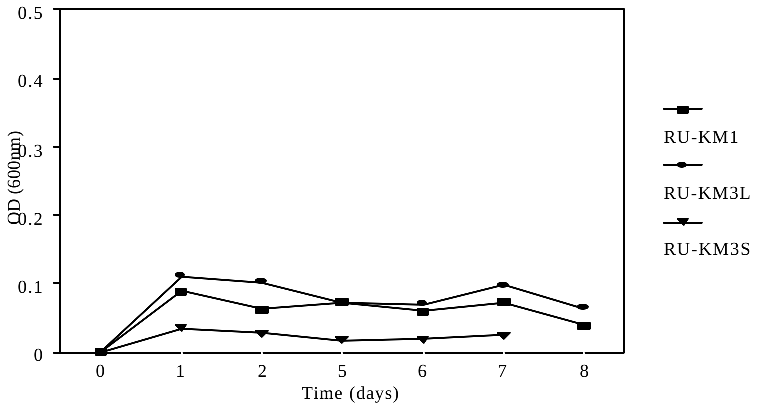


Figure 4.3.4 Growth of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ in benzonitrile minimal medium

The results showed that benzonitrile was a poor source of nitrogen for these strains, as they were unable to grow in minimal medium containing this nitrile as the sole sole of nitrogen. None of the nitriles tested were good sources of nitrogen. It was suggested that the ability of these nitriles to grow in nutrient broth supplemented with nitrile (acrylonitrile) be examined, as this would give an indication of the ability of the strains to grow in the presence of nitrile compounds. Growth under these conditions could mean that the strains do possess enzymes that are able to break down these compounds, at least to non-toxic levels.

4.3.2 Growth of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ in nutrient broth with acrylonitrile

The ability of the three hydantoinase-producing bacteria to grow in nutrient broth supplemented with 0.1% acrylonitrile was investigated. The results showed that these bacteria were unable to grow in the presence of 0.1% acrylonitrile. For subsequent experiments nutrient broth supplemented with 0.01% acrylonitrile was used.

4.3.3 Detection of ammonia produced from nitrile hydrolysis by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$, using protocol 1 (Maestracci *et al.*, 1988)

The hydrolysis of acrylonitrile and propionitrile by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ was investigated. Production of ammonia from nitrile hydrolysis was determined using protocol 1. The results are shown in Table 4.3.1.

Table 4.3.1 Production of ammonia from acrylonitrile by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$, as determined by protocol 1 (Maestracci et al., 1988)

Strain	Ammonia (mM)	% Conversion
RU-KM1	3.60"0.02	14.4
RU-KM3$_L$	2.59"0.01	10.36
RU-KM3$_S$	3.02"0.02	12.08

The results showed very low ammonia production from acrylonitrile by all 3 strains. The low nitrile hydrolyzing activity could be related to one of two factors. Firstly, a precipitate was observed in the assay mixture, after addition of reagents, possibly carbonate which was used in the preparation of one of the reagents. This could have interfered with the accurate quantification of ammonia. Secondly, definite colour development was observed with the naked eye, but the spectrophotometer readings did not correspond with the colour change observed. Hence, the possible use of a modified version of this method was investigated.

4.3.4 Detection of ammonia produced from nitrile hydrolysis by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$, using protocol 2 (Pereira *et al.* 1998)

This protocol was tested due to problems encountered with the first protocol. The results of this experiment are shown in Table 4.3.2.

Table 4.3.2 Production of ammonia from 25mM acrylonitrile by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$, as determined by protocol 2

Strain	Ammonia (mM)	% Conversion
RU-KM1	14.44±0.08	57.76
RU-KM3$_L$	5.42±0.14	21.68
RU-KM3$_S$	14.42±0.17	57.68

The amount of ammonia produced when detection protocol 2 was used was higher than that observed with protocol 1. No precipitation was observed in the assay mixtures, and the colour difference between the controls and the reaction samples was high. This method was chosen for use in subsequent experiments, owing to the fact that the results were considered to be more reliable.

The total conversion values show that RU-KM1 and RU-KM3$_S$ achieved higher acrylonitrile-degrading activity than RU-KM3$_L$, the conversion yields being 57.76%, 57.68%, and 21.68%, respectively. It was interesting to note that there was a difference in conversion rates between RU-KM3$_L$ and RU-KM3$_S$, since both are *Pseudomonas putida* strains. This further confirms that these two strains are not identical.

4.3.5 Inducer dependence of nitrile hydrolysis by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$

An experiment was carried out to investigate whether nitrile hydrolysis in RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ depended on the presence of a nitrile in the growth medium. Acetonitrile, at a concentration of 0.01%, was added to nutrient broth as an inducer. The results are shown in Figure 4.3.5.

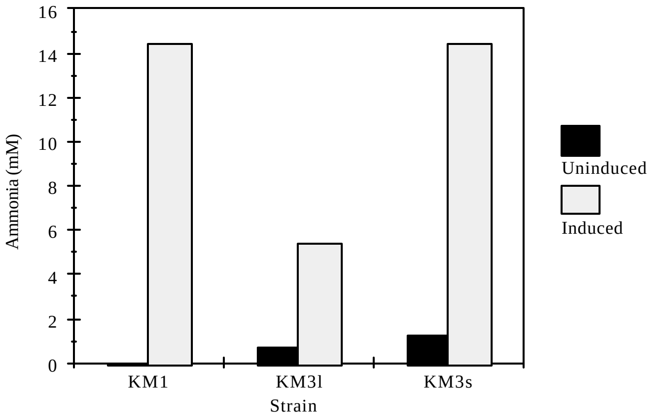


Figure 4.3.5 Inducer dependence of nitrile hydrolysis by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$, using acrylonitrile as a substrate. (**Key:** KM1 = RU-KM1; KM3l = RU-KM3$_L$; KM3s = RU-KM3$_S$; Induced = Cells cultured in the presence of 0.01% acrylonitrile; Uninduced = Cells cultured in the absence of acrylonitrile. The data represent a mean ("SEM) of triplicate determinations, with standard errors # 0.1)

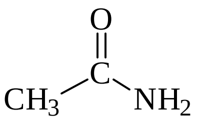
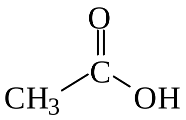
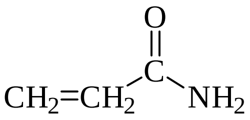
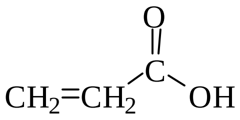
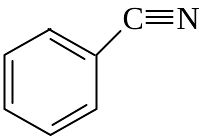
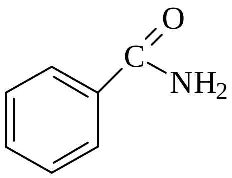
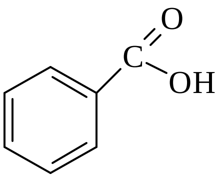
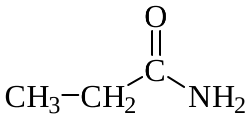
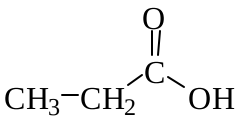
The results showed that higher nitrile-converting activity occurred when the bacteria were cultured in nitrile-containing medium. Induced RU-KM1 resulted in 14.44mM ammonia (57.76% nitrile conversion), whereas non-induced RU-KM1 was unable to hydrolyze acrylonitrile. Non-induced RU-KM3$_L$ produced 0.66mM ammonia (2.64% acrylonitrile conversion), whereas upon induction 5.43mM was formed, i.e., 21.72% conversion was achieved. Non-induced RU-KM3$_S$ also hydrolyzed acrylonitrile to a lesser extent than when induced, and the conversion rates were 4.92% (1.23mM) without induction, and 57.68% (14.42mM) upon induction with acrylonitrile.

For non-constitutive enzyme systems, the presence of a substrate or a substrate analogue in the growth medium is required for expression of the enzyme responsible for metabolism of that substrate. This is generally observed with enzyme systems involved in the metabolism of complex substrates, which are poor nutrient sources. Since it has been clearly indicated that the nitrile-hydrolyzing enzymes of these bacteria are non-constitutive, acrylonitrile was added to the growth medium in subsequent experiments.

4.3.6 Substrate selectivity of the nitrile-hydrolyzing enzymes of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$

This experiment was conducted to investigate the range of nitrile substrates which could be hydrolyzed by the different bacterial strains. This would shed light on whether the bacteria had broad substrate selectivities, which is an important characteristic often required of enzymes in order to be considered for industrial application. The substrates tested were acetonitrile, acrylonitrile, propionitrile, and benzonitrile, at a starting concentration of 25mM. Table 4.3.3 shows the structures of the nitrile substrates used in this investigation, and the results are shown in Figure 4.3.6.

