CHARACTERISATION OF THE HYDANTOIN-HYDROLYSING ACTIVITY OF *Pseudomonas putida* STRAIN RUKM3s AND DEVELOPMENT OF A BIOCATALYST FOR AMINO ACID PRODUCTION

by

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ABSTRACT

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This study tested the hypothesis that the hydantoin-hydrolysing enzymes of a novel *Pseudomonas putida,* RUKM3s, with high-levels of activity of a non-stereoselective hydantoinase, and an L-selective *N*-carbamyl amino acid amidohydrolase (NCAAH), could be optimally extracted, partially purified for characterisation, stabilised by immobilisation, and applied as a biocatalyst for production of amino acids from 5-mono-substituted hydantoin substrates.

Experiments were devised to optimise conditions for the production of RUKM3s biomass with high levels of hydantoin hydrolysing activity, and to evaluate techniques of protein extraction, enzyme isolation, purification and characterisation. The NCAAH of RUKM3s is a dimer of approximately 60 kDa, with two subunits of approximately 30 kDa each. The hydantoinase is approximately 210 kDa. Methods of enzyme immobilisation were investigated and operational parameters of the immobilised biocatalysts were evaluated. Stabilisation of biocatalysts by immobilisation revealed that among five methods of immobilisation used, covalent coupling to Eupergit® C provided the most suitable biocatalyst formulation of the RUKM3s enzymes.

A model of the hydantoinase reaction based on the stabilised biocatalyst was developed and tested by empirical studies in a bioreactor system. In the system, the high hydantoinase activity from RUKM3s was coupled with the high NCAAH activity of a mutant *Agrobacterium tumefaciens* strain, RUOR-PN1, to enhance the overall product yield. It was demonstrated that the combined bioreactor system could achieve close to 100 % conversion yields of amino acid, operating in a continuous substrate-feed mode.

SUMMARY

The aim of the research reported in this thesis was to develop a stabilised biocatalyst for the production of amino acids from 5-monosubstituted hydantoins. A novel *Pseudomonas putida* strain, RUKM3s, which has high hydantoinase activity, was used as the source of the biocatalyst. The *P. putida* strain RUKM3s produces a non-stereoselective hydantoinase that converts 5-monosubstituted hydantoins to *N*-carbamyl amino acids, and an L-selective *N*-carbamoyl amino acid amidohydrolase (NCAAH) that converts *N*-carbamyl amino acids to amino acids. It therefore has potential for use in stereoselective synthesis. L-selectivity among *Pseudomonas* spp. is rare, which makes the strain RUKM3s novel.

Experiments were devised to optimise conditions for the production of RUKM3s biomass with high levels of hydantoin hydrolysing activity, and to evaluate techniques of protein extraction, enzyme isolation, purification and characterisation. Methods of enzyme immobilisation were investigated and operational parameters of the immobilised biocatalysts were evaluated. A model of the hydantoinase reaction based on the stabilised biocatalyst was developed and tested by empirical studies in a bioreactor system. In the system, the high hydantoinase activity from RUKM3s was combined with the high NCAAH activity of a mutant *Agrobacterium tumefaciens* strain, RUOR-PN1, to enhance the overall product yield. It was demonstrated that the combined bioreactor system could achieve close to 100 % conversion yields operating in a continuous substrate-feed mode.

The optimal fermentation conditions for production of RUKM3s biomass were found to be 28 °C, pH 7 and 25-40% relative dissolved oxygen (DO_2) saturation. Grown under optimal conditions, RUKM3s cells had the highest hydantoinase activity after approximately 16 hours of growth and the highest NCAAH activity after 10 hours of growth. The biomass concentration of RUKM3s on the basis of dry cell weight was 11.6 g /L in a specially-formulated '*Pseudomonas putida* medium' (PP2) and 3.3 g/L in nutrient broth supplemented with hydantoin (HNB medium). Sonication was found to be the best method of protein extraction in terms of the amount of protein extracted and the amount of hydantoinase activity measured in the cell-free supernatant after treatment.

When conditions were optimised for the biocatalytic activity of the enzymes, it was found that the optimal temperature for substrate incubation for both hydantoinase and NCAAH was 40 °C, while the optimal pH for NCAAH activity during substrate incubation was 9 and the

optimal pH for hydantoinase activity was 9-10. The amount of product from NCAAH was highest after 5 hours of incubation, while the amount of product from hydantoinase was highest after 3 hours of incubation.

After the conditions for biomass production, enzyme synthesis, and protein extraction had been optimised, various methods were used to isolate and purify the hydantoin hydrolysing enzymes of RUKM3s. The purified enzymes were characterised in terms of molecular weight, number and size of subunits and stereoselectivity. SDS-PAGE analysis and size exclusion chromatography indicated that the NCAAH of RUKM3s is a dimmer of approximately 60 kDa, with two subunits of approximately 30 kDa each. The hydantoinase was found to be approximately 210 kDa. The characterisation of the chiral selectivity of the enzymes of RUKM3s indicated that the system has a non-selective or D-selective hydantoinase activity and an L-selective *N*-carbamoylase activity.

Stabilisation of biocatalysts by immobilisation revealed that among the five methods of immobilisation used, covalent coupling to Eupergit® C provided the most suitable biocatalyst formulation of the RUKM3s enzymes. The matrix coupled with 63% of the solubilised protein and could achieve yields of 57.2 % *N*-carbamyl glycine and 21.6 % glycine when operated in batch reactions. The biocatalyst could be re-used in 18 cycles before activity levels declined to 50% in the case of NCAAH activity, and in 28 cycles in the case of the hydantoinase activity. During storage, the biocatalyst exhibited a half-life of 2-3 weeks. Covalent immobilisation on Eupergit® C was also used to produce a biocatalyst from the enzymes of *Agrobaterium tumefaciens* strain RUOR-PN1. The protein binding yield of RUOR-PN1 was found to be 75 %. The yield from hydantoinase was 45.2 % *N*-carbamyl glycine and that from NCAAH was 40% glycine.

The activities measured for the biocatalysts from RUKM3s and RUOR-PN1 were used to model the hydantoinase reaction and empirical data from their performances in batch and continuously operated reactors were used to design a two-stage bioreactor system that could combine the high hydantoinase activity of RUKM3s and the high NCAAH activity of RUOR-PN1 to achieve high conversion efficiencies. The model of the hydantoinase reaction proposed was used successfully to predict production of NCG and glycine in the combined bioreactor. The modelled values closely approximated the experimental values obtained for the combined bioreactor system. The total conversion of hydantoin to NCG for the combined

bioreactor system was 91 % and the conversion of NCG to glycine was 66.5 %. The process could feasibly be scaled-up and utilised in larger bioreactors in an industrial process. The innovation of bringing together the biocatalytic activity of two different enzymes from two different microbial strains in a dual column bioreactor system, to produce amino acids, has not been reported previously.

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ABBREVIATIONS

ATPS	Aqueous Two Phase System
CSIR	Council for Scientific and Industrial Research
Da	Dalton
D-	*Dextro* rotatory
DO	Dissolved oxygen
EC	Enzyme Commission
EDAC	1-Ethyl-3- (dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylene Disodium-Tetrachloro-acetic Acid
HMM	Hydantoin minimal Media
HNB	Nutrient Broth (supplemented with 0.1% Hydantoin)
HPG	Hydroxyphenyl Glycine
HPH	Hydroxyphenyl Hydantoin
HPLC	High Performance Liquid Chromatography
h	Hour(s)
kDa	KiloDalton
Kd	Distribution coefficient
L-	*Laevo* rotatory
µg	Microgram
mM	Millimolar
min	Minutes
NB	Nutrient broth
NCAAH	*N*-Carbamyl Amino acid Amidohydrolase
NCG	*N*-Carbamylglycine
ND-PAGE	Non-Denaturing Polyacrylamide Gel Electrophoresis
PEG	Polyethylene Glycol
PP2	*Pseudomonas putida* medium, recipe 2
RU	Rhodes University
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
s	Seconds
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Micrograph

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CHAPTER 1
REVIEW OF LITERATURE

1.1 INTRODUCTION

In 1999, amino acids represented an industry worth $5.7 billion worldwide. Forecasts on world demand for amino acids up to 2004 predicted an annual growth of over five percent, driven by burgeoning applications in animal feed, pharmaceutical and nutraceuticals industries. In the United States of America alone, nineteen different amino acids were sold to the value of $1.3 billion in 2000. This figure was expected to grow at 2.9% annually to $1.5 billion in 2005 (Myskens, 2001). The high level of commercial interest in amino acids is a result of a growing understanding of the properties of amino acids, and the many biological functions that they and their derivatives have. This understanding has resulted in an expansion of current commercial usage and potential applications for the future. New production technologies in stereo-selective bioconversion have expanded possibilities for production of enantiomerically pure amino acids, and higher efficiencies in downstream processing have made large-scale production more economical.

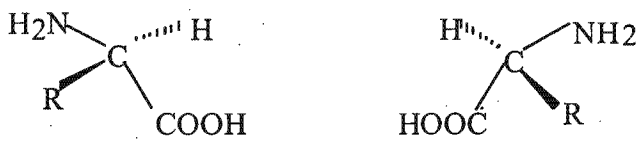
Amino acids are widely used as additives in the food, feed, pharmaceutical and cosmetic industries (Demain and Solomon, 1986); as intermediates in the production of semi synthetic antibodies such as penicillins and cephalosporins (Grifantini *et al.*, 1998); as starting materials for the synthesis of chemicals, peptides and peptide hormones (Lee and Kim, 1998); and as building blocks in the synthesis of pesticides (May *et al.*, 1998b). For food and other nutritional applications, only the proteogenic L-isomers of amino acids are useful (Demain and Solomon, 1986). The largest uses of amino acids are in animal feeds, followed by flavorants and specialty uses, which include feeding solutions and other medical applications. There are many new uses now being developed for amino acids that present opportunities for current and potential new products (Myskens, 2001).

Various methods are currently in use for the production of amino acids from natural and synthetic substrates. Available methods range from exclusively chemical processes to fully enzyme-catalysed biological processes. Enzymatic processes have the ability to produce optically active amino acids. The optical activity of biomolecules is due to their chirality, which

is critical to functionality because enantiomers of the same molecule can possess disparate properties with respect to their effects in biological systems.

1.2 NATURE, FUNCTIONS AND APPLICATIONS OF AMINO ACIDS

Amino acids (also known as aminocarboxylic acids) are organic acids comprising an amino (NH_2) group and a carboxylic acid (COOH) group. Amino acids are classified as α-, β-, γ- ..., depending on the position of the carbon bearing the amino group with respect to the carboxylic group. Fig. 1.1 shows the general structure of α-amino acids.



L-α-amino acid D-α-amino acid

Fig.1.1 Structure of L- and D-α-amino acids

Except in the simplest case where R = H, an amino acid contains a chiral centre (the α-carbon) and hence can exist as one of a pair of enantiomers. The two enantiomeric forms differ so little in structure that of all the physical properties that can be measured only one can distinguish between them. The structures in Fig. 1.1 rotate the plane of polarised light in opposite directions. When prepared from an optically inactive substrate and optically inactive reagents, an amino acid is obtained as a mixture of equal amounts of the two enantiomers, that is, as a racemic modification. Naturally occurring amino acids are not racemic, but optically active, occurring in the L- or D-forms.