Table 4.3.3 Structures of the nitriles and the products obtained from their hydrolysis

Nitrile substrate	**Amide**	**Carboxylic acid**
Acetonitrile $CH_3-C\equiv N$	**Acetamide**  $CH_3-\overset{\overset{O}{\|}}{C}-NH_2$	**Acetic acid**  $CH_3-\overset{\overset{O}{\|}}{C}-OH$
Acrylonitrile $CH_2{=}CH_2-C\equiv N$	**Acrylamide**  $CH_2{=}CH_2-\overset{\overset{O}{\|}}{C}-NH_2$	**Acrylic acid**  $CH_2{=}CH_2-\overset{\overset{O}{\|}}{C}-OH$
Benzonitrile 	**Benzamide** 	**Benzoic acid** 
Propionitrile $CH_3-CH_2-C\equiv N$	**Propionamide**  $CH_3-CH_2-\overset{\overset{O}{\|}}{C}-NH_2$	**Propionic acid**  $CH_3-CH_2-\overset{\overset{O}{\|}}{C}-OH$

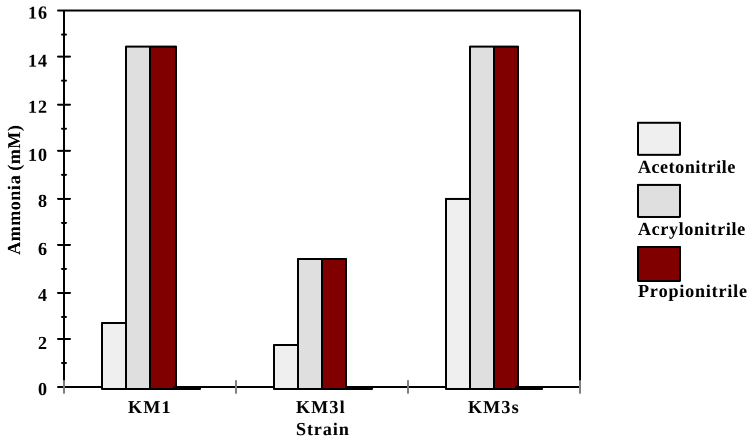


Figure 4.3.6 Substrate selectivity of the nitrile-hydrolyzing enzymes of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$

(**Key:** KM1 = RU-KM1; KM3l = RU-KM3$_L$; KM3s = RU-KM3$_S$. The data represent a mean ("SEM) of triplicate determinations, with standard errors # 0.17)

Three of the 4 nitriles tested were hydrolyzed by all four strains, *viz.*, acetonitrile, acrylonitrile, and propionitrile. Benzonitrile was not hydrolyzed by any of the strains, suggesting that these strains preferentially hydrolyze aliphatic nitriles. This should be confirmed by testing hydrolysis of alternative aromatic nitriles by these strains. Aromatic nitrile-degradation is not as common as aliphatic nitrile-degradation, and the reaction is generally catalyzed by nitrilases. Aliphatic nitrile-degradation, on the other hand, is generally catalyzed by nitrile hydratases (Cowan *et al.*, 1998). RU-KM1 and RU-KM3$_S$ were observed to convert acrylonitrile and propionitrile to a greater extent than RU-KM3$_L$, and RU-KM3$_S$ achieved higher conversion of acetonitrile than the other strains. The total conversions of acetonitrile, acrylonitrile, and propionitrile by RU-KM1 were 10.92% (2.73mM ammonia formed), 57.76% (14.44mM ammonia formed), and 46.88% (11.72mM ammonia formed), respectively. RU-KM3$_L$ achieved conversion yields of 7.16%

(1.79mM), 21.72% (5.43mM), and 9.44% (2.36mM), with acetonitrile, acrylonitrile, and propionitrile, respectively. RU-KM3_S achieved conversion yields of 32.12% (8.03mM), 57.68% (14.44mM), and 55.32% (13.83mM), with acetonitrile, acrylonitrile, and propionitrile, respectively.

RU-KM3_L and RU-KM3_S again demonstrated differences with respect to enzyme activity. Nitrile hydrolyzing activity was much higher in RU-KM3_S than in RU-KM3_L, which further confirms the difference between these *P. putida* strains.

At this stage, it was not yet clear whether ammonia was produced from nitrile hydrolysis by a nitrilase, or by a nitrile hydratase in combination with amidase. The results observed with benzonitrile suggest that the enzyme system responsible for nitrile hydrolysis in these bacteria is the nitrile hydratase-amidase system, since this system is highly unlikely to be able to hydrolyze aromatic substrates. However, further investigations were required to confirm this, as described in the next section.

4.3.7 The effect of urea on nitrile hydrolysis by RU-KM1, RU-KM3_L, and RU-KM3_S

This experiment was conducted to determine the enzyme system responsible for nitrile hydrolysis in RU-KM1, RU-KM3_L, and RU-KM3_S. Urea is a known inhibitor of the amidase enzyme involved in nitrile hydrolysis (Cramp and Cowan, 1999). Thus, incorporation of urea in the reaction mixture allows for the detection of ammonia released specifically from nitrile hydrolysis, and not that from amide hydrolysis as this would be inhibited. In an organism possessing the two-step pathway, one would expect the amount of ammonia produced in the presence of urea to be about half that produced in its absence.

The effect of urea on nitrile hydrolysis by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ was investigated using 25mM acrylonitrile as a substrate, and urea was added to the reaction mixtures and controls at a concentration of 25mM. The results of this experiment are shown in Fig. 4.3.7.

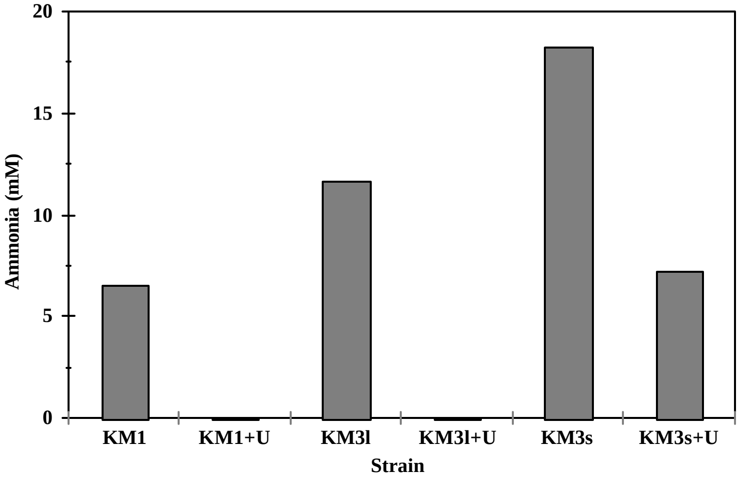


Figure 4.3.7 The effect of urea on the hydrolysis of acrylonitrile by RU-KM1, RU-KM3$_L$, and RU-KM3$_S$

(**Key:** KM1= RU-KM1; KM1 + U = RU-KM1 + Urea; KM3l = RU-KM3$_L$; KM3l + U = RU-KM3$_L$ + Urea; KM3$_S$ = RU-KM3$_S$ + Urea. The data represent a mean ("SEM) of triplicate determinations, with standard errors # 0.17)

Addition of urea to the reaction mixtures of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$, using acrylonitrile as a substrate, resulted in a decrease in the amount of ammonia produced. No ammonia was detected when urea was added to reaction mixtures of RU-KM1 and RU-KM3$_L$ with acrylonitrile, whereas RU-KM3$_S$ produced less ammonia in the presence of urea. This suggests that the system responsible for nitrile hydrolysis in these bacteria is the two-step process involving a nitrile hydratase and an amidase, since ammonia production by this system is supposed to decrease as a result of the inhibition of the amidase enzyme.

4.4 CONCLUSIONS

The presence of a nitrile-hydrolyzing system in strains RU-KM1, RU-KM3$_L$, and RU-KM3$_S$ has been demosntrated. RU-KM1 and RU-KM3$_S$ showed higher nitrile-hydrolyzing activity than RU-KM3$_L$. Nitrile hydrolysis in these strains appears to involve the two-step system, which consists of a nitrile hydratase and an amidase. However, further investigations are necessary to confirm this. One of the ways in which this may be achieved is by purification of the enzymes and characterization of the activities of the purified enzymes.

The effect of benzonitrile on enzyme activity in resting cells reactions for hydrolysis of other nitriles by these strains may also shed light on the enzyme system responsible for nitrile hydrolysis in these bacteria. This is based on previous reports that benzonitrile inhibits some nitrile hydratases (Cramp *et al.*, 1998).

The stereoselectivities of the nitrile-hydrolyzing enzymes of these strains also need to be determined, as production of compounds such as profens, which are non-steroidal anti-inflammatory drugs, from nitrile compounds, requires enantioselective processes because the enantiomers generally have different biological activities (Yamamoto *et al.,* 1996).

The conditions for nitrile hydrolysis by RU-KM1, RU-KM3_L, and RU-KM3_S may be improved by determining the effects of parameters such as temperature, pH, and reaction time. However, the purpose of this preliminary study was only to investigate whether RU-KM1, RU-KM3_L, and RU-KM3_S could catalyze nitrile hydrolysis or not.

The investigation described in this chapter has suggested the presence of an amidase enzyme, which is involved in nitrile hydrolysis, in RU-KM1, RU-KM3_L, and RU-KM3_S. The next chapter deals with the investigation of the ability of these bacteria to hydrolyze amides, which would demonstrate whether they are able to produce amidases.

CHAPTER 5

Investigation of the amide-hydrolyzing activity of RU-KM1, RU-KM3$_L$, and RU-KM3$_S$

5.1 INTRODUCTION

Amidases have potential value in the development of commercial processes for the production of useful chemicals, such as 2-arylpropionic acids, which are used as non-steroidal non-inflammatory drugs (Gilligan *et al.*, 1993; Wu *et al.*, 1998). The ability of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR to hydrolyze various amides was investigated, with the main objective of broadening the potential usefulness of these hydantoinase-producing strains in biocatalytic applications. The advantage of doing this kind of investigation is that one could discover biocatalytic applications other than that which the microorganisms had been isolated for, and thus obtain a biocatalyst for a number of processes from a single isolation. Amide-hydrolyzing activity was chosen particularly because it involves hydrolysis of amide bonds, which is effectively the same reaction that occurs with hydantoin- ring hydrolytic cleavage.

Amidase (linear amide hydrolase) [EC 3.5.1] is an enzyme that hydrolyzes the C-N bond of aliphatic and aromatic amides (Kagayama and Ohe, 1990), converting the amides to carboxylic acids and ammonia (Maestracci *et al.*, 1988). Amides may be converted chemically, but there are a number of disavantages associated with this route, the major one being the production of racemic products (Hayashi *et al.*, 1997). Enzymatic amide hydrolysis, on the other hand, is considered to be advantageous over chemical hydrolysis, mainly due to the enantioselectivity of enzymes, which ensures that the products obtained are enantiomerically pure (Hirrlinger *et al.*, 1996).

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

The amide substrates: acrylamide, propionamide, acetamide, and prolinamide, were all purchased from Sigma-Aldrich. Sodium hydroxide and sodium nitroprusside were obtained from Saarchem (Pty) Ltd. (S.A), whereas phenol was purchased from NT Laboratory Supplies (S.A.). Sodium hypochlorite was purchased from Associated Chemical Enterprises (S.A.).

5.2.2 Culture conditions

The four bacterial strains were each inoculated into 50mL minimal medium (Appendix 1.3), in 250mL conical flasks, containing either acrylamide or propionamide (20mM) as the sole nitrogen source. These were incubated at 28°C on an orbital shaker at 200rpm. Bacterial growth was monitored by measuring optical density at 600nm, every 24 hours on a Shimadzu UV-Visible spectrophotometer.

5.2.3 Product detection from amide hydrolysis reactions

The hydrolysis of amides was quantified colorimetrically by using the phenol-hypochlorite method (indophenol assay), to measure the concentration of ammonium ions released by the hydrolysis of amides (Pereira *et al.*, 1998). After the reaction time had elapsed, the reaction mixtures and controls were transferred to 1.5mL eppendorf tubes and microfuged for 5 minutes at 13 000g. The assay was then carried out as follows:

- A 100mL aliquot of each of the supernatants was transferred to another set of eppendorf tubes.

- 400mL of solution A (0.59M phenol and 1mM sodium nitroprusside) was added
- the mixture was vortexed to mix
- 400mL of solution B (0.11M sodium hypochlorite and 2M sodium hydroxide) was added
- Samples were allowed to stand at room temperature for 30 minutes
- Absorbance at 600nm was measured spectrophotometrically

The concentration of ammonia in the samples was calculated from a standard curve prepared using ammonium sulfate (Appendix 3.3).

5.2.4 Hydrolysis of propionamide by cells cultured in propionamide minimal medium

The four bacterial strains were each cultured in 200mL of propionamide minimal medium, in 1L conical flasks, until they reached the exponential phase of growth. The cells were harvested by centrifugation at 7000g for 10 minutes, after which the pellet was resuspended in half the initial culture volume of cold 0.1M potassium phosphate buffer, pH 7.0. This cell suspension was then centrifuged at 7000g for 10 minutes. The pellet was again resuspended in half the initial culture volume of buffer and centrifuged at 8000g. The supernatant was discarded and the cells resuspended in potassium phosphate buffer, pH 7.0, to give a cell concentration of 0.1g/mL. Biocatalytic reaction mixtures consisting of 1mL of this cell suspension and 1mL of a 50mM propionamide solution were prepared in triplicate. Controls (1mL appropriate cell suspension plus 1mL buffer; 1mL of 50mM propionamide plus 1mL buffer) were also prepared. The reaction mixtures and controls were incubated at 30°C for 3 hours, with shaking at 200rpm.

5.2.5 Investigation of the inducer dependence of the amidase enzymes of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR were each inoculated into 200mL nutrient broth and into 200mL nutrient broth supplemented with 0.1% propionamide, in 1L conical flasks. These were incubated at 28°C until the bacteria reached the exponential growth phase (as indicated by OD_{600}), after which they were harvested by centrifugation at 7000g for 10 minutes. The pellets thus obtained were resuspended in half the initial culture volume of cold 0.1M potassium phosphate buffer, pH 7.0, and subsequently centrifuged at 7000g for 10 minutes. The cells were washed once more with 0.1M potassium phosphate buffer, pH 7.0, and centrifuged at 8000g for another 10 minutes. Cell suspensions of each bacterial strain were prepared in 0.1M phosphate buffer to get a final concentration of 0.1g/mL cells. Biocatalytic reaction mixtures, which consisted of 1mL of the appropriate cell suspension and 1mL of 50mM acrylamide, were prepared in triplicate. Cells and substrate controls (1mL cell suspension plus 1mL buffer; 1mL substrate solution plus 1mL buffer) were also prepared. The biocatalytic reaction mixtures were incubated at 30°C for 3 hours, with shaking at 200rpm.

5.2.6 Investigation of the substrate selectivity of the amidase enzymes of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

The four bacterial strains were each inoculated into 400mL of nutrient broth supplemented with 0.1% propionamide, in 2L conical flasks. These were incubated at 28°C until they reached the exponential phase. Cells were harvested as above (Section 5.2.5) and 0.1g/mL cell suspensions were prepared in 0.1M potassium phosphate buffer, pH 7.0. Solutions of amide substrates: acetamide, acrylamide, propionamide, and prolinamide, were prepared at a concentration of

50mM. A 1mL aliquot of each substrate was reacted with 1mL appropriate cell suspension at 30°C for 3 hours, with shaking at 200rpm. Reactions were prepared in triplicate. Cell controls and substrate controls were also prepared and incubated at the same time as the reaction mixtures.

5.3 RESULTS AND DISCUSSION

5.3.1 Growth of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR in amide minimal media

The ability of RU-KM1, RU-KM3$_L$, RU-KM3$_S$ and RU-OR to utilize amides as the sole source of nitrogen for growth was investigated using 10mM acrylamide and propionamide. The results of these experiments are shown in Figures 5.3.1 and 5.3.2, below.

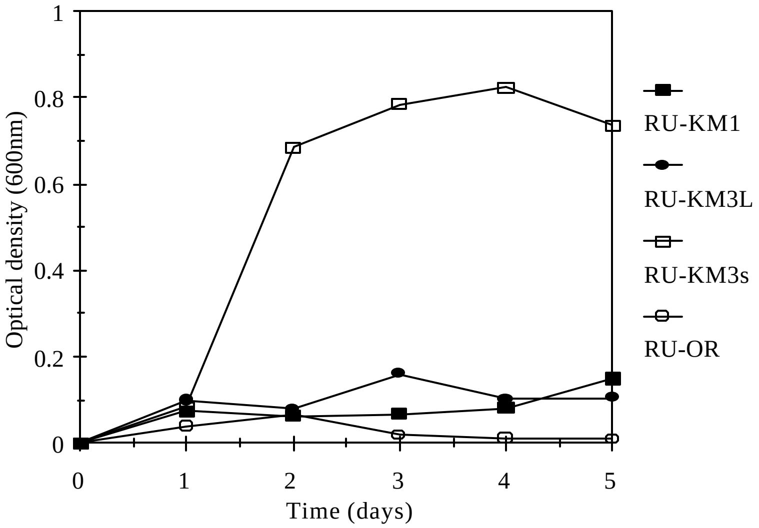


Figure 5.3.1 Growth of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR in acrylamide minimal medium

When acrylamide was used as the sole source of nitrogen in the growth medium, only RU-RU-KM3$_S$ was observed to grow (Figure 5.3.1, above). RU-KM1, RU-KM3$_L$, and RU-OR did not grow, and this suggests that this amide does not support the growth of these bacteria. RU-KM1 and RU-KM3$_L$ only grew up to an OD$_{600}$ of 0.1, after which no further growth was observed because the OD$_{600}$ remained approximately the same from 1 day up to 5 days of incubation. RU-OR, however, appeared to have reached the death phase after 2 days of incubation, because the optical density decreased to zero at day 3.

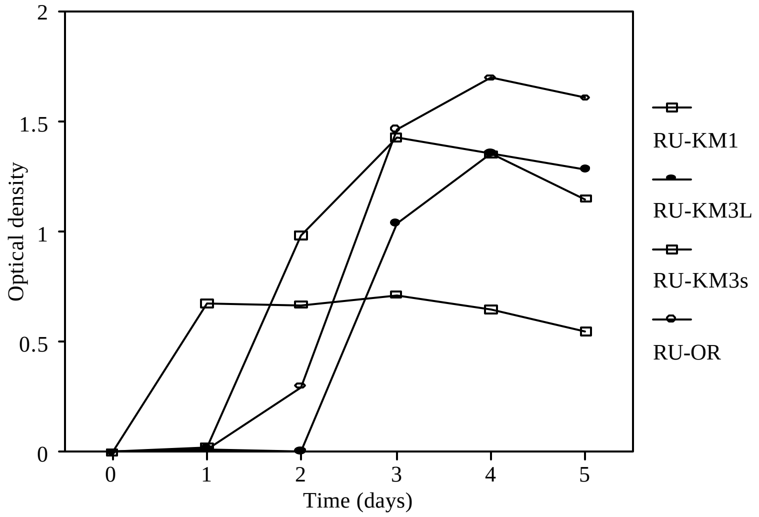


Figure 5.3.2 Growth of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR in propionamide minimal medium

The investigation of the ability of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR to utilize propionamide as the sole nitrogen source showed that all 4 strains were all able to assimilate nitrogen from this amide (Figure 5.3.2). However, RU-KM1 utilized this amide less readily for growth, since the optical density did not increase after 1 day of incubation, and it remained the same until day 5. Thus, all four strains apparently utilized propionamide more readily than acrylamide, and hence, it was used in the subsequent experiments to determine amide-hydrolyzing ability. However, due to the low yields of biomass from growth in propionamide minimal medium, nutrient broth supplemented with 0.1% propionamide as inducer was used as the culture medium in subsequent experiments, unless otherwise stated.

5.3.2 Hydrolysis of propionamide by cells cultured in propionamide minimal medium

The hydrolysis of propionamide by the different bacteria, cultured in propionamide minimal medium, was investigated. The results obtained are illustrated in Table 5.3.1.