The α-amino acids, as components of proteins and peptides, and in their free form, are one of the most important classes of organic substances in cells of living organisms. They are the monomers from which proteins and peptides are derived. There are twenty naturally occurring amino acids. Table 1.1 presents a list of the twenty common amino acids, their structural formulae and molecular weights of the side groups. Nineteen of these are optically active, having the L-configuration. Fourteen of the L-amino acids are essential for nutrition in the diet of human

beings (Rozzell and Wagner, 1992). In animals, proteins are the principal material of the skin, hair, muscles, tendons, nerves, blood, enzymes, antibodies and hormones.

Table 1.1 Structural formulae and molecular weights of side groups of twenty common amino acids.

Name (symbol)	Structural formula	Relative molecular weight of side group
Alanine (Ala or A)	CH_3-CH (NH_2)-COOH	15
Arginine (Arg or R)	HN=C(NH_2)-NH-(CH_2)$_3$-CH(NH_2)-COOH	100
Asparagine (Asn or N)	H_2N-CO-CH_2-CH(NH_2)-COOH	58
Aspartic acid (Asp or D)	HOOC-CH_2-CH(NH_2)-COOH	59
Cysteine (Cys or C)	HS-CH_2-CH(NH_2)-COOH	47
Glutamic acid (Glu or E)	H_2N-CO-(CH_2)$_2$-CH(NH_2)-COOH	73
Glutamine (Gln or Q)	HOOC-(CH_2)$_2$-CH(NH_2)-COOH	72
Glycine (Gly or G)	NH_2-CH_2-COOH	1
Histidine (His or H)	NH-CH=N-CH=C-CH_2-CH(NH_2)-COOH	81
Isoleucine (Ile or I)	CH_3-CH_2-CH(CH_3)-CH(NH_2)-COOH	57
Leucine (Leu or L)	(CH_3)$_2$-CH-CH_2-CH(NH_2)-COOH	57
Lysine (Lys or K)	H_2N-(CH_2)$_4$-CH(NH_2)-COOH	72
Methionine (Met or M)	CH_3-S-(CH_2)$_2$-CH(NH_2)-COOH	75
Phenylalanine (Phe or F)	Ph-CH_2-CH(NH_2)-COOH	91
Proline (Pro or P)	NH-(CH_2)$_3$-CH-COOH	41
Serine (Ser or S)	HO-CH_2-CH(NH_2)-COOH	31
Threonine (Thr or T)	CH_3-CH(OH)-CH(NH_2)-COOH	45
Tryptophan (Trp or W)	Ph-NH-CH=C-CH_2-CH(NH_2)-COOH	130
Tyrosine (Tyr or Y)	HO-*p*-Ph-CH_2-CH(NH_2)-COOH	107
Valine (Val or V)	(CH_3)2-CH-CH(NH_2)-COOH	43

Proteins are composed of up to 16 percent nitrogen. This fact creates the distinguishing difference between proteins and two other basic nutrient groups- carbohydrates and fatty acids. After water, proteins constitute the second greatest portion of the human body by mass. Amino

acids which are not produced by organs and glands of the body are called essential amino acids. These amino acids must be obtained through ingestion of food material that contains them. The essential amino acids include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The remaining amino acids are referred to as nonessential. This does not imply that they are not of essential function; rather it means that they do not need to be obtained through diet, because the body can produce them by itself (Borgeson and Janshekar, 1999). Although it is not common to take supplements containing amino acid formulas, they are available. Amino acid formulas are usually combined with multivitamin formulas in nutritional supplements. Most amino acid supplements are derivatives of animal, yeast, or vegetable protein (Borgeson and Janshekar, 1999).

Several amino acids of the D-configuration also occur in nature; D-alanine, D-glutamic acid and D-2-aminoadipic acid are three such examples found as components in the cell walls of microorganisms or in some of their secondary metabolites. Unnatural D-amino acids such as D-phenylglycine and *p*-hydroxy-D-phenylglycine have applications as side chains for pharmaceutical products such as β-lactam antibiotics (Rozzell and Wagner, 1992).

Methods for the production of optically active amino acids include extraction from plant and animal hydrolysates, chemical synthesis, conversion of carbohydrate substrates such as sugars and molasses, and enzymatic catalysis using stereo-selective enzymes (biocatalysts) to convert amino acid amides (Skepu, 2000). Enantiomerically pure compounds can also be prepared by other methods which include resolution of racemates using enzymes, optically active acids or bases forming diastereomeric salt pairs or chromatographic systems (Drauz, 1997).

There are numerous examples of specific uses of amino acids. D-phenylglycine and D-*p*-hydroxyphenylglycine are important building blocks for semi-synthetic antibiotics (Chien and Hsu, 1996). Others are L-valine which is used in the production of cyclosporin A (Hermes *et al.*, 1993), an immunosuppressant, iso-valine which is a natural substituent of several antibiotics and D-valine which is used as an intermediate in the production of fluvalinate, an insecticide (van der Tweel *et al.*, 1993; Skepu, 2000).

1.3 SYNTHESIS OF AMINO ACIDS

1.3.1. Chemical synthesis of amino acids

Many methods exist for the chemical synthesis of amino acids from a variety of starting materials. The most common of these methods is amination of α-halo-acids. In various modifications, this method may take the form of direct ammonolysis (approximately 70% yield) or indirect halogenation by Heu-Volhard–Zelinsky halogenation or by modification of malonic ester synthesis (35% yield). Yet better yields can be obtained by the Gabriel phthalimide synthesis or by the pthalimidomalonic ester method (Morrison and Boyd, 1983).

Another common method used for chemical synthesis of amino acids is the Strecker amino acid synthesis from aldehydes via α-aminonitrile intermediates (Wagner and Rozzell, 1992). Alkaline or acid hydrolysis can also be used for the chemical production of amino acids from hydantoin substrates. Most of these chemical methods yield synthetic amino acids that are optically inactive and need to be resolved if optically active products are desired. Stereospecific chemical synthesis of unnatural amino acids has been reported on (Mzengeza *et al.*, 2000; Cho *et al.*, 2003). There is also growing interest in synthesis in chiral media to yield optically active amino acids (Syldatk *et al.*, 1990).

Hydantoins and their derivatives were first recognised by Bucherer and Steiner as important synthons in the production of amino acids. Hydantoins are very stable, and therefore, very stringent conditions are required for their chemical hydrolysis. During acid hydrolysis, 60% sulphuric acid at 120-130 °C is required, and in alkaline hydrolysis barium hydroxide is used to achieve the conversion. This renders the chemical processes hazardous and uneconomic. The chemical reactions lead to a racemic mixture of L- and D-amino acids that requires resolution (Syldatk *et al.*, 1990).

1.3.2. Chemoenzymatic synthesis of amino acids using hydantoin-cleaving enzymes

In addition to acid and alkaline hydrolysis, hydantoins can be converted to natural and synthetic amino acids using chemoenzymatic synthesis (Takahashi *et al.*, 1979; Yamada *et al.*, 1987) which combines the hydantoin-cleaving activity of enzymes such as amidases, acylases, and

dehydrogenases with a chemical reaction step. In one example of chemoenzymatic synthesis, a hydantoinase is used to convert 5-monosubstituted hydantoins to *N*-carbamoyl amino acids in the first stage. The second stage is decarbamoylation by chemical diazotisation. The reaction uses nitrite and hydrochloric acid to convert the intermediates to amino acids (Kim and Kim, 1993; Lee *et al.*, 1995). Yields from the chemical reactive step using nitrite and hydrochloric acid are generally low (Olivieri *et al.*, 1978; Grifantini *et al.*, 1996), and large amounts of unreacted intermediate go to waste (Kim and Kim, 1994).

Another example of a chemoenzymatic method of preparing enantiomerically pure amino acids is based on the synthesis of amino acids using a hydantoinase system, followed by a cofactor-dependent enzymatic reductive amination of α-keto acids to L-amino acids. Examples of products obtained this way include some L- and D-amino acids and peptides used for synthesizing drugs and chiral auxiliaries (Drauz, 1997).

1.3.3. Enzymatic synthesis of amino acids by hydantoin hydrolysis

Hydantoins (Fig. 1.2) derive their name from **hyd**rogenation of the naturally occurring compound all**antoin** (-also a product of uric acid degradation) which is used to produce 5-ureidohydantoin. The hydrogenation reaction, which uses hydroiodic acid, was first invented and described by A. von Baeyer in 1861. Besides allantoin, carboxymethylenehydantoin and carboxyethylenehydantoin are the only other naturally occurring hydantoin derivatives described in literature. Modern usages of the name hydantoin and the names 2,4-Imidazolidinedione; 2,4-Imidazolinedione; glycolylurea; and Imidazolidine-2, 4-dione or 2,4-diketotetrahydroimidazole refer to von Baeyer's original compound and the group name of various hydantoin derivatives, including members of a group of substances with anticonvulsant activity used to treat epilepsy (Ware, 1950).

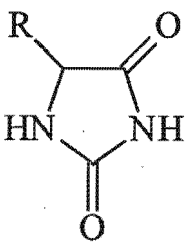


Fig. 1.2 Structure of a 5-monosubstituted hydantoin

Important hydantoin derivatives include the 5-monosubstituted hydantoins used as precursors in the synthesis of D,L-amino acids, and 5,5-disubstituted hydantoins, which are of pharmacological importance as hypnotic and narcotic agents. Various hydantoin derivatives report to a number of different biochemical functions including bactericidal, virucidal, fungicidal, herbicidal and tuberculostatic activities. Due to their high nitrogen content, hydantoins can be biomineralised and used as fertilizer (Skepu, 2000).

Enzymatic conversion of hydantoins to amino acids under optimized conditions can achieve high yields with minimal waste and under mild reaction conditions. Interest in the biotechnological application of hydantoin hydrolysis heightened in the 1970s when the potential industrial production of amino acids as side chains for semi-synthetic penicillins and cephalosporins was recognized. The publication in the mid-70s of evidence that partially purified fractions of dihydropyrimidinase from calf liver could produce *N*-carbamoyl-D-amino acids indicated that the enzyme could find application in the preparation of optically active amino acids (Dudley *et al.*, 1974).

Optically pure amino acids can be synthesized by the stereo-selective enzymatic hydrolysis of 5-monosubstituted hydantoins to their corresponding amino acids by two-step carboxylic acid amide hydrolysis reactions (Fig. 1.3). Various amino acids can be synthesized depending on the nature of the side group, R. Table 1.2 shows a list of some 5-monosubstituted hydantoins and the amino acids produced from them.