Table 5.3.1 Production of ammonia from 25mM propionamide by bacteria cultured in propionamide minimal medium

MICROORGANISM	Ammonia (mM)	% Conversion
RU-KM1	6.84 " 0.03	27.36
RU-KM3$_{L}$	24.42 " 0.08	97.68
RU-KM3$_{S}$	24.15 " 0.05	96.6

Amidase activity was assayed when the bacteria reached the mid-exponential phase in accordance with previous reports that the enzyme is expressed in this phase of growth (Asano *et. al.*, 1982; Collins and Knowles, 1983). The results from the investigation of the hydrolysis of propionamide by propionamide-cultured cells (Table 5.3.1) showed that RU-KM3$_{L}$ and RU-KM3$_{S}$ were able to achieve almost 100% conversion of this amide (97.68% and 96.6%, respectively). However, RU-KM1 only achieved a 27.36% conversion of this amide. RU-KM3$_{L}$

and RU-KM3$_S$ hydrolyzed propionamide almost to the same extent, thus indicating that the activity of the amidases of these two bacterial strains is similar. This observation could be due to the fact that both are *Pseudomonas putida* strains (G.F. Matcher, 1997). The low activity of RU-KM1 with propionamide may be interpreted in two ways: one is that the amidase of RU-KM1 has low activity; and the second interpretation is that the amidase of RU-KM1 does not preferentially hydrolyze this particular amide.

5.3.3 Inducer dependence of amide hydrolysis by RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

The inducer dependence of the amidase of RU-KM1, RU-KM3L, RU-KM3S, and RU-OR for amide hydrolysis was investigated by determining the amount of ammonia production from acrylamide by induced and non-induced cells. The growth media used were nutrient broth (no induction) and nutrient broth supplemented with 0.1% propionamide (induction). The results are shown in Figure 5.3.3 and Table 5.3.2.

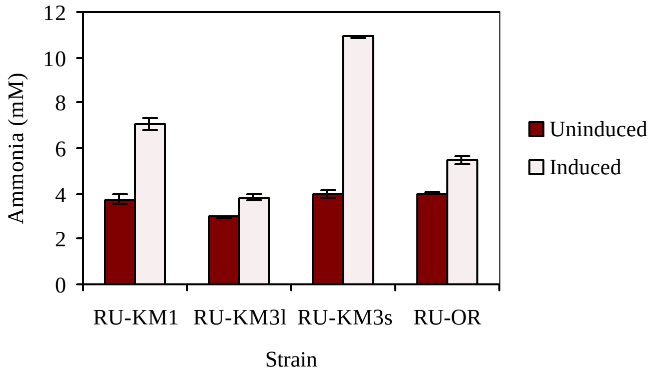


Figure 5.3.3 Hydrolysis of acrylamide by noninduced and induced RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR (Uninduced = Ammonia produced from acrylamide by uninduced cells; Induced = ammonia produced from acrylamide by propionamide-induced cells)

Table 5.3.2 Total acrylamide conversion (%) by induced and non-induced RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

Strain	**% Acrylamide converted**	
	Non-induced	**Induced**
RU-KM1	15.0	28.24
RU-KM3$_L$	11.84	15.28
RU-KM3$_S$	15.84	43.6
RU-OR	15.96	22.0

The results showed a marked difference in the amount of ammonia produced by RU-KM1 and RU-KM3$_S$ non-induced and induced cells, with the induced cells producing far more ammonia from acrylamide than non-induced cells (Figure 5.3.3 and Table 5.3.2). Non-induced cells of RU-KM1 produced 3.75mM ammonia (15% conversion), whereas induced cells produced 7.06mM ammonia (28.24% conversion). RU-KM3$_S$ produced 3.96mM ammonia (15.84% conversion) without induction, and 10.89mM ammonia (43.6% conversion) upon induction. Induced RU-KM3$_L$ and RU-OR cells also hydrolyzed acrylamide to a greater extent than non-induced cells. Non-induced RU-KM3$_L$ produced 2.96mM ammonia (11.84%), whereas induced cells produced 3.82mM (15.82%). Non-induced RU-OR produced 3.96mM ammonia (15.96%), whereas induced cells produced 5.50mM (22% conversion).

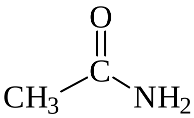
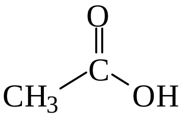
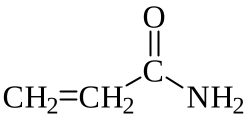
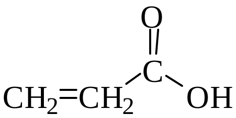
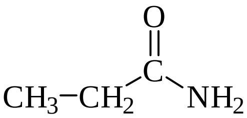
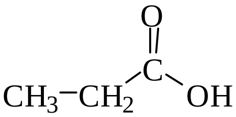
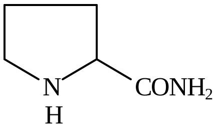
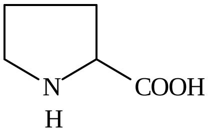
These results indicate that the hydrolysis of amides by these bacteria is increased by the presence of an amide in the growth medium, although they still showed some conversion in the absence of an inducer. A number of amidases have been reported in the literature to be inducer dependent

(Asano *et. al.*, 1982; Ciskanik *et. al.*, 1995; Gilligan *et al.*, 1993; Hayashi *et al.*, 1997). Various amides are used for induction of amidase activity, for example, ketoprofen amide (Hirrlinger *et al.*, 1996), benzyl cyanide (Effenberger and Graef, 1998), crotonamide (Gilligan *et al.*, 1993) and pyrazinamide (Kagayama and Ohe, 1990). In this study, propionamide was chosen as an inducer due to its support of the growth of these bacteria. Inducer-independent amidases have also been reported (Kagayama and Ohe, 1990; Skouloubris *et al.*, 1997; Fournand *et al.*, 1998).

5.3.4 The substrate selectivity of the amidases of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

The hydrolysis of various amide substrates (acrylamide, acetamide, propionamide, and prolinamide) by whole cells of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR was investigated. Table 5.3.3 shows the structures of the amide substrates used in this investigation. Figure 5.3.4 and Table 5.3.4 show the results obtained.

Table 5.3.3 Structures of amide substrates used and hydrolysis products

Amide	**Carboxylic acid**
Acetamide  $CH_3-C(=O)-NH_2$	**Acetic acid**  $CH_3-C(=O)-OH$
Acrylamide  $CH_2{=}CH_2-C(=O)-NH_2$	**Acrylic acid**  $CH_2{=}CH_2-C(=O)-OH$
Propionamide  $CH_3-CH_2-C(=O)-NH_2$	**Propionic acid**  $CH_3-CH_2-C(=O)-OH$
Prolinamide 	**Proline** 

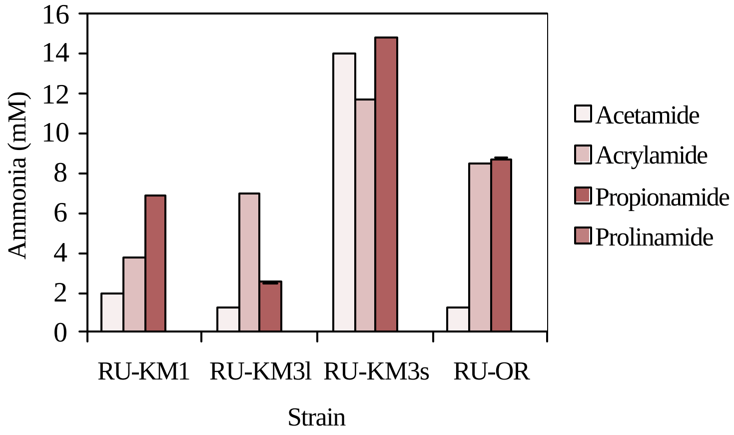


Figure 5.3.4 The substrate selectivity of the amidases of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

(The results represent the mean of triplicate determinations, with standard errors # 0.05)

Table 5.3.4 Total conversion (%) of amide substrates by RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

Strain	**Substrate conversion (%)**			
	Acetamide	**Acrylamide**	**Propionamide**	**Prolinamide**
RU-KM1	7.76	15.16	27.4	0.00
RU-KM3$_L$	49.2	27.8	10.0	0.00
RU-KM3$_S$	57.24	46.64	59.2	0.00
RU-OR	5.08	33.92	34.8	0.00

From the results of this investigation, it was concluded that none of the four strains were able to hydrolyze prolinamide, since no ammonia production was detected after reaction with this amide. This is presumably due to a steric effect as prolinamide is a cyclic amide, whereas the other are aliphatic amides. Prolinamide was also not hydrolyzed by the amidase from *Comamonas acidovorans* KPO-2771-4 (Hayashi *et al.*, 1997).

The bacteria showed different selectivity patterns as far as the other amides were concerned (Fig. 5.3.4 and Table 5.3.3). The preferred substrate for RU-KM1 was propionamide, with 27.4% conversion, whereas acetamide was the least hydrolyzed, with 7.76% conversion. Acrylamide hydrolysis was achieved by RU-KM1 with a conversion rate almost double that of acetamide hydrolysis, (15.16%). RU-KM3$_L$ converted acrylamide (27.8%) more readily than the other two amides (4.92% for acetamide, and 10% for propionamide), and in this case acetamide was again the least hydrolyzed. RU-KM3$_S$ showed high activity and little selectivity with the three amides tested. Acetamide and propionamide were hydrolyzed to similar extents, 56.24% and 59.2%, respectively, whereas acrylamide was the least hydrolyzed (46.64%), but considerably more ammonia was produced by this bacterium from acrylamide than the amount produced by the other bacteria. RU-OR produced approximately equal amounts of ammonia from acrylamide and propionamide, 33.92% and 34.8%, respectively, but produced the least ammonia from acetamide (5.08%).

It may be suggested that the amidases of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR prefer straight chain substrates, owing to the fact that prolinamide, which is a cyclic amide, was not hydrolyzed. The substrate selectivity of RU-KM1 is similar to that reported for the aliphatic amidase of *Helicobacter pylori*, where propionamide was the preferred substrate, followed by acrylamide, and lastly by acetamide (Skouloubris *et al.*, 1997). These results show that the three bacteria show relatively relaxed substrate specificities with respect to aliphatic substrates. Another broad spectrum amidase was reported in *Pseudomonas chlororaphis* B23, which hydrolyzed aliphatic amides as well as some aromatic amides (Ciskanik *et al.*, 1995). A narrow spectrum amidase was reported by Asano *et al.* (1982), from *Arthrobacter* sp. J-1.

Acrylic acid, which is produced from the hydrolysis of acrylamide, is used in the manufacture of a variety of polymers, including flocculants, adhesives, and thickeners (Brennan *et al.*, 1995).

5.4 CONCLUSION

This investigation has illustrated that RU-KM1, RU-KM3_L, RU-KM3_S and RU-OR all possess amide hydrolyzing activity. Of these, RU-KM3_S seems to have higher amide-hydrolyzing activity under the conditions employed. However, amide hydrolysis by these bacteria still needs to be optimized. The conditions used in this study were based on those reported in the literature for other microorganisms, and may not be optimal for these bacteria. Various reaction conditions need to be examined, such as the effects of parameters such as pH, temperature, and other inducers. However, the purpose of the present study was to elucidate whether these bacteria possess amidase enzymes, and this has been achieved.

The presence of amidases in these microorganisms has important implications in that it shows that these bacteria may be used for the production of a variety of industrially important compounds. They may be used for the production of amino acids from hydantoins, using hydantoinases (see Chapter 3), or, partially, for production of compounds such as 2-

arylpropionic acids from amides, using amidases. RU-KM3$_S$ has again shown its suitability as a biocatalyst, in that, among the four strains tested, it had the highest amidase activity.

The stereoselectivity of the amidases of these strains needs to be determined, as this is important for biocatalytic application of these enzymes since they may be used for production of pharmaceutically active compounds such as 2-arylpropionic acids, which are anti-inflammatory drugs whose enantiomers have different biological activities.

The discovery of the amidase activity in these microorganisms led to the investigation of another amide-bond hydrolyzing enzyme system, the imide-hydrolyzing enzyme system. This system involves an imide-hydrolyzing enzyme as well as an amidase, and this investigation is described in the next chapter.

CHAPTER 6

Investigation of the imide-hydrolyzing activity of RU-KM1, RU-KM3$_L$, RU-KM3$_S$ and RU-OR

6.1 INTRODUCTION

In this study, the ability of four bacterial strains, namely RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR, to hydrolyze cyclic imides was investigated. These bacteria were previously selected for their ability to hydrolyze hydantoin and its derivatives, as was the bacterial strain in which microbial imide hydrolysis was first reported (Ogawa *et al.*, 1996). The main objective of this investigation was to determine the presence of alternative potential biocatalytic ability in these microorganisms.

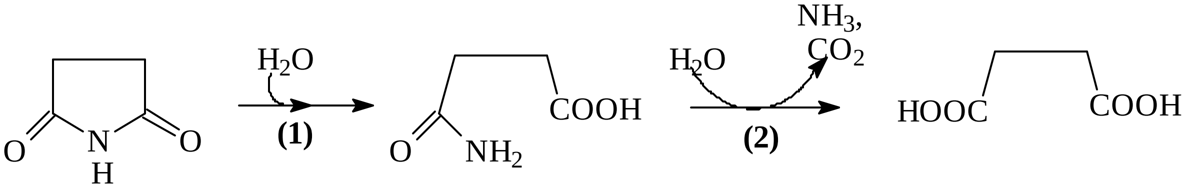
Imide-hydrolyzing activity involves two enzymes, an imidase (or hydantoinase) and an amidase. The presence of hydantoinase and amidase enzymes has already been demonstrated in these bacteria. Since some hydantoinases have been found to possess the ability to hydrolyze complex cyclic imides as well as hydantoins (Soong *et al.*, 1999), an attempt was made to elucidate whether the imide-hydrolyzing activity in these bacteria was related to the hydantoin-hydrolyzing activity known to be present in these strains.

The structures of cyclic imides are similar to those of cyclic ureides, and they are known to be hydrolyzed by mammalian dihydropyrimidinase, which is involved in pyrimidine metabolism (Dudley *et al.*, 1974; Ogawa *et al.*, 1996). Microbial hydrolysis of cyclic imides was first reported by Ogawa *et al.*, (1996). Imidase, which specifically hydrolyzes simple cyclic imides and sulfur-containing cyclic imides, catalyzes the first step of cyclic imide metabolism in microorganisms (Soong *et al.*, 1999). This enzyme is different from cyclic ureide-hydrolyzing enzymes (Soong *et al.*, 1999). Cyclic imide hydrolyzing activity was reported to be widely distributed in microorganisms, *viz.*, bacteria, yeasts and moulds (Soong *et al.*, 1998).

Cyclic imide **Cyclic ureide**

Figure 6.1.1 Diagram illustrating the structures of cyclic imides and cyclic ureides

Imidase catalyzes the conversion of imides to monoamidated dicarboxylates, which are then converted to dicarboxylates by an amidase (Figure 6.1.2).



Succinimide Succinamic acid Succinate

Figure 6.1.2 Enzymatic hydrolysis of cyclic imides

(Key: (1) = imidase; (2) = amidase)

6.2 MATERIALS AND METHODS

6.2.1 Culture conditions for the investigation of imide hydrolysis by RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR

Bacteria were inoculated from hydantoin minimal medium agar plates into 100mL each of minimal medium containing 20mM succinimide (Sigma-Aldrich) as the sole source of nitrogen

(Appendix 1.4). These were incubated at 28°C on an orbital shaker at 200rpm. Bacterial growth was monitored by measuring the increase in optical density at 600nm on a Shimadzu UV-Visible spectrophotometer.

6.2.2 Resting cell reactions of RU-KM1 and RU-OR with succinimide

Resting cell reactions were carried out with RU-KM1 and RU-OR only, since they showed appreciable levels of growth in succinimide minimal medium. Cells were harvested by centrifugation at 7000g for 10 minutes at 4°C after they had reached the early stationary phase of growth in succinimide minimal medium (4 days). The pellet thus obtained was washed by resuspension in 50mL of cold potassium phosphate buffer (0.1M, pH 7.0), and centrifugation at 7000g for 10 minutes at 4°C. The cells were resuspended in 0.1M potassium phosphate buffer and centrifuged for another 10 minutes at 8000g at 4°C. Cell suspensions containing 0.1g/mL cells were prepared in potassium phosphate buffer (0.1M, pH 7.0). Biocatalytic reaction mixtures were prepared by mixing a 1mL aliquot of the appropriate cell suspension with 1mL of a 50mM solution of succinimide, also in potassium phosphate buffer (0.1M, pH 7.0). Cells and substrate controls (1mL cell suspension plus 1mL buffer; 1mL substrate solution plus 1mL buffer) were also prepared. Biocatalytic reaction mixtures and controls were incubated at 30°C for 3 hours, with shaking at 200rpm.

6.2.3 Product detection from imide hydrolysis by RU-KM1 and RU-OR

The second step of both hydantoin and succinimide hydrolysis results in the release of ammonia from an amide substrate by an amidase enzyme. Thus the phenol-hypochlorite method (see Chapter 5, Section 5.2.1.5) was used to determine hydrolysis of the substrate. The concentration of ammonia produced was determined from a standard curve prepared using ammonium sulphate (Appendix 3.3).

6.2.4 Investigation of inducer dependence of imide hydrolysis by RU-KM1 and RU-OR

RU-KM1 and RU-OR were each inoculated into 200mL of either nutrient broth or nutrient broth supplemented with 0.1% succinimide, in 1L conical flasks. They were incubated at 28°C on an orbital shaker at 200rpm, until they reached the early stationary phase ("24 hours in nutrient broth). Cells were harvested by centrifugation at 7000g for 10 minutes, and washed with potassium phosphate buffer (0.1M, pH 7.0). The pellets obtained were used to prepare 0.1g/mL cell suspensions using 0.1M potassium phosphate buffer, pH7.0. Biocatalytic reaction mixtures were prepared using 1mL of appropriate cell suspension (either RU-KM1 or RU-OR) and 1mL of 50mM succinimide, prepared in 0.1M potassium phosphate buffer, pH 7.0. All biocatalytic reactions were conducted in triplicate. Controls were prepared using either 1mL cell suspension (cells control) or 1mL 50mM succinimide (substrate control) plus 1mL 0.1M potassium phosphate buffer. The biocatalytic reaction mixtures were incubated together with the controls at 30°C for 3 hours. The extent of hydrolysis of succinimide was determined by measuring the concentration of ammonia produced in the supernatant after centrifugation of the biocatalytic reaction mixtures.

6.2.5 Differentiation between imidase and hydantoinase reactions in RU-KM1 and RU-OR

In order to investigate whether imide hydrolysis in RU-KM1 and RU-OR was carried out by hydantoinase or by a separate enzyme system, i.e. an imidase, the two bacterial strains were each inoculated into 200mL nutrient broth supplemented with either hydantoin or succinimide as an inducer, in 1L conical flasks. These were incubated at 28°C, with shaking at 200rpm, until they reached the stationary phase ("24 hours). Cells were harvested by centrifugation at 7000g for 10 minutes. The pellets were washed with 0.1M potassium phosphate buffer, pH 7.0 (for succinimide-induced cells), and pH 8.0 (for hydantoin-induced cells).

A 0.1g/mL cell suspension of hydantoin-induced RU-KM1 was prepared using 0.1M potassium phosphate buffer, pH 7.0, and this was used for biocatalytic reactions with succinimide. A 0.1g/mL of hydantoin-induced RU-KM1 was prepared using 0.1M potassium phosphate buffer, pH 8.0, and this was used for biocatalytic reactions with hydantoin. RU-OR cell suspensions were prepared as above. Biocatalytic reactions consisted of 1mL cell suspension (succinimide- or hydantoin-induced RU-KM1 or RU-OR) and 1mL of 50mM substrate solution, either succinimide or hydantoin.