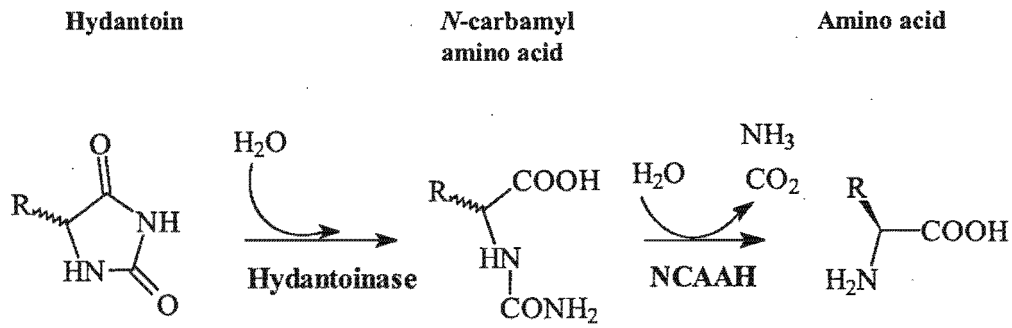
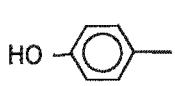


Fig. 1.3 Hydantoinase reaction (Grifantini *et al.*, 1998)

Table 1.2 Amino acids synthesized from 5-monosubstituted hydantoins

R- group	Hydantoin derivative	Amino acid
H-	Hydantoin	Glycine
CH_3-	5-Methylhydantoin	Alanine
$(CH_3)_2CH$-	5-Isopropylhydantoin	Valine
$(CH_3)_2$-CH-CH_2-	5-Isobutylhydantoin	Leucine
CH_3CH_2 CH_2 CH_2-	5-*n*-butylhydantoin	*nor*-Leucine
$(CH_3)_3C$-	5-*tert*-butylhydantoin	*tert*-leucine
HO 	*p*-Hydroxyphenyl Hydantoin	p-Hydroxyphenyl Glycine

In hydantoin hydrolysis, an L- or D-selective or non selective hydantoinase converts a 5-monosubstituted hydantoin to an L- or D- (or mixture of L- and D-) *N*-carbamyl amino acid(s). Thereafter, an L- or D- selective NCAAH converts the *N*-carbamylamino acid to an L- or D-amino acids. The stereoselectivity of the reactions may arise from the activity of either the hydantoinase or the NCAAH, or it may be substrate-dependent. A number of microorganisms that produce both hydantoinase and NCAAH have been described in literature (Yokozeki *et al.*, 1987).

At a technological level, the enzymatic conversion of substituted hydantoins to amino acids can be accomplished in one step if both enzymes needed for the biotransformation are present in the same system. Kim and Kim (1994) achieved a one-step enzymatic production of D-*p*-hydroxyphenylglycine from 5-substituted hydroxyphenylhydantoin using the strain *Agrobacterium* sp I-671 which possessed D-hydantoinase and *N*-carbamoylase activity.

However, in many multi-enzyme biotransformation systems, the activity of one of the enzymes can be rate limiting. In a study aimed at improving D-HPG production from D-HPH using a recombinant *Escherichia coli*, it was found that D-hydantoinase activity was increased 2.57-fold but carbamoylase activity remained constant, which resulted in only a 30% increase in the reaction rate, suggesting that hydrolysis by *N*-carbamoylase was the rate-limiting step of the reaction (Chao *et al.*, 1999). In many microbiological systems that have hydantoin hydrolysing enzymes, the conversion of hydantoins to *N*-carbamyl amino acids and amino acids is accompanied by the interconversion of D- and L- forms of the molecules, which is mediated by racemases. The presence of racemases in hydantoin cleaving systems is variable (Watabe *et al.*, 1992).

1.4 ENZYMES IN BIOCATALYSIS

1.4.1 Enzyme structure and function

Enzymes are functional proteins of high molecular weight (15 000 to several million Daltons) that act as catalysts, mediating cellular reactions. The available range of potential biocatalysts is quite wide. It is estimated that the total enzyme genetic diversity may be as great as 10^{13} distinct functional sequences (Burton *et al.*, 2002). Even though this number is substantially reduced on account of the high homology between enzymes from related and unrelated species, it is a fact of the extensive genetic diversity of the microorganisms, plants and animals from which enzymes are derived that there is a high number of potential biocatalysts as yet unexploited. Latest advances in recombinant DNA technologies add to the range of potential biocatalysts by enabling pre-selection of 'ideal characteristics' of desired biocatalysts to suit 'ideal' process conditions of choice. Examples of biocatalyst characteristics that can be improved include turnover, enatioselectivity, functionality, conversion of non-natural substrates, enzyme stability and enzyme activity (Burton *et al.*, 2002).

As biocatalysts, enzymes are versatile and very effective catalysts because they are capable of working under conditions of mild pH, temperature and pressure, and they present fewer safety and pollution concerns compared to chemical catalysts. In biological systems, enzymes are organized into complexes which carry out multi-step conversions constituting biochemical pathways where a product from one reaction becomes substrate for the next, until a full conversion from one substrate to a required product is accomplished. Many different biochemical pathways are identifiable in living systems (Alcamo, 1996).

Enzymes acting on their natural substrates are generally very selective in terms of chemoselectivity, regioselectivity, diastereoselectivity and enantioselectivity. Enantioselectivity derives from the fact that enzymes are chiral reagents, exclusively constituted from L-amino acids, and any chirality present in the substrate molecule leads to the formation of diastereomeric enzyme-substrate (product) complexes, thus resulting in a highly stereoselective reaction (Azerad, 1995).

As microbial products, enzymes are easy to produce in high yields. A wide variety of enzymes can, potentially, be obtained from one culture. The wide range of environments in which microorganisms exist leads to a diversity of enzymes with a wide range of stabilities and activities. However, under certain conditions, enzymes can have disadvantages that include limited operational and storage stability, poor productivity, lower conversion yields and narrow substrate specificity.

1.4.2 Development of a biocatalytic process

The classical approach to the development of a biocatalyst for industrial bioconversion is a multi-stage process that involves the development of analytical methods for qualitative and quantitative detection of substrate and product; screening for a suitable biocatalyst (an enzyme) to achieve the desired conversion; optimisation of conditions for microbial growth; induction and optimisation of enzyme synthesis; optimisation of conditions for bioconversion; extraction, isolation and characterisation of enzyme; immobilisation and reuse of free enzyme or resting cells; isolation and purification of the product; and laboratory-scale process development (Syldatk *et al.*, 1992). This scheme of activities is presented in Fig. 1.4 as a model for the

development of a bioconversion process. The model incorporates a variety of techniques that enable genetic manipulation of sources of enzymes such as microorganisms, and the design of proteins (enzymes) of 'ideal' functionality to suit processes of choice where natural forms of the biocatalysts are unavailable.

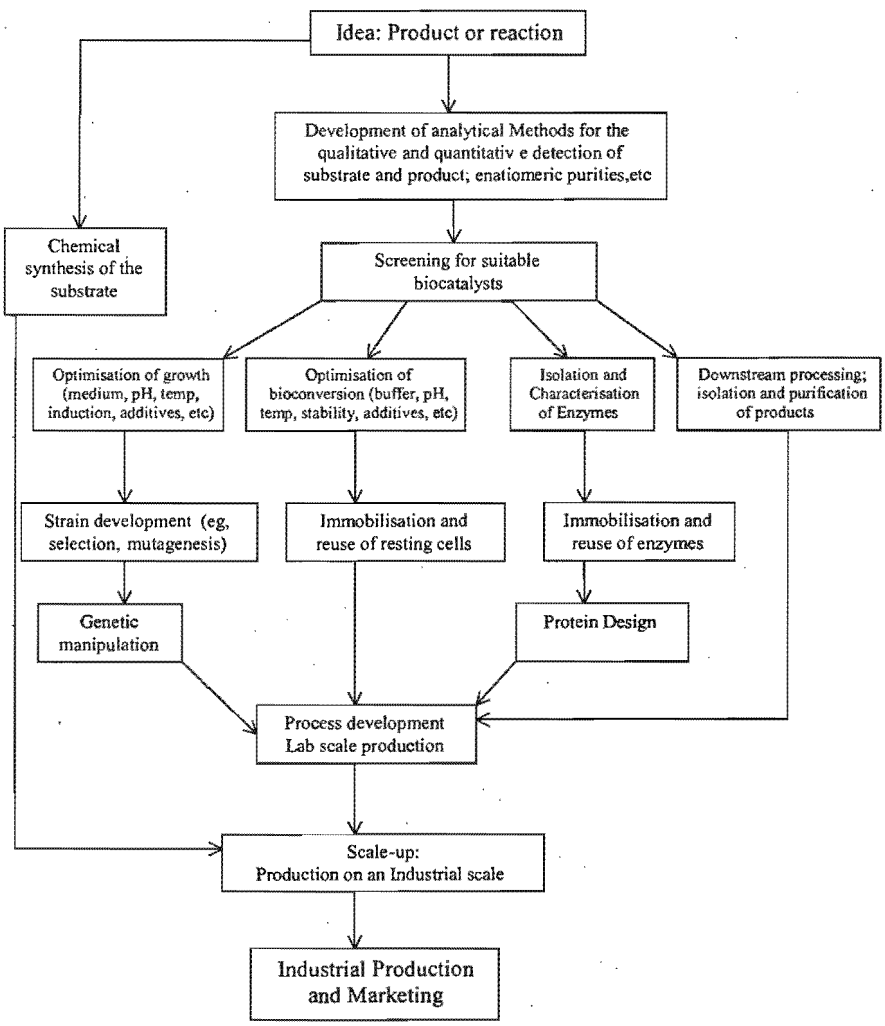


Fig. 1.4 Development of an industrial bioconversion process (taken from *Rozzell and Wagner, 1992*)

In some enzyme-based processes, it is possible to use two enzymes as components of the same biocatalyst. The efficiency of a dual-enzyme process can be limited if the enzymes have different reaction rates, and one of the reactions is slower than the other. It can also be difficult to find common optima of biocatalytic reaction conditions for a two-enzyme system in terms of enzyme activity and stability if their properties differ significantly. It has been postulated that these problems can be overcome by separation of the two component reactions through a two-step process that uses free or immobilised forms of each of the enzymes in one or the other of the two stages.

Predicting the future use of biocatalysts for the transformation of natural substrates and other organic compounds needs to take many factors into account. To date, relatively few biotransformations have been developed to an industrial scale. This is primarily because there has been little economic incentive to replace existing successful chemical processes with biocatalysts, despite their inherent problems of instability, lack of selectivity and narrow operational range. However, advances that improve biocatalyst performance, coupled with an increasing emphasis on 'chirotechnology' are driving the development of biocatalysts as a complementing, and not a rival technology to existing chemical approaches (Faber and Fransen, 1993). Like all industrial processes, the competitiveness of enzymatic processes is affected by costs of substrate; costs of producing or isolating enzymes; possible space or time yields and costs of isolating products. The biocatalyst can often represent a significant proportion of process operating costs (Burton *et al.*, 2002). All these factors are dependent on the nature of the desired product (Tramper, 1985).