6.3 RESULTS AND DISCUSSION

6.3.1 Growth of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR in succinimide minimal medium

The ability of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR to utilize succinimide as a source of nitrogen was investigated by growing the microorganisms in minimal medium containing 10mM succinimide. Figure 6.3.1 shows the results obtained.

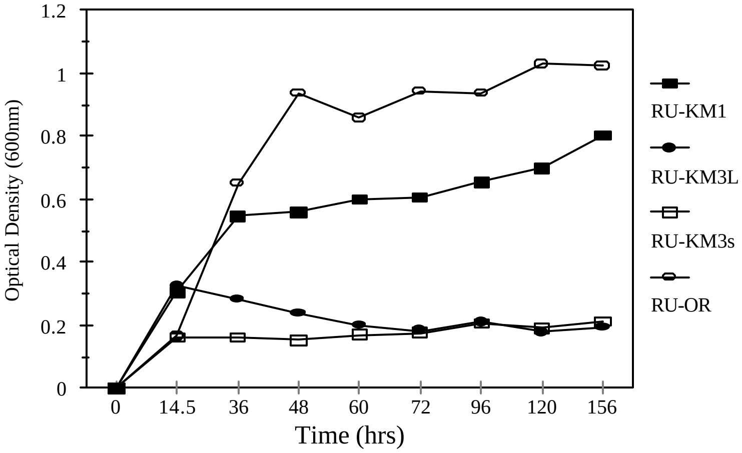


Figure 6.3.1 Growth curves of RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR in succinimide minimal medium

No lag phase was observed in any of the four cases, as indicated by the optical density, which had already increased after 14 hours of incubation. The RU-KM3$_L$ and RU-KM3$_S$ cultures reached optical density values of 0.15 and 0.3, respectively, after 14.5 hours of incubation, but after that no further increase was observed. The RU-KM1 culture reached an optical density of 0.8 after an incubation period of 6.5 days, while RU-OR reached an optical density of 1.0 after 5 days of incubation. To our knowledge, this is the first report on the ability of microorganisms to utilize an imide as the sole source of nitrogen. Other microorganisms have been reported to have the ability to utilize succinimide as the sole source of carbon (Ogawa *et al.*, 1996; Soong *et al.*, 1998). Due to the fact that these two bacteria showed significant growth in succinimide minimal medium, they were selected for further experiments on imide hydrolysis.

6.3.2 Hydrolysis of succinimide by RU-KM1 and RU-OR cultured in succinimide minimal medium

The ability of RU-KM1 and RU-OR, which both grew in succinimide minimal medium, to hydrolyze succinimide and thus produce ammonia, was investigated using resting cells of these strains, cultured in minimal medium containing succinimide as the sole source of nitrogen. 25mM succinimide was used as the substrate for the biocatalytic reaction. Table 6.3.1 shows the results obtained.

Table 6.3.1 Hydrolysis of succinimide by RU-KM1 and RU-OR cultured in succinimide minimal medium

Strain	**Ammonia (mM)***	**Total conversion (%)**
RU-KM1	11.13"0.01	44.52
RU-OR	12.32"0.02	49.28

(Ammonia produced from 25mM succinimide)

The results of this investigation showed that RU-OR was slightly more effective than RU-KM1 with respect to succinimide hydrolysis. This was indicated by the fact that RU-OR achieved 49.28% conversion of succinimide, whereas RU–KM1 achieved 44.52%. However, these conversion yields are not a complete representation of succinimide hydrolysis by these bacteria, since the method of detection used quantifies only the activity of the second enzyme involved in this biocatalytic reaction, the amidase. The total amount of succinimide hydrolyzed would be represented by the total amount of succinamic acid produced (from succinimide-ring-hydrolysis) plus the amount of ammonia produced from succinamic acid by the amidase enzyme (See Fig.

6.1.2 for structure). However, further investigations are required to develop a method for the detection of succinamic acid produced from succinimide hydrolysis.

6.3.3 Inducer dependence of imide hydrolysis in RU-KM1 and RU-OR

The importance of the presence of succinimide in the growth medium of RU-KM1 and RU-OR for succinimide hydrolysis was investigated. The growth media used were nutrient broth (non-induced) and nutrient broth supplemented with 0.1% succinimide (induced). The results are shown in Figure 6.3.2.

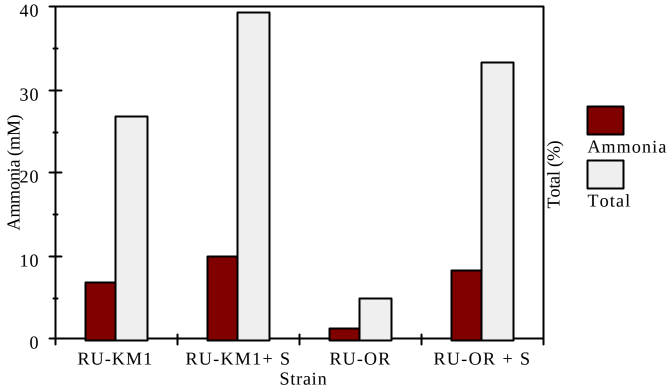


Figure 6.3.2 Hydrolysis of succinimide by induced and non-induced RU-KM1 and RU-OR

(RU-KM1 + S = Induced RU-KM1; RU-OR + S = Induced RU-OR; Ammonia = Amount of ammonia produced from 25mM succinimide; Total = % Conversion of succinimide. The data represent the mean of triplicate determinations, with standard errors # 0.05)

When the hydrolysis of succinimide by induced and non-induced cells of RU-KM1 and RU-OR was investigated, it was observed that both RU-KM1 and RU-OR hydrolyzed this substrate to a greater extent when induced than when succinimide was absent from the growth medium (Figure 6.3.2). RU-KM1 showed a succinimide conversion yield of 26.88% by cells grown in the absence of an inducer, whereas 39.36% conversion was achieved by cells grown in the presence of an inducer. RU-OR achieved 4.88% conversion by cells grown in the absence of an inducer, whereas 33.44% conversion was achieved by cells grown in the presence of succinimide as an inducer. However, fairly high levels of ammonia were detected when non-induced RU-KM1 was reacted with succinimide. This suggests that an inducer is not strictly required for imide hydrolysis in this bacterium, i.e. for enzyme expression, but it does enhance succinimide-hydrolyzing activity. To our knowledge, this is the first report of inducer-independent imide hydrolysis in a microorganism. The imidase of *Blastobacter* sp. Strain A17p-4 was reported to be induced by cyclic imides such as succinimide and glutarimide (Ogawa *et al.*, 1996). A different observation was made with RU-OR, however. There were significantly lower levels of ammonia when non-induced RU-OR was reacted with succinimide, than when RU-OR was induced. This suggests that the presence of an imide is required for imidase enzyme expression in this bacterium.

6.3.4 Investigation of the enzyme system responsible for imide hydrolysis by RU-KM1 and RU-OR

The hydrolysis of hydantoin and succinimide by cells which had been induced with either of these substrates was investigated. Table 6.3.2 shows total hydantoin and succinimide hydrolysis (%) by succinimide- and hydantoin-induced RU-KM1 and RU-OR. Table 6.3.2 and Figure 6.3.3 show the production of ammonia by RU-KM1 and RU-OR from succinimide and hydantoin, after induction with either succinimide or hydantoin.

Table 6.3.2 Conversion (%) of succinimide or hydantoin by RU-KM1 and RU-OR induced with hydantoin or succinimide

Strain	Substrate conversion (%)	
	Hydantoin	**Succinimide**
RU-KM1 + H	30.4	21.4
RU-KM1 + S	32.2	22.84
RU-OR + H	10.64	29.56
RU-OR + S	10.64	23.5

(**Key**: RU-KM1 + H = RU-KM1 induced with hydantoin; RU-KM1 + S = RU-KM1 induced with succinimide; RU-OR + H = RU-OR induced with hydantoin; RU-OR + S = RU-OR induced with succinimide. The starting substrate concentration was 25mM)

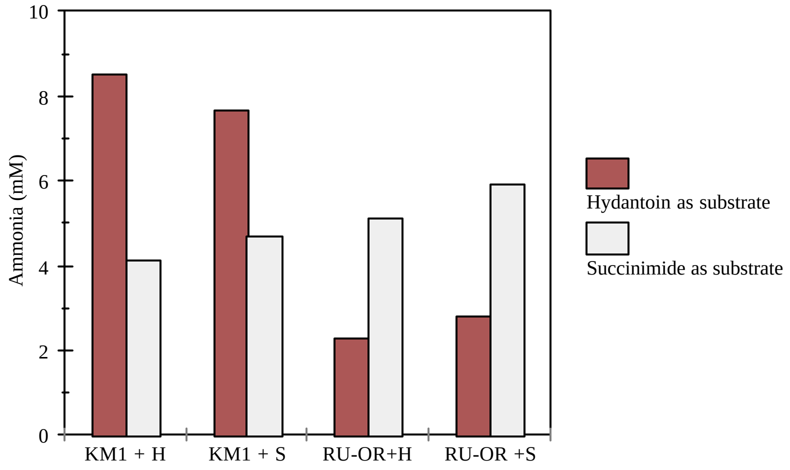


Figure 6.3.3 Ammonia production by RU-KM1 and RU-OR induced with hydantoin or succinimide, and using succinimide or hydantoin as substrate.

(**Key:** KM1 + H = Hydantoin-induced RU-KM1; KM1 + S = Succinimide-induced RU-KM1; RU-OR + H = Hydantoin-induced RU-OR; RU-OR + S = Succinimide-induced RU-OR. The data represent the mean of triplicate determinations, with standard errors # 0.082)

The hydantoinase of *Blastobacter* sp. Strain A17p-4 has recently been reported to have the ability to hydrolyze cyclic imides, as well as hydantoins (Soong *et al.*, 1999). RU-KM1 and RU-OR were also initially isolated for their ability to hydrolyze hydantoin, thus it was necessary to determine whether succinimide hydrolysis in these two strains was catalyzed by the hydantoinase enzyme, or by a different enzyme, possibly imidase. The method used was based on the suggestion that hydantoin-induced cells would hydrolyze succinimide less efficiently, in the same way that succinimide-induced cells would be less able to hydrolyze hydantoin. This suggestion would possibly hold only if these bacteria could produce both enzymes independently of each other.