1.5 HYDANTOINASES

1.5.1 History of hydantoinases

The earliest reports of hydantoinases in literature date from the 1930s. In 1926 Gaebler and Ketch investigated the metabolism of hydantoin derivatives. By 1950 Eadie *et al.* had reported the presence of hydantoinase in plants. Characterisation of hydantoinases started in the 1950s when the role of dihydropyrimidinase in pyrimidine metabolism was first published. Liebermann and Kornberg revealed the existence of 5-carboxymethylhydantoinase during an investigation of

the metabolism of orotic acid in microorganisms in 1954. By 1958, the value of hydantoinases in the production of optically pure amino acids was still not yet known. The main focus of that era was investigations of metabolic pathways of natural hydantoins and the fate of their derivatives. Many of those derivatives were anti-convulsant drugs used in the treatment of epilepsy (Rozzell and Wagner, 1992).

Investigating the metabolism of 5-phenylhydantoin and its 3-ethyl and 3-methyl derivatives, Dudley *et al.* (1970) described how 5-phenylhydantoin specifically produced D-phenylhydantoic acid. They also proposed spontaneous or enzymatic racemisation of the unhydrolysed L-phenylhydantoin. Bius and Dudley later confirmed the D-specificity of the hydantoinase from calf liver and the chemical racemisation of phenylhydantoin under the reaction conditions used (Cecere *et al.*, 1975).

Interest in hydantoinases for amino acid production grew in the 1970s. The first patent applications on hydantoinases were lodged in the years 1976-1978 due to growing demand for unnatural amino acids such as D-phenylglycine, D-*p*-hydroxyphenylglycine and D-phenylglycine used as side chains for semi synthetic antibiotics. In one of the earliest patents Dinelli *et al.* (1976) laid claim to a 'process for the preparation of L-carbamylamino acids and the corresponding L-amino acids' in United States Patent No. 3,964,970. In this process L-selective hydantoinases from calf liver were used to produce L-carbamoylamino acids and L-amino acids. In the following year, in US Patent No. 4,016,037, Mitsugi *et al.* (1977) patented a method for producing L-amino acid by fermenting hydantoin substrates with *Flavobacterium aminogenes*. There have since been many patents issued for novel processes, novel strains and novel enzymes that are used to produce amino acids.

From the late 1970s to the early 1990s, the distribution of D-and L- specific hydantoinases from different microorganisms and other natural sources was explored and efforts were made to optimise process conditions, as well as isolate and characterise the enzymes for industrial use. It was around this time that *N*-carbamoylases were discovered, allowing hydrolysis of *N*-carbamylamino acids to their amino acids without chemical decarbamoylation. Hydantoin racemases were also discovered, enabling the interconversion of D- and L- isomers. The

emergence of intense biotechnological interest in hydantoinases in this period marginalized the earlier investigations into their metabolic roles (Rozzell and Wagner, 1992).

Functionally related amidohydrolases seem to share a close evolutionary relationship. Kim *et al.* (1997) cloned the gene coding for the thermostable D-hydantoinase from *Bacillus stearothermophilus* and completely determined its nucleotide sequence. The D-hydantoinase protein showed amino acid sequence homology (20-28%) with other hydantoinases and functionally related allantoinases and dihydroorotases. More evidence of this close evolutionary relationship among functionally related amidohydrolases was published by Kim and Kim (1998b). Unclassified hydantoinase-related enzymes include imidases, which degrade succinimide, and carboxyethylhydantoinase, which is involved in the degradation of histidine.

Hydantoin hydrolysing enzymes are very diverse. Individual hydantoinases, NCAAHs and racemases are almost as unique and varied as the different microbial, plant and animal species from which they derive. For example, it is known that among hydantoinases from microbial sources, the hydantoin cleaving enzymes of *Bacillus* spp and *Clostridium* spp have properties and substrate specificities distinctly different from one another and from those of *Arthrobacter* spp and *Flavobacterium* spp (Rozzell and Wagner, 1992).

1.5.2 Classification of hydantoinases

Hydantoinases and *N*-carbamoylases are classified in the Enzyme Commission (E.C.) nomenclature as hydrolases (E.C. 3) that catalyze carboxylic acid amide hydrolysis (E.C. 3.5), also known as amidohydrolases or amidases. Amidohydrolases are sub-divided into *N*-carbamoylamino acid amidohydrolases (*N*-carbamoylases, E.C. 3.5.1), which hydrolyze linear amides, and hydantoin amidohydrolases (hydantoinases, E.C 3.5.2), which hydrolyze cyclic amides.

In 1957, Wallach and Grisola proposed that hydantoinases were functionally identical to the cyclic amide dihydropyrimidinase (E.C. 3.5.2.2). These authors demonstrated that an 80% purified hydantoinase hydrolysed hydantoin, dihydrouracil and dihydrothymine. However, in 1958 Campbell found that an enzyme from *Clostridium uracilium* hydrolysed dihydrouracil but neither dihydrothymine nor hydantoin. In 1993, Runser and Meyer isolated an enzyme from an

Agrobacterium sp. that hydrolysed hydantoin and 5-monosubstituted hydantoin derivatives but not dihyropyrimidine. Later, Ogawa *et al.* (1995a, 1997) reported an enzyme thought to be an imidase from a *Blastobacter* sp. that hydrolysed dihydropyrimidines and hydantoin but had different metabolic function from dihydropyrimidinases. These findings established that dihydropyrimidinases and hydantoinases are not necessarily the same enzymes.

Three other cyclic amide amidohydrolases are described in literature: allantoinase (E.C. 3.5.2.5) which hydrolyses allantoin; carboxymethylhydantoinase (E.C. 3.5.2.4) which catalyses the branching reaction of the pyrimidine degradation pathway; and *N*-methylhydantoinase (E.C. 3.5.2.14) which degrades creatinine. All four share the catalytic function of acting on the cyclic amide ring.

The second enzyme, NCAAH, exists in D- and L- enantioselective forms, both of which used to be classified as functionally similar to β- ureidopropionase (E.C. 3.5.1.6), which hydrolyses *N*-carbamoyl alanine to alanine. The D- and L-selective isomers of NCAAH are now individually classified as *N*-carbamoyl-D-amino acid amidohydrolase (E.C. 3.5.1.77) (Ogawa *et al.*, 1993) and *N*-carbamoyl-L-amino acid amidohydrolase (E.C. 3.5.1.87) (Ogawa *et al.*, 1995).

1.5.3 Stereoselectivity of hydantoin-hydrolysing enzymes

D-stereoselectivity among hydantoinases is widely reported in the literature. It is useful in the enzymatic production of optically pure *N*-carbamyl-D-amino acids or D-amino acids from DL-5-mono substituted hydantoin derivatives. Microorganisms with enzymes that have the ability to convert hydantoins to L-amino acids are less usual. Liebermann and Kornberg first described L-selective cleavage of a hydantoin derivative in the 1950s. They found that the enzymes from the anaerobic bacterium *Clostridium oroticum,* involved in the orotic acid degradation pathway, were able to produce L-aspartic acid from the corresponding L-5-carboxymethylenehydantoin.

L-selective cleavage of hydantoins was shown to be different from D-selectivity by Akamatsu in inducer experiments in 1960. Tsugawa *et al.* (1966) reported the L-selective cleavage of carboxyethylene hydantoins in the 1960s. In screening experiments, Tsugawa *et al.* (1966) isolated strains of the genera *Pseudomonas*, *Micrococcus*, *Aerobacter*, *Achromobacter* and *Bacillus*, able to produce L-glutamic acid from D,L-5-hydantoin propionic acid. *Bacillus brevis*

ATCC8185 was the first microorganism used for the 90% biotechnological conversion of a racemic hydantoin derivative to an L-amino acid, L-glutamic acid (Syldatk and Pietzsch, 1995).

Ishikawa *et al.* (1997) studied the mechanism of stereospecific conversion of D,L-5-substituted hydantoins to the corresponding L-amino acids by a strain of *Pseudomonas*. The results indicated that the hydantoinase catalysed the hydrolysis reaction of D- and L-5-(2-methylthioethyl) hydantoin, and that the hydrolysis of the L-enantiomer proceeded preferentially compared with that of the D-enantiomer. On the basis of these findings, it was speculated that the mechanism consisted of an initial exclusive conversion of DL-5-substituted hydantoins to the L-forms of the corresponding *N*-carbamyl-amino acids by the hydantoinase in combination with a hydantoin racemase, followed by the conversion of the *N*-carbamyl-L-amino acids to L-amino acids by the *N*-carbamyl-L-amino acid amidohydrolase (Ishikawa *et al.*, 1997).

L-selective hydantoin cleavage has been reported in bacteria of the genera *Arthrobacter*, *Bacillus*, *Clostridium*, *Flavobacterium*. Those reported include a *Flavobacterium* sp that converts aromatic substrates with non-selective hydantoinase and L-selective NCAAH, *Bacillus brevis* with L-selective hydantoinase and NCAAH and *Pseudomonas* strain NS671 which is able to catalyse the conversion of 2-methylthioethylhydantoin to L-methionine using a non-selective hydantoinase, L-selective NCAAH and a racemase which converts D-hydantoins to their L-isomers (Rozzell and Wagner, 1992).

1.5.4 Hydantoinases in biocatalysis

Chemoenzymatic hydrolysis of hydantoins utilises enzymatic hydrolysis followed by a chemical decarbamoylation step. Fully enzymatic hydrolysis accomplishes both steps stereospecifically, without the need for chemical reagents and avoids any loss of product that may be caused by chemical reactants. In instances where the hydantoinase is not enantiospecific, a selective carbamoylase in the second step can assure a high degree of enantiomeric purity of the amino acid produced.

The usefulness of biological catalysts can be enhanced by immobilisation of the enzymes, crude extracts or cells. There are numerous reports on the use of immobilised enzymes in hydantoin-hydrolysing reactions (Dinelli *et al.*, 1978; Olivieri *et al.*, 1979; Lee *et al.*, 1996). In this regard,

the use of immobilised cells is more common than the use of enzymes (Syldatk *et al.*, 1999). Except for a process that uses dihyropyrimidinase from calf liver (Cecere, 1977) most processes use free or immobilised resting cells rather than isolated enzymes.

Rapid racemisation of many hydantoins can allow up to 100% conversion of racemic hydantoins into optically pure products in one step. Yields can be increased through chemical racemisation of unconverted enantiomers. Keil *et al.* (1995) prepared a series of 14 D-α-amino acids in high chemical and optical yields from the corresponding racemic hydantoins by employing two novel hydantoinases from thermophilic microorganisms. These thermostable D-hydantoinases catalysed the highly enantioselective hydrolysis of hydantoins with wide structural variety.

Several methods have been used to purify hydantoinases from microbial sources. The general procedure typically follows the steps of cell disruption, separation of crude extract from cell debris, crude precipitation, separation of protein pellet, and purification and characterisation of the active protein. Most purified hydantoinase proteins are ultimately kept as solutions, freeze–dried extract powder or protein crystals (Abendroth *et al.*, 2000).

A common problem with many purification protocols is that for many intracellular enzymes, including hydantoinases, much of the activity is lost during purification. Morin (1986) applied a number of purification methods to hydantoinase enzymes from *Pseudomonas putida* strain DSM 84, and found that much of the hydantoinase activity of the strain, which was high and stable in crude extract, was lost during separation from the other proteins (Morin *et al.*, 1986).

1.5.5 Reported characterisation of purified hydantoinases

Most reported characterisations of the hydantoinase and *N*-carbamoylase enzymes indicate that whereas the sources and conditions for the optimal activity of hydantoinases may differ, the molecular weight and subunit structure of the enzymes are quite similar. However, this is not to suggest that such enzymes are identical. According to most reports, the molecular weight of hydantoinase is between 200 and 230 kDa, and that of the *N*-carbamoylase is 55-60 kDa.

Kauz and Shnackerz (1989) isolated a 5,6-dihydropyrimidine amidohydrolase from an acetone powder of calf liver and purified the enzyme to homogeneity. The purification made use of heat

treatment, ammonium sulphate fractionation and chromatography on chelating Sepharose and DEAE-Sepharose with 44% recovery of total activity. The native enzyme had a molecular mass of 217 kDa consisting of four subunits with a molecular mass of 54 kDa each (Kauz and Shnackerz, 1989).

Kikugawa *et al.*, (1994) purified another dihydropyrimidinase over 564-fold from a rat liver extract, using heat, ammonium sulphate fractionation, and chromatography by DEAE-Sepharose CL-6B, carboxymethyl-Sepharose CL-6B, hydroxyapatite and Sephacryl S-300. The purified enzyme was shown to be homogeneous by gel electrophoresis both in the presence and absence of SDS. Its molecular mass, determined by gel filtration, was 215 kDa and the subunit mass was 54 kDa. The enzyme catalysed the reversible cyclization of 5,6-dihydrouracil to *N*-carbamoyl-α-alanine, or 5,6-dihydrothymine to *N*-carbamoyl-α-aminoisobutyric acid.

Luksa *et al,* (1997) purified a D-hydantoinase to homogeneity from *Bacillus circulans*. A purification of 243-fold was achieved with an overall yield of 12%. The relative molecular mass of the native enzyme was 212 kDa and that of the subunit was 53 kDa. The enzyme was sensitive to thiol reagents and required metal ions for its activity. The optimal conditions for the hydantoinase activity were pH 8.0-10.0 and a temperature of 75 °C. The enzyme was most stable in a pH range of 8.5-9.5 and temperature up to 60 °C, and it was significantly stable not only at high temperatures, but also on treatment with the protein denaturant SDS.

Morin *et al.* (1986) purified a hydantoinase from a strain of *Pseudomonas fluorescens* by hydrophobic interaction chromatography on phenyl-Sepharose, gel filtration on Sephacryl S-400, and preparative electrophoresis. Molecular weight values of 230 and 60 kDa for the native enzyme and each of the four subunits were estimated for the hydantoin-hydrolysing enzyme. The hydantoinase was stable at temperatures up to 40 °C but showed an optimal activity at 55 °C. The enzyme was markedly inhibited by copper, *p*-hydroxymercuribenzoate, 8-hydroxyquinoline, and 2,2-dipyridyl but not by zinc, and poorly by EDTA and *o*-phenanthroline. The hydantoin-hydrolysing activity could be reactivated by ferrous ions. Dihydrouracil was the most readily hydrolysed substrate. The enzyme could also hydrolyse 5-substituted hydantoins such as isopropylhydantoin (to *N*-carbamoyl-D-valine) continuously for 10 days in a membrane reactor with 30% conversion (Morin *et al.*, 1986).

Lee *et al.* (1995) purified a thermostable hydantoinase from *B. stearothermophillus.* The relative molecular mass of the hydantoinase was determined to be 226 kDa by gel-filtration chromatography, and a value of 54 kDa was obtained as the molecular mass of the subunit on analytical SDS-PAGE. The hydantoinase was strictly D-specific and metal-dependent. The optimal pH and temperature were about 8.0 and 65 °C respectively, and the half-life of the D-hydantoinase was estimated to be 30 min at 80 °C, indicating the most thermostable enzyme so far (Lee *et al.*, 1995).

In 1997, Graf *et al.* reported finding a new amidohydrolase capable of deacetylating several *N*-acetyl-1-phenylethylamine derivatives (R)-specifically, in a strain of *Arthrobacter aurescens.* The amidohydrolase had a molecular mass of 220 kDa estimated by gel filtration. SDS-PAGE showed two subunits with molecular masses of 16 kDa and 89 kDa. The optimum pH and temperature were pH 8 and 50 °C, respectively. The enzyme was stable in the range of pH 7-9 and at temperatures up to 30 °C (Graf *et al.*, 1997).

1.5.6 Strain improvement for hydantoin hydrolysis

Microbiological strain improvement through selective screening of organisms, excision of relevant genes and cloning them into appropriate vectors for expression in suitable hosts is an important part of the process of developing biocatalysts. While hydantoin-hydrolysing enzymes from some microorganisms have been identified as potential biocatalysts for the commercial production of amino acids, the enzymes are mostly produced in heterologous hosts such as Escherichia coli due to the fact that the activity of the enzymes in the native strains are tightly regulated by growth conditions (Hartley *et al.*, 2001). However, a few mutants of native strains such as RUOR-PN1F9, a regulatory mutant of *Agrobacterium tumefaciens* have native enzyme production levels equivalent to those achieved by heterologous expression systems (Hartley *et al.*, 2001).

Various efforts have been made to clone and express the genes for hydantoin-cleaving activity with significant levels of success. Buson *et al.* (1996) obtained a clone positive for D-carbamoylase activity by screening a genomic library of *Agrobacterium radiobacter* in *E. coli.* The D-carbamoylase gene named *cau*A was placed under the control of a T7 RNA-dependent

promoter and expressed in *E. coli*. After induction with isopropyl-thio-α-D-galactopyranoside, the synthesis of D-carbamoylase in *E.coli* reached about 40% of the total protein. The expressed protein was shown to possess a molecular mass on SDS-PAGE of 36 kDa and showed an enhanced stability compared to that of the wild-type enzyme derived from *A. radiobacter*.

Chao *et al.* (1999) successfully cloned the genes encoding D-hydantoinase and carbamoylase from *Agrobacterium radiobacter* into *E. coli*. They found that by over-expressing both D-hydantoinase and carbamoylase, recombinant *E. coli* strains were able to convert D,L-hydroxyphenyl hydantoin (D,L-HPH) to D-*p*-hydroxyphenylglycine (D-HPG) with a conversion yield of 97%, accounting for productivity 5 times higher than that achieved by the *A. radiobacter* native enzymes. Immobilizing the recombinant cells with *kappa*-carrageenan resulted in conversions of 93%, while *A. radiobacter* cells gave 20% conversion within the same period. Since the reaction substrate (HPH) is highly insoluble, achieving sufficient agitation was an important issue in this heterogeneous system (Chao *et al.*, 1999).

Chien *et al.* (1998) cloned a DNA fragment containing the gene for D-hydantoinase from *Pseudomonas putida* into *E. coli*. Under the control of a T5*lac* promoter, and lactose induction, the D-hydantoinase activity of the transformed *E. coli* was found to be about 20-fold higher than that of the wild type (Chien *et al.*, 1998).

In an attempt to select more efficient biocatalysts, Grifantini *et al.* (1998) cloned the hydantoinase and carbamoylase genes from *Agrobacterium tumefaciens* (formerly *A. radiobacter*) in *E. coli*. The genes were assembled to give two operon-type structures, one having the carbamoylase gene preceding the hydantoinase gene and the other with the carbamoylase gene following the hydantoinase gene. The recombinant strains stably and constitutively produced the two enzymes and efficiently converted the corresponding hydantoins into *p*-hydroxyphenylglycine and phenylglycine. The order of the genes within the operon and the growth temperature of the strains turned out to be important for both enzyme and D-amino acid production. The configuration with the carbamoylase gene preceding the hydantoinase gene was the most efficient one when the biomass was grown at 25 °C rather than 37 °C. This biomass produced D-amino acid twice as efficiently as the industrial strain of *A. tumefaciens*. The efficiency was found to be correlated with the level of carbamoylase produced, indicating that

the concentration of this enzyme is the rate-limiting factor in D-amino acid production under the conditions used on an industrial scale (Grifantini *et al.*, 1998).

In 1996 Ishikawa *et al.* reported that an *N*-carbamyl-L-amino acid amidohydrolase was purified from cells of *E.coli* in which the gene for *N*-carbamyl-L-amino acid amidohydrolase of a strain of *Pseudomonas* sp. was expressed. The results of gel filtration chromatography and SDS-PAGE suggested that the enzyme was a dimeric protein with 45 kDa identical subunits. The optimum pH and temperature were 7.5 and 40 °C respectively, with *N*-carbamyl-L-methionine as the substrate. The enzyme activity was inhibited by ATP, and was lost completely in the presence of *p*-chloromercuribenzoate (1mM). The enzyme was strictly L-specific and showed broad substrate specificity for *N*-carbamyl-L-amino acids.

1.5.7 Influence of metal ions on enzyme activity

Many enzymes require metal ions as cofactors in order to have high activity. However, this effect is not universal, and the activity of some enzymes may be inhibited by the presence of some metal ions. A number of reports in literature indicate that hydantoinases may have a metal-dependent inductive mechanism. In a study of a 5,6-dihydropyrimidine amidohydrolase isolated from calf liver and purified to homogeneity, Kauz and Shnackerz (1989) found that the enzyme was a tetramer with subunits that contained one zinc atom per subunit, and the enzyme was slowly inactivated by chelating-agents (Kauz and Shnackerz, 1989).

Gross *et al.* (1990) found that hydantoinase from a strain of *Arthrobacter* spp. could be stabilised over a 24 hour period at 50 °C by the addition of 0.5 mM Mn^{2+} ions. This stabilising role of Mn^{2+} ions was also observed by Ishikawa *et al.* (1996) who found that an *N*-carbamyl-L-amino acid amidohydrolase purified from cells of a recombinant strain of *E.coli* required Mn^{2+} ion (above 1 mM) for optimal activity. In contrast, the metals Cu^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} inhibited the enzyme activity of amidohydrolase in a strain of *Arthrobacter aurescens,*. This inhibition was reversed by addition of the chelating agent EDTA (Graf *et al.*, 1997). In a different study, an *N*-carbamoylsarcosine amidohydrolase, purified from *Pseudomonas putida* 77, was strongly inhibited by Cu^{2+}, Hg^{2+}, and Ag^{2+} ions as well as some thiol reagents (Kim *et al.*, 1986). When characterising *N*-carbamyl-L-amino acid amidohydrolase (E.C. 3.5.1.87), Ogawa *et al.* (1995)

found that Mn^{2+}, Ni^{2+} and Co^{2+} enabled reactivation of enzyme that had been treated with a chelating agent.