The results of this investigation showed that both RU-KM1 and RU-OR could hydrolyze succinimide and hydantoin, whether induced with hydantoin or succinimide (Fig. 6.3.3). It was also observed that RU-KM1 was more active with hydantoin after induction by either inducer, as indicated by the fact that this bacterium produced more ammonia from hydantoin, whether induced with hydantoin or with succinimide. The hydantoin conversion yields were 30.4% and 32.2% for RU-KM1 induced with hydantoin and succinimide, respectively. The succinimide conversion percentages were 21.4% and 22.8% for RU-KM1 induced with hydantoin and succinimide, respectively.

RU-OR, on the other hand, was observed to show higher biocatalytic activity with succinimide than with hydantoin, whether induced with hydantoin or succinimide. RU-OR induced with hydantoin gave 10.6% hydantoin conversion, whereas succinimide conversion was 29.5%. RU-

OR induced with succinimide gave 10.6% hydantoin conversion, and 23.5% succinimide conversion.

These results suggest that the hydantoin hydrolysis and succinimide hydrolysis systems, in both RU-KM1 and RU-OR, may be related to each other, since similar levels of activity were observed with both substrates were observed, whether hydantoin or succinimide was used as an inducer. There is also a possibility that both hydantoin and succinimide induce the expression of the same enzyme, i.e. hydantoinase, since these substrates have similar chemical structures (Fig. 6.1.1). However, this does not exclude the possibility that hydantoin and succinimide hydrolysis by RU-KM1 and RU-OR are catalyzed by two different enzymes, *viz.*, hydantoinase and imidase, which, due to the similarity of their substrates, are inducible by either substrate.

Table 6.3.3 NCG Production by RU-KM1 and RU-OR induced with hydantoin or succinimide

Strain	**NCG (mM)**	**% Conversion**
RU-KM1 + H	3.70 "0.028	14.8
RU-KM1 + S	5.32 " 0.01	21.28
RU-OR + H	1.49 " 0.12	5.96
RU-OR + S	0.00 " 0.009	0.00

(**Key:** + H = induced with hydantoin; + S = induced with succinimide)

In a further attempt to elucidate the enzyme system responsible for imide hydrolysis by RU-KM1 and RU-OR, the amount of NCG produced from hydantoin hydrolysis by hydantoin or succinimide induced cells was determined using Ehrlich's assay (Chapter 2). The results showed that RU-KM1 was able to catalyze the production of NCG from hydantoin, whether induced with hydantoin or succinimide (Table 6.3.1). NCG produced by hydantoin-induced RU-KM1 was 3.70mM, whereas that produced by succinimide-induced RU-KM1 was 5.32mM. Low levels of NCG were detected when RU-OR was induced with succinimide. The amount of NCG detected by hydantoin-induced RU-OR was 1.49mM. This supports the suggestion that hydantoin and succinimide hydrolysis in these bacteria may be at least partially independent of each other, since NCG production in RU-KM1 was induced to different levels by hydantoin and succinimide.

However, there is also a possibility that NCG production was higher than that detected, as both strains produce an *N*-carbamylase enzyme, which hydrolyzes NCG to glycine. The structures of the intermediates of hydantoin hydrolysis (*N*-carbamylamino acids) and imide hydrolysis (monoamidated dicarboxylates) are similar, therefore these compounds might be hydrolyzed by the same enzyme, i.e., *N*-carbamylase. Future work on imide hydrolysis should therefore include investigation of this possibility. Thus, to further compare hydantoinase activity in RU-KM1 and RU-OR when induced with succinimide or hydantoin, glycine production has to be determined.

6.4 CONCLUSIONS

Cyclic imide hydrolysis has been studied for the first time in an *Agrobacterium tumefaciens* strain (RU-OR) and in a *Pseudomonas* sp. (RU-KM1). The reaction conditions still need to be optimized for each of these bacteria. It would be of great importance to investigate the effects of parameters such as pH, and temperature on the imide-hydrolyzing activities of RU-KM1 and RU-OR. It would also be useful to determine the growth phase where maximum enzyme expression occurred. A simple technique for quantification of monoamidated dicarboxylates produced from cyclic imide-ring opening, e.g. succinamic acid, also needs to be developed, since this, together with ammonia production, would give an indication of the total cyclic imide conversion. Further work is also required to differentiate between hydantoinase and imidase activity in these strains, and one of the ways in which this may be achieved is by purification of the enzymes.

The stereoselectivity of the imide-hydrolyzing enzymes of RU-KM1 and RU-OR also needs to be determined. Conversion yields in the results reported in this study were always less than 50%, which suggests that the enzymes of the enzymes of these strains are possibly highly stereoselective, since 50% (or less) conversion of a substrate could mean that only one isomer was hydrolyzed by the enzymes. However, a conclusion cannot be drawn from this observation alone, as the yields reported here do not include the intermediate of imide hydrolysis.

This investigation is not complete, as it was a preliminary investigation to determine whether these strains could hydrolyze imides or not. However, some novel findings have resulted from this study. Firstly, the utilization of succinimide as a nitrogen source, the ability of RU-KM1, a *Pseudomonas* sp., to hydrolyze imides without induction, i.e., it is non inducer-dependent, and thirdly, the study of imide-hydrolyzing activity in an hydantoinase-producing *Agrobacterium tumefaciens*. Soong *et al.* (1998) mentioned an *Agrobacterium tumefaciens* and a *Pseudomonas* sp. which had imide-hydrolyzing activity, however, no mention was made on either induction characteristics or which enzyme system was catalyzing hydrolysis of imides in these bacteria. A publication reporting these results is in preparation.

CHAPTER 7

General Discussion

7. 1 GENERAL DISCUSSION

The main objective of this study was to develop biotransformations with potential application in the production of enantiomerically pure amino acids and related compounds, using four previously isolated hydantoinase-producing bacteria, *viz*., RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR.

The first task undertaken was characterization of the hydantoin-hydrolyzing enzymes of RU-KM3$_S$ in crude extract preparations, since this strain was the only one among the four which had only been characterized in whole cells. The effects of various parameters on 5-methylhydantoin hydrolysis by RU-KM3$_S$ crude extract were investigated. The biocatalytic reaction time required for maximum substrate conversion was found to be between 3 and 5 hours, with detectable levels of activity observed even after 1 hour of reaction. The enzymes of RU-KM3$_S$ retained most of their activity after incubation for 3 hours at 40°C. This finding demonstrates the potential of this microorganism for industrial production of amino acids, as stability is one of the prerequisites when assessing the feasibility of using a specific biocatalyst. Hydantoin hydrolysis in RU-KM3$_S$ was, to an extent, metal-dependent, with metal ions such as Mg^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} activating the enzymes, and Cu^{2+} and Fe^{2+} inactivating the enzymes. However, further investigations are required to determine the effects of these metal ions on the activity of the activity of the individual enzymes, and this could be achieved by purifying the enzymes.

The hydantoinase of RU-KM3$_S$ could hydrolyze D- and L-5-methylhydantoin to the same extent, and was thus characterized as non-stereoselective. However, the *N*-carbamylase was found to be L-selective. This was an novel finding which has positive implications because most of the hydantoin-hydrolyzing microorganisms which have been reported are D-selective, and thus there is a need for biocatalysts that are able to produce L-amino acids. This is the first report of an L-amino acid-producing *Pseudomona putida*. Further work, such as isolation of amino acid products, is however required to confirm this finding. Publication of these results is in progress.

The four hydantoinase-producing bacterial strains were all demonstrated to have the ability to hydrolyze a wide range of hydantoin substrates, resulting in the production of a wide range of amino acids, namely, glycine, alanine, valine, leucine, norleucine, *t*-leucine, and *p*-hydroxyphenylglycine, which all have important industrial (pharmaceutical and agricultural) applications. This has led to our laboratory being granted an Innovation Fund by the South African D.A.C.S.T to conduct further development work on amino acid production by these bacteria.

RU-KM1, RU-KM3_L, and RU-KM3_S, were demonstrated to have the ability to hydrolyze nitriles, in addition to hydantoins. All four strains were also demonstrated to be able to hydrolyze amides, which suggests that they also produce amidases. Further work needs to be undertaken to further characterize these enzyme activities in these bacteria. Both nitrile hydrolysis and amide hydrolysis were found to be inducible in these strains, but in this study only one compound was used as an inducer. Thus, effects of various inducers on enzyme activity need to be determined, as certain compounds may act as better inducers of enzyme activity than others. The growth phase at which most enzyme production, and thus activity, occurs also needs to be established in these strains. Effects of parameters such as temperature and pH also need to be determined.

RU-KM1 and RU-OR were demonstrated to possess imide-hydrolyzing activity. This is the first report on the sutdy of imide hydrolysis in either a *Pseudomonas* sp. or an *Agrobacterium* sp. These microorganisms were demonstrated to have the ability to utilize succinimide as a nitrogen source, which is a novel finding because utilization of imides by microorganisms as nitrogen sources has not been reported before. A publication of these results is in preparation.

This activity needs to be further characterized by investigating optimal conditions for maximum product conversion, such as temperature and pH, and growth phase at which maximum enzyme production occurs. The substrate selectivity of the imide-hydrolyzing enzymes of these bacteria has yet to be investigated. Further investigations are also required to determine the enzyme system responsible for imide hydrolysis in these strains, i.e., determine whether it is the hydantoinase or imidase system. This may be achieved by investigating the substrate selectivity of these strains, as hydantoinases only hydrolyze complex imides whereas imidases only hydrolyze simple imides. However, a more confirmatory investigation would involve the purification of these enzymes.