May *et al.* (1998a) analysed the metal dependency of a hydantoinase from a strain of *A. aurescens* on the basis of kinetic studies of metal-chelator-caused enzyme inactivation, denaturation and metal-induced reactivation, accompanied by the identification of specific metal binding ligands. The enzyme was found to be inactivated by metal chelating agents and completely dissociated into its subunits. Enzyme activity could be restored in re-associated monomers by the addition of cobalt, manganese or zinc ions, whereas nickel and magnesium were ineffective. Zinc seemed to play an essential role for the catalytic activity as well as the stabilisation of the active quaternary structure of the hydantoinase. Histidine-specific chemical modification of the enzyme caused a complete loss of the catalytic activity and revealed that the histidine residues are the putative zinc binding ligands. It was found to be very likely that at least one metal-ion acts specifically near or at the active site of the enzyme (May *et al.*, 1998a).

1.5.8 Selectivity, specificity, and influence of substrate and product

The synthesis of enzymes can be induced by the presence of their target substrates during cell growth. When the *Agrobacterium tumefaciens* strain RU-OR was studied at Rhodes University, it was found that the strain converted D,L-*p*-hydroxyphenylhydantoin to D-*p*-hydroxyphenylglycine using an enzyme system that involved a racemace, a hydantoinase and a D-selective *N*-carbamylamino acid amidohydrolase. The *N*-carbamoylase was induced by growth in media containing 2-thiouracil. However, some mutant strains with inducer-independent production of the enzymes and/or altered response to substrates have also been isolated. A mutant strain of the *Agrobacterium tumefaciens* (RU-ORL5) was isolated, which expressed the hydantoinase and the NCAAH in the absence of inducer (Hartley *et al.*, 1998). Another strain with great potential for industrial application was RUOR-PN1F9, in which hydantoinase and NCAAH enzyme activity were found to be inducer-independent, and sensitive to nitrogen repression or ammonia shock like the native RUOR strain (Hartley *et al.*, 2001).

Products, by-products and substrate can inhibit the activity of some hydantoin-cleaving enzymes. In a study on inhibition of activity, Kim and Kim (1994) found that *N*-carbamoylase was

severely inhibited by ammonium ions, which are co-produced when D-hydroxyphenylhydantoin is converted to D-hydroxyphenylglycine. In order to increase the conversion yield of the amino acid, simultaneous removal of the inhibitory by-product from reaction mixture was carried out using adsorbents of ammonium ions. The yield reached about 98% in the presence of adsorbents in 27 hours, while only 50 % conversion had been observed in the absence of adsorbents.

In a subsequent study, Kim and Kim (1995) partially purified the same D-hydantoinase and *N*-carbamoylase and then determined the optimal ratio of the D-hydantoinase to *N*-carbamoylase to minimise the accumulation of intermediate (*N*-carbamoyl-D-p-hydroxyphenylglycine) in the direct enzymatic production of D-hydroxyphenylglycine. The sequential reaction was numerically simulated, and the simulation results coincided well with experimental data and the optimal ratio between D-hydantoinase and *N*-carbamoylase was found to be about 1:3 on a weight basis. This implies that the rate of reaction of the hydantoinase is about three-times faster than that of the *N*-carbamoylase.

Other important factors in hydantoin conversion are substrate selectivity and stereospecificity. A D-hydantoinase from *A. tumefaciens* was shown to exhibit distinctive substrate specificity, being most active on 5,6-dihydrouracil and D,L-5-methylhydantoin, but only slight activity on D,L-benzylhydantoin. Extracts or whole cells of *A. tumefaciens* were used as biocatalysts for the stereospecific conversions of D,L-phenylhydantoin and D,L-5-methylhydantoin to the respective *N*-carbamyl D-amino acids. The D-hydantoinase from *A. tumefaciens* was shown to have stereoselectivity in the transformation of a racemic substrate into a product of therapeutic value, ACPA, an excitatory amino acid antagonist (Durham and Weber, 1996).

Kim *et al.* (1986) purified an *N*-carbamoylsarcosine amidohydrolase involved in the microbial degradation of creatine in *Pseudomonas putida* 77. These authors found that ammonia, carbon dioxide, and sarcosine were formed from *N*-carbamoylsarcosine through the action of the purified enzyme preparation. *N*-Carbamoyl derivatives of D-tryptophan, D-phenylalanine, D-phenylglycine and *p*-hydroxy-D-phenylglycine were also hydrolysed by the enzyme. However, reaction of the L-isomers of the *N*-carbamoyl amino acids gave no production of ammonia, carbon dioxide, or the corresponding amino acids, showing that the enzyme was stereoselective for the D-isomers of the substrates.

1.6 SCREENING FOR HYDANTOIN-CLEAVING ACTIVITY

The identification of microbial sources of biocatalytic enzymes useful in biotransformation typically starts with the development of protocols for screening of microorganisms from the environment. Many research groups have isolated and screened microbes for hydantoin-cleaving activity. Hydantoinase-catalysed reactions based on both whole cells and crude extracts of various microorganisms are extensively described in literature. Many of the strains found to be positive for hydantoin-cleaving activity belong to *Pseudomonas* spp. (Sun 1983; Yamada and Kumagai 1978). Degradation of amides by *Pseudomonas* spp. has been extensively documented, even though the substrate specificity of the amidases seems to be variable, suggesting evolutionary differentiation (Ogawa and Shimizu, 1997). *Pseudomonas* spp. offer a great wealth of biochemical variations in amino acid biosynthetic pathways and their regulatory mechanisms. However, most amino acids are also excellent carbon sources for *Pseudomonas* spp., and may be readily utilised for growth. Therefore, the use of whole cells in the production of amino acids, and in the elucidation of the biochemical pathways and their regulation, can be complicated (Ogawa and Shimizu, 1997). Besides hydantoin hydrolysis, many other hydrolases have been identified in *Pseudomonas* spp., including proteases, amylases, PHP depolymerases, xylanases, pectin hydrolases, glycosidases and cellulases (Demain and Solomon, 1985).

Morin (1987) described a method for the isolation of soil microorganisms with high hydantoinase activity. Cultures were plated on different media and cultured under different conditions, and then enriched in liquid media. Rapid, efficient and reliable detection and selection of hydantoinase-positive organisms was achieved by a spot-test based on the direct detection of *N*-carbamylamino acids in agar. Hydantoinase-producing strains growing in solid media were found to produce a yellow colour after 5-10 seconds of reaction, indicating the presence of *N*-carbamylamino acids (Morin *et al.*, 1987).

Immunological techniques can also be used in screening. Siemann *et al.* (1993) used antibodies as screening tools for hydantoin-hydrolysing activity in *Arthrobacter aurescens*. They used a colony transfer procedure for the rapid detection of highly active strains. Polyclonal antibodies were produced against highly purified L-hydantoinase, hydantoin-racemase and L-*N*-carbamoylamino acid amidohydrolase of *Arthrobacter aurescens*.

Gokhale *et al.* (1996) screened 125 strains of *Pseudomonas* spp. from their culture collection for production of hydantoinase activity using D,L-phenylhydantoin as a substrate. A strain of *Pseudomonas desmolyticum* was found to be the best hydantoinase producer. The enzymatic reactions were performed with an 18-20 hour culture grown in nutrient broth and with 5-phenylhydantoin as the substrate. Upon optimization of the biotransformation reaction, the optimum pH and temperature for D-*N*-carbamoylphenylglycine production were found to be 9.5 and 30 °C, respectively. Under these conditions complete conversion of 27.0 gL^{-1} of D,L-phenylhydantoin to 26.5 gL^{-1} of *N*-carbamoyl phenylglycine within 24 hours, with a molar yield of 90% was achieved. The product was pure D-*N*-carbamoyl phenylglycine, which implies that the hydantoinase involved in the transformation process was D-stereospecific. The pure product was further chemically converted to D-phenylglycine using nitrous acid with an 80% chemical yield. Thus, the overall conversion efficiency of D,L-5-phenylhydantoin to D-phenylglycine was found to be 65-68% (Gokhale *et al.*, 1996).

Lee *et al.* (1995) screened one thousand thermophiles isolated from soils for hydantoinase activity and its thermostability. The thermophilic bacterium that showed the highest thermostability and activity was identified as a strain of *B. stearothermophillus* according to morphological and physiological characteristics.

In 1997, Graf *et al.* reported finding a new amidohydrolase capable of deacetylating several *N*-acetyl-1-phenylethylamine derivatives (R)-specifically, in a strain of *Arthrobacter aurescens*. The strain was isolated from a wet haystack by enrichment culture with (R)-*N*-acetyl-1-phenylethylamine as the sole carbon source. (R) and (S)-*N*-acetyl-1-phenylethylamine did not serve as inducers for acylase formation, but improved the growth rate resulting in a 47-fold increase in enzyme production.

Ikenaka *et al.* (1998.) isolated a thermotolerant bacterium that produced *N*-carbamyl-D-amino acid amidohydrolase from soil by enrichment culture at 45 °C with *N*-carbamyl-D-amino acid as the sole nitrogen source. The enzyme activities and substrate specificities of these strains were examined by the resting cell biocatalytic reactions. One of the enzymes, produced by a *Pseudomonas* sp. was purified and characterised and the amino acids of its *N*-terminal region were sequenced.

1.7 RU HYDANTOINASES

Earlier studies by the 'Hydantoinase Research Group' at Rhodes University, Grahamstown, South Africa, have included: screening for hydantoin-cleaving activity; isolation of novel strains RUKM3s, RUKM1, RUKM3$_L$, and RUOR; optimisation of conditions for growth; optimisation of bioconversion of 5-monosubstituted hydantoins; and the development of colorimetric methods for the qualitative and quantitative detection of products. A characterisation of the hydantoin-hydrolysing activity of the enzymes of RUKM3s in resting cells (Buchanan, 1996; Phehane, 1998) and the characterisation of the amide bond hydrolysis in hydantoinase-producing bacteria, including RUKM3s (Skepu, 2000) were also conducted. Among the microbial strains with hydantoin-hydrolysing enzymes isolated at Rhodes University, RUKM3s was found to have potential for use in producing L-amino acids because it has a non-selective hydantoinase and an L-selective *N*-carbamoyl amino acid amidohydrolase (Burton *et al.*, 2001; Matcher *et al.*, 2004). As far as we know, there are no other reports of L-selective carbamoylases from *Pseudomonas* sp., and therefore, the strain RUKM3s is be novel.

A further study on RUKM3s aimed at identifying the hydantoinase and N-carbamoylase-encoding genes in this strain used transposon mutagenesis and insertional inactivation of two genes, *dhp* and *bup*, encoding a dihydropyrimidinase and β-ureidopropionase respectively (Matcher *et al.*, 2004). The insertion of a transposon (plasposon pTnMod-OKm) resulted in loss of hydantoinase and N-carbamoylase activity, indicating that these genes were responsible for hydantoin hydrolysis in RUKM3s.