The stereoselectivities of the amide-hydrolyzing enzymes, nitrile-hydrolyzing enzymes, and imide-hydrolyzing enzymes of the four strains need to be determined, as stereoselectivity is one of the major requirements for the successful application of biocatalysts to industrial processes for production of valuable compounds, particularly biologically active compounds.

In conclusion, the four strains, RU-KM1, RU-KM3$_L$, RU-KM3$_S$, and RU-OR, have been demonstrated to have a broad range of amide bond-hydrolyzing enzymes. This has positive implications for the industrial application of these biocatalysts, as they may be used for production of a number of different commercially important products, which are mainly carboxylic acids resulting from amide hydrolysis. Products include unusual amino acids such as *tert*-leucine, which is used in the production of a variety of antiviral, anti-tumor, and anti-inflammatory drugs, and valine, which is used in the production of some insecticides. Other products include acrylic acid, which is used in the synthesis of a number of polymers, including flocculants, adhesives, and thickeners. These strains may also be applied to the production of 2-arylpropionic acids, which are non-steroidal, anti-inflammatory drugs produced from hydrolysis of nitriles.

APPENDIX AND BIBLIOGRAPHY

1. Media

1.1 Hydantoin minimal medium

ddH_2O	115mL
1M $CaCl_2$	200µL
1M $MgCl_2$	200µL
4% Hydantoin	250mL
40% Glucose	25mL
10x M9 Salts[a]	100mL
Trace elements[b]	10mL

10x M9 Salts[a]

Na_2PO_4	300g
KH_2PO_4	150g
NaCl	25g

(per litre ddH_2O, autoclaved seperately)

Trace elements[b]

Boric acid	50mg
$MnSO_4 \cdot 7H_20$	40mg
$ZnSO_4$	40mg
$(NH_4)_6MO_7O_{24} \cdot 4H_2O$	20mg
KI	10mg
$CuSO_4$	4mg

Dissolve all components in 990mL ddH_2O and autoclave.

Add 20mg $FeCl_3$ to 10mL ddH_2O, autoclave separately. Add to the solution above.

1.2 Nitrile minimal media

1% Acrylonitrile minimal medium

- Components prepared as in 1.1, except 4% acrylonitrile used in place of hydantoin

Minimal media containing 10mM nitrile

- Components prepared as in 1.1, except 40mM nitrile used in place of hydantoin

1.3 Amide minimal media

- Components prepared as in 1.1, except 40mM amide used in place of hydantoin

1.4 Succinimide minimal medium

- As in 1.1, except 40mM succinimide used in place of hydantoin

2. Reagents

2.1 Ehrlich's reagent

Make up 10% *p*-dimethylaminobenzaldehyde solution in 6N HCl.

2.2 Ninhydrin reagent

Dissolve 0.8g Ninhydrin and 0.12g hydrindantin in 30mL methoxyethanol. Add 10mL 4M acetate buffer, pH5.5. Prepare fresh, in a brown bottle.

2.3 Phenol-hypochlorite assay reagents

Solution 1: 20g Phenol in 100mL 95% ethanol

Solution 2: Dissolve 25g calcium hypochlorite in 300mL hot ddH_2O. Add 135mL of a 200g/L K_2CO_3 solution. Heat and make up to 500mL with ddH_2O.

3. Analytical

3.1 Colorimetric quantification of *N*-carbamylamino acids

Prepare *N*-carbamylglycine standards ranging from 0 – 50mM using a 100mM stock solution. Aliquot 1mL of each into test tubes, and add 0.5mL of 12% trichloroacetic acid. Add 3mL ddH2O, and then 0.5mL Ehrlich's reagent. Allow to stand at room temperature for 20 minutes. Read absorbance at 420nm. Product concentrations in samples calculated using Quatrro Pro. v.7.0.

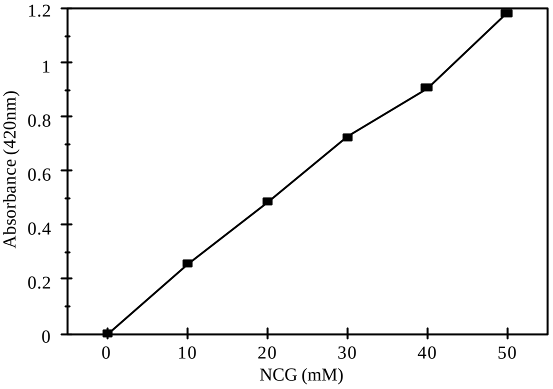


Figure 1. NCG standard curve for determination of *N*-carbamylamino acid production. $R^2 = 0.99797$. (The data represent the mean ± SEM of duplicate determinations)

3.2 Colorimetric quantification of amino acids

Prepare a 1mM stock of alanine, and use this to prepare standards ranging from 0 – 0.5mM. Add 20μL of each to 0.980mL of 0.1M potassium phosphate buffer, pH 8.0. Add 1mL ninhydrin reagent. Boil for 15 minutes and cool at room temperature. Add 3mL 50% ethanol and allow to stand at room temperature for 10 minutes. Read absorbance at 570nm.

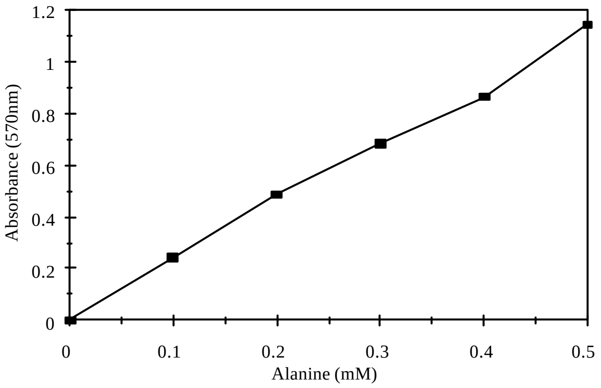


Figure 2. Alanine standard curve used for determination of amino acid production. $R^2 = 0.9967$. (The data represent the mean ± SEM of duplicate determinations)

3.3 Colorimetric quantification of ammonia (Phenol-hypochlorite protocol 2)

Standard solutions ranging from 0 – 25mM ammonium sulfate prepared using a 50mM stock solution. To 100μL of each, add 400μL of solution A (0.59M phenol and 1mM sodium nitroprusside), vortex, and then add 400μL of solution B (0.11M sodium hypochlorite and 2M NaOH). Leave at room temperature for 30 minutes, then read absorbance at 600nm.

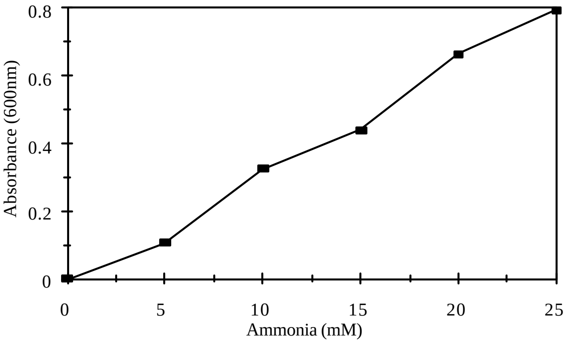


Figure 3. Standard curve used for determination of ammonia production.

$R^2 = 0.9916$. (The data represent the mean ± SEM of duplicate determinations)

4. ^{1H}NMR Spectra of synthesized 5-monosubstituted hydantoins

5-Methylhydantoin:

(* 10.5 (O=C-N$\underline{H}$-C=O); * 7.8 (O=C-N$\underline{H}$-CH); * 4.0 (q, H_3C-C$\underline{H}$); * 1.2 (d, $\underline{H}_3$C-CH))

5-Isopropylhydantoin:

(* 10.5 (O=C-N$\underline{H}$-C=O); * 7.9 (O=C-N$\underline{H}$-CH); * 3.9 (NH-C$\underline{H}$-C=O); * 1.O, (m, H_3C-C$\underline{H}$-CH_3); * 0.8 (2xd, $\underline{H}_3$C-CH-C$\underline{H}_3$)

5-*n*-Butylhydantoin:

(* 7.7 (O=C-N$\underline{H}$-C=O); * 5.5 (O=C-N$\underline{H}$-CH); * 3.9 (m, H_2C-C$\underline{H}$-C=O); *1.4(m,H_2C-C$\underline{H}_2$-CH); * 1.2 (q, H_3C-C$\underline{H}_2$-C$\underline{H}_2$), * 0.8 (t, $\underline{H}_3$C-CH_2))

5-Isobutylhydantoin:

(* 10.5 (O=C-N$\underline{H}$-C=O); * 8.0 (O=C-N$\underline{H}$-CH); * 4.0 (q, NH-C$\underline{H}$-CH_2); * 1.7 (m, $(H_3C)_2$-C$\underline{H}$-CH_2); * 1.3 (m, CH-C$\underline{H}_2$-CH) * 0.8 (m, $\underline{H}_3$C-CH-C$\underline{H}_3$))

5-*t*-Butylhydantoin:

(* 10.5 (O=C-N$\underline{H}$-C=O); * 7.9 (O=C-N$\underline{H}$-CH); * 3.6 (NH-C$\underline{H}$-$C(CH_3)_3$); * 1.1 ($\underline{H}_3$C-$C(CH_3)_2$); * 0.9 (H_3C-$(C\underline{H}_3)_2$-CH))

5-*p*Hydroxyphenylhydantoin:

(* 10.6 (O=C-N$\underline{H}$-C=O); * 9.4 (O$\underline{H}$-Phe); * 8.2 (O=C-N$\underline{H}$-CH); * 7.1 (d, OH-C$(C\underline{H}_2)_2$); * 6.7 (d, $(C\underline{H}_2)_2$-CH); * 5.0 (NH-C$\underline{H}$-Phe))

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