The strain RUKM3s was originally isolated from soil samples in Grahamstown, South Africa. After isolation and screening for hydantoinase activity, it was identified as a *Pseudomonas putida* by 16s rRNA (RU Hydantoinase Research Group, *Annual Report* 1998). The strain is now deposited with the American Type Culture Collection (accession number ATCC BAA-919). *Pseudomonas* spp. are rod-shaped, gram-negative polar-flagellated, non-sporulating aerobic bacteria. They are very common in soil and in water where they are saprophytes (Palleroni, 1984).

In other studies at Rhodes University, the hydantoin-hydrolysing activity of an *A. tumefaciens* strain RUOR was studied (Hartley, *et al.*, 1998). It was found that the strain converts D, L-*p*-hydroxyphenylhydantoin to D- *p*-hydroxyphenylglycine using an enzyme system that involves a racemace, a hydantoinase and a D-selective *N*-carbamylamino acid amidohydrolase. The *N*-carbamoylase was active at alkaline pH, and was not inhibited by *N*-carbamyl amino acids. Enzyme activity was induced by growth in media containing 2-thiouracil. A mutant strain (RUOR-L5) was isolated, which expressed the hydantoinase and the NCAAH in the absence of inducer (Hartley *et al.*, 1998). A constitutive mutant strain, RUOR-PN1 was found to have a relatively higher *N*-carbamoylase activity than most other strains. In the present work, an attempt was made to produce an immobilised biocatalyst system that combined the high hydantoinase activity of RUKM3s and the high *N*-carbamoylase activity of RUOR-PN1 in continuously operated packed-bed bioreactors.

1.8 PROJECT PROPOSAL

In the present work, a novel species of *Pseudomonas putida,* RUKM3s, with high levels of activity of a non-stereoselective hydantoinase, and an L-selective *N*-carbamyl amino acid amidohydrolase (NCAAH) isolated from soil samples, was studied. Specific research activities in the project included optimisation of growth conditions, optimisation of conditions for enzyme synthesis, determination of conditions for optimal biocatalytic activity, investigation of enzyme extraction, isolation and characterisation; and immobilisation of the enzymes for use as a biocatalyst for the production of amino acids. A model of the operational parameters of the biocatalyst is proposed.

Hypothesis

The hydantoin-hydrolysing enzymes of RUKM3s can be optimally extracted, isolated, purified and stabilized for industrial application as a biocatalyst for stereospecific amino acid production from 5-monosubstituted hydantoin substrates.

Specific Project Objectives

a) **Development and Optimisation of biocatalytic reactions**

On the basis of work previously done, to improve and optimise the yields of *N*-carbamyl amino acids and amino acids using hydantoin-hydrolysing enzymes from RUKM3s, and to obtain information on the optimised conditions for production of the amino acids.

b) **Investigations with a range of substrates**

To test the ability of the enzymes to hydrolyse selected alternative substrates and produce alternative products.

c) **Development of improved and stabilized biocatalyst**

To investigate methods for immobilization and stabilization of enzymes and cells to produce stable biocatalysts, and for application in alternative media.

d) **Isolation, purification and characterization of enzymes**

To investigate advances in methods for isolation, purification, and biochemical characterisation of enzymes, as well as analysis of products.

e) **Modeling the hydantoinase reaction**

To generate data suitable for modeling of the hydantoinase reaction using cells, crude extracts, isolated enzymes and immobilized biocatalysts in order to develop an efficient bioreactor system for the production of amino acids.

CHAPTER 2

OPTIMISATION OF BIOMASS PRODUCTION AND PROTEIN EXTRACTION FOR HYDANTOIN-HYDROLYSING ENZYME ACTIVITY IN RUKM3s

2.1 INTRODUCTION

The objective of the work described in this chapter was to optimise conditions for biomass production, and evaluate the efficacy of a number of cell disruption methods in the extraction of protein, from *Pseudomonas putida* RUKM3s cells. Optimal conditions for biomass production were evaluated on the basis of the amount of biomass produced, and the levels of enzyme activity expressed in the biomass. The optimisation of conditions for extraction of protein was evaluated in terms of the amount of protein released into the supernatant after disruption or permeabilisation of the cells, and on the basis of enzyme activity present in the supernatant after centrifugation to separate cell debris.

Microbial growth and optimal yield of biomass are dependent on a variety of factors that mainly fall into the broad categories of chemical composition of the growth medium and the physical conditions of the growth environment. The chemical components of growth media include macronutrients that supply the elements carbon, nitrogen and phosphorus; electron acceptors such as oxygen and sulphur; and micronutrients such as vitamins and minerals (including trace elements) which are required as essential growth factors. Such components of the growth media need to be optimised to favour the highest yield of biomass for each microbial species.

Physical conditions in the growth environment affect the amount of microbial growth. These attributes include conditions such as temperature, pH and osmotic pressure of media. Temperature and pH affect enzyme activity in a microrganism's metabolic pathways. When the temperature is too low, metabolic activity is reduced, and when it is too high, microbial enzymes and other cell components may become deactivated. A pH that is too high or too low can lead to precipitation of cellular components, reduced metabolic activity and possible cell death. If the osmotic pressure or salt concentration of the medium exceeds the optimum for the microbial cytoplasm, dehydration and shrinkage occur, and if it is less, the microorganism swells and cells

may burst. The physical environment of the growth medium has to be optimised in order to achieve high yields of biomass (Primrose, 1987).

In this study, the effects of using various carbon sources were investigated. The effect of addition of various concentrations of hydantoin on growth of RUKM3s was evaluated. In concurrent collaborative work at Bio/Chemtek, CSIR, Modderfontein (Johannesburg, South Africa) on medium optimisation, PP2 medium was developed for cultivation of RUKM3s biomass. In this study a comparison of biomass concentration from PP2 and HNB media was undertaken. In addition, controlled experiments were carried out in fermenters to determine the optimal temperature, pH and dissolved oxygen (DO_2) conditions for RUKM3s.

The hydantoin-hydrolysing enzymes of RUKM3s are intracellular. Ideally, when using intracellular enzymes in biocatalytic processes, it would be cost effective to use resting cells without additional protein extraction steps. However, some bioconversion products are also excellent nutritional sources for the resting cells of organisms whose enzymes catalyse their formation and they may be utilised for growth. Therefore, the use of whole cells in bioconversion, and in the elucidation of the biochemical pathways and their regulation can become complicated (Stainer, 1966; Ogawa and Shimizu, 1997). Extraction of intracellular enzymes from bacterial cells is achieved by cell disruption, cell lysis or cell permeabilisation. Various techniques exist for the extraction of intracellular enzymes. These include vigorous treatments with ultrasound, high pressure, mechanical abrasion, lytic enzymes, freeze-thawing, osmolysis and detergent treatment. In this chapter, an evaluation of these techniques for isolating RUKM3s hydantoin hydrolysing enzymes is presented.

A rapidly growing aerobic microbial cell culture has a very high demand for dissolved oxygen. Oxygen is used as a terminal electron acceptor during the process of oxidative phosphorylation and is therefore a pre-requisite for energy generation in aerobic microorganisms. Oxygen must therefore be present in sufficient amounts in the direct environment of the organisms, in the growth medium, so that it can interact with the membrane electron transport system to effect oxidation of reduced pyridine nucleotide co-factors (Primrose, 1987).

A major problem with supplying oxygen is that it is only sparingly soluble in aqueous systems. The solubility of oxygen decreases when temperature increases, and in the presence of dissolved

solutes. This means that with limited amounts of dissolved oxygen available in liquid cultures the rate of oxygen utilisation can exceed the rate of oxygen transfer (OTR) to the liquid phase within a short time. The efficiency of oxygen transfer is dependent on the surface area to volume ratio of the air bubbles and the residence time of the bubbles in the liquid broth (Primrose, 1987).

For shake-flask cultures in small volumes, it is possible to supply adequate amounts of oxygen by growing microorganisms with constant agitation. To achieve high cell densities in large-scale cultures the oxygen requirement of the culture can be met only by forced aeration. This is achieved by pumping air into the culture through a sparger with small orifices and agitating the culture vigorously with impellers, normally in a fermentor or a bioreactor (Fig. 2.1). Regulated batch cultures, with controls for pH, DO_2, and temperature are often used in research and applied areas to cultivate cells under optimised growth conditions.

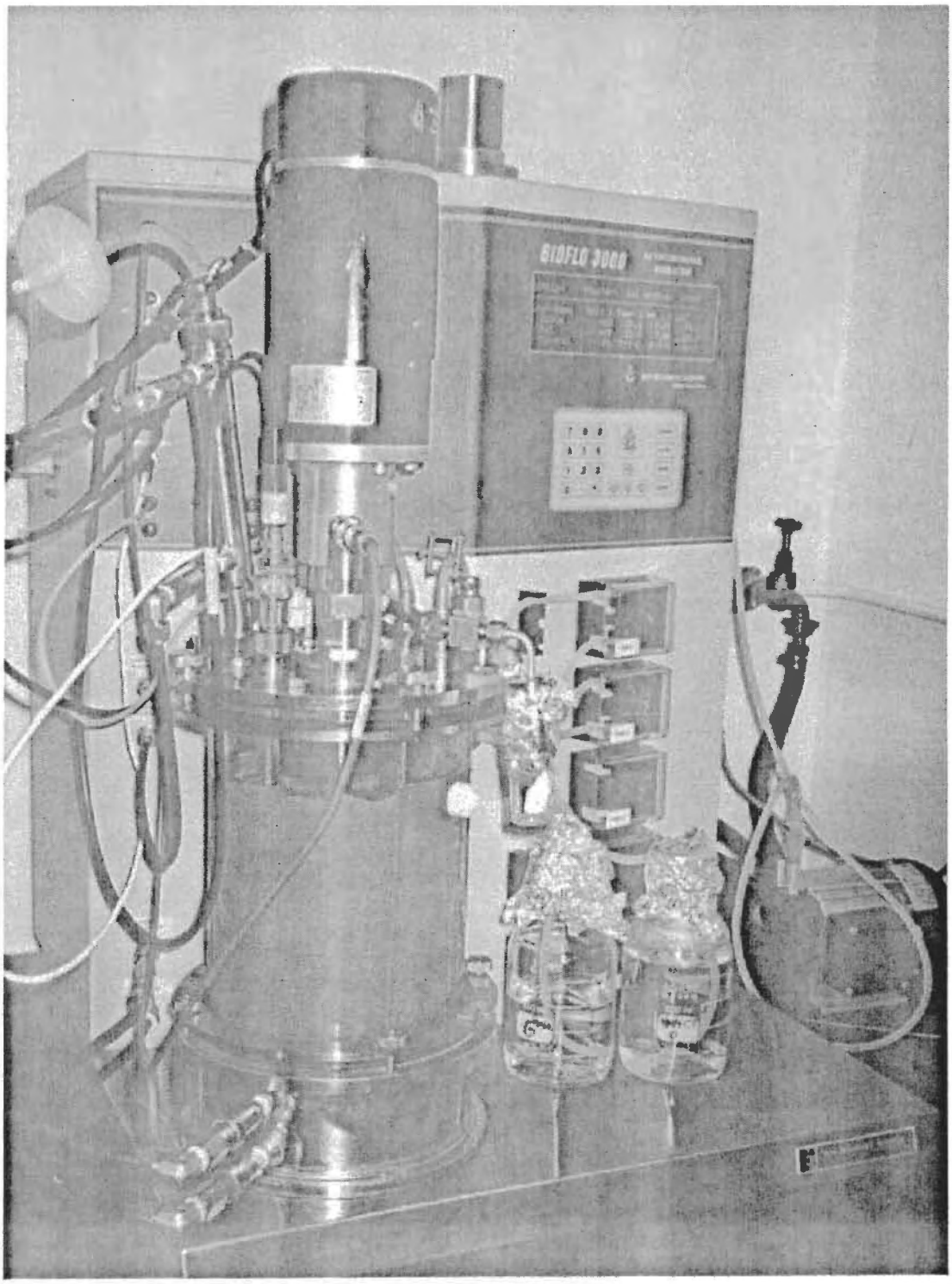


Fig. 2.1 Laboratory scale bioreactor

There are a number of methods that are suitable for disruption of bacterial cells (Table 2.1).

Table 2.1 Techniques and principles of cell disruption for bacteria (Demain and Solomon, 1985).

Technique	Principle
Cell lysis	Osmotic pressure
Enzyme digestion	Cell wall digestion
Chemical solubilisation	Cell wall/membrane solubilised
Homogeniser	Mechanical shear
Grinding with abrasive	Mechanical shear
French press (high pressure)	High pressure disruption
Ultrasonication	Shear and cavitation
Bead milling	Mechanical shear

Most enzymes in current use in bulk quantities are extracellular, whereas the majority of enzymes are either intracellular or membrane-bound (Wiseman, 1995). In order to exploit the full potential of enzymes, cheap efficient methods of extracting and purifying such enzymes have to be investigated. Intracellular enzymes are usually released from cell membranes or from cell organelles by mechanical or chemical disruption, or permeabilization of the cell membranes to allow leakage. In order to release enzymes from cell debris, the homogenates may require solubilisation to weaken the forces binding the proteins to the cell material, which can be achieved by treatment of the disrupted cells with detergent. Gram-positive bacteria are susceptible to enzyme digestion with lysozyme. Gram-negative species are less susceptible to lysozyme and do need prior treatment, such as with non-ionic detergents (Schwinghamer, 1980).

In order to maintain the activity of the enzyme, low temperature must be maintained during cell disruption. If the desired enzyme is susceptible to proteolytic attack, it maybe necessary to add protease inhibitors to the cell suspension before cell disruption. Once the aqueous extract has been separated from insoluble residues by centrifugation or filtration, the solution is clarified. Bacterial extracts may be viscous due to the presence of DNA and may contain ribosomal

material. The viscosity can be reduced by addition of DNAse when making the extract. All nucleic material can be precipitated by aggregation with polycationic macromolecules.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Na_2HPO_4, KH_2PO_4, NaCl, $MgCl_2$, $CaCl_2$, Boric acid, $MnSO_4.2H_2O$, $ZnSO_4$, $(NH_4)Mo_2O_{24}.4H_2O$, KI, $CuSO_4$, agar, glucose, glycerol, glycine, mannitol, nutrient broth and yeast extract were purchased from Merck Chemicals (South Africa). Antifoam 204, Durapol®, Bradford's reagent, hydantoin, hydrindantin, ninhydrin and *N*-carbamylglycine were purchased from Sigma Chemical Co. (St Louis, MO., USA). Sunflower oil was purchased from local supermarkets.

2.2.2 Cell growth

2.2.2.1 Screening and isolation of bacterial cells

Pseudomonas putida RUKM3s cells, previously isolated from the soil samples and screened for hydantoinase activity by members of the Rhodes University's Hydantoinase Research Group, were used for this project (Phehane, 1998).

2.2.2.2 Storage of cultures

RUKM3s cultures were kept refrigerated on Hydantoin Minimal Media (HMM) agar plates for short-term storage and as glycerol stock solutions at –70 °C for long-term storage. HMM agar comprised, per L of distilled, deionised water: 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 10 g glucose, 10 g Hydantoin, 0.02 g $MgCl_2$, 0.02 g $CaCl_2$, 0.5 mg Boric acid, 0.4 mg $MnSO_4.2H_2O$, 0.4 mg $ZnSO_4$, 0.2 mg $(NH_4)Mo_2O_{24}.4H_2O$, 0.1 mg KI, 0.04 mg $CuSO_4$ and 20 g Agar. To prepare glycerol stocks, 1 mL of starter culture in HMM broth was added to 450 µL of a sterile mixture (1:1) of HMM and glycerol in a sterile eppendorf tube. The final concentration of glycerol was 15.5 v/v %. HMM broth (containing 10g Glucose/ Litre) was constituted the same way as the HMM agar, less the 20 g of agar.

2.2.2.3 Plate cultures

Isolates of RU-KM3s were streaked onto plates of HMM agar using sterile technique and the plates were incubated at 25-28 °C for 3-4 days. Sample cultures of RUKM3s were previously identified by 16S rRNA and biochemical analysis (RU Hydantoinase Research Group, 1998). In the present study, the cells were submitted for scanning electron microscopy (SEM) (at Rhodes University) for further characterisation.

2.2.2.4 Seed culture

A single colony of RU-KM3s from a 3-4 day old HMM agar plate was inoculated into 50 mL HMM broth supplemented with 1% hydantoin as the sole nitrogen source and 1% glucose as the sole carbon source. The broth was then incubated at 28 °C with shaking at 200 rpm until early stationary phase (3-4 days).

2.2.3 Optimisation of conditions for biomass production

An inoculum from the starter culture was transferred into 200 mL nutrient broth (NB also abbreviated as HNB when hydantoin has been added) containing 0.1% hydantoin as an inducer, to give an initial OD_{600} of 0.02 absorbance units. The growth medium was then incubated with shaking at 200 rpm until early stationary phase (18-24 h). For larger scale production of the biomass, a 200 mL inoculum was added to a 5-litre fermentor containing 4.5 L growth medium (NB or PP2). PP2 was *Pseudomonas putida* medium formula 2, prepared by mixing per 1.4 L distilled, deionised water: 1.12 g Citric acid, 1.96 g $MgSO_4.7H_2O$, 5.60 g KH_2PO_4, 14.00 g Yeast Extract (or 22.4 g nutrient broth), 1.4 g Sunflower Oil, 1.40 g hydantoin, 0.7 mg boric acid, 0.56 mg $MnSO_4.2H_2O$, 0.56 mg $ZnSO_4$, 0.28 mg $(NH_4)Mo_2O_{24}.4H_2O$, 0.14 mg KI, 0.056 mg $CuSO_4$, and 1.40 ml antifoam (Antifoam 204 or Durapol®). The 5 L cultivation of biomass was conducted in BioFlo 3000 and BioFlo 110 desktop fermentors (New Brunswick Scientific, USA).

2.2.3.1 Effect of different carbon sources on biomass production

RUKM3s cells were grown in nutrient broth, nutrient broth supplemented with 0.1% hydantoin, HMM supplemented with glucose, HMM supplemented with glycerol and HMM supplemented

with mannitol. Growth in the different media was monitored over 24 h by measurement of OD_{600nm} at 2 h intervals.

2.2.3.2 Effect of hydantoin as supplemental nitrogen source on biomass production

OD_{600} readings of cultures growing in nutrient broth, nutrient broth supplemented with 0.01% hydantoin and nutrient broth supplemented with 0.1% hydantoin, were recorded at 2 h intervals.

2.2.3.3 Optimum temperature for biomass production and enzyme expression

Six bioreators containing PP2 medium and set at the following temperatures; 25, 28, 30, 32, 33 and 35 °C, were inoculated with an RUKM3s seed culture. Growth of the cells at the different temperatures was monitored by collection of samples at regular intervals. The biomass in the samples was measured on dry cell weight basis. Duplicate 1 mL portions of the samples in pre-weighed eppendorf tubes were centrifuged for 5 min at 13000 rpm in a microfuge (Biofuge), the pellet washed in 0.1% HCl, centrifuged again and dried in an oven at 80 °C. The dry cell weight of the biomass was determined by difference.

2.2.3.4 Optimum pH for biomass production

Three fermentors containing PP2 medium and set to pH values 6.5, 7 and 7.5, were inoculated with RUKM3s seed culture. The pH was controlled by additons of dilute solutions of NaOH and HCl. The production of RUKM3s biomass was monitored on the basis of dry cell weight as in section 2.2.2.3 above.

2.2.3.5 Optimum dissolved oxygen (DO_2) for biomass production

Four fermentors containing PP2 medium and set to DO_2 levels 10, 25, 40 and 50 %, were inoculated with RUKM3s seed culture. The agitation was set to cascade and aeration level was set at 20 mmol O_2/L-air/hr. The production of RUKM3s biomass was monitored on the basis of dry cell weight as in section 2.2.2.3 above.

2.2.3.6 Enzyme activity relative to biomass during growth of RUKM3s

After the optimisation of conditions for biomass production, the enzyme activity of the biomass was monitored during growth under optimised conditions. The hydantoinase and NCAAH activities were measured by the Erhlich's and Ninhydrin assays respectively.

2.2.4 Cell harvesting

Whole cells were harvested in early stationary phase by centrifugation at 7000 rpm for 10 min in pre-weighed 250 or 500 mL centrifuge tubes at 4 °C, The supernatant was then discarded and the pellet washed by resuspension in half the original volume of cold KH_2PO_4 buffer (0.1M, pH 8.0). The suspension was then centrifuged at 8000 rpm for 10 minutes at 4 °C. The supernatant was discarded and the centrifuge tubes weighed. The wet cell mass of the pellet was determined by difference. The cells were stored at -20°C for short-term storage until required. For long term storage freeze-drying or lyophilisation was used. The viability and storage stability of the cells were tested by inoculating 200 mL HNB with 0.2 g of freeze-dried cells after 1, 2 and 3 weeks of storage, and incubation at 25-28° C.

2.2.5 Optimisation of protein extraction

Cells of RUKM3s were resuspended in phosphate buffer to give a final wet-cell concentration of 100 mg/ 2.5 mL and then subjected to cell disruption by various methods.

2.2.5.1 French pressing

Resuspended cells were disrupted by a single 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 crude extract was centrifuged at 8000 rpm for 10 minutes and the concentration of protein in the cell-free supernatant determined. The crude enzyme extract was collected and used in biocatalytic reactions for amino acid production.

2.2.5.2 Ultrasonication

A 40 mL sample of cell suspension was transferred to 50 mL centrifuge tubes and maintained at 4 °C in an ice jacket. The cells were then subjected to intermittent bursts of ultrasound from a

Vibra Cell sonicator (Sonics & Materials Inc. Danbury, CT., USA) with the amplitude set to 60% of full scale. The enzyme activity and the concentration of protein in cell-free supernatant were monitored at 2 min intervals over a period of 10 min.

2.2.5.3 Glass bead-milling

A suspension of cells was exposed to short alternate bursts of milling in a glass bead mill in the presence of glass beads (1:2 v/v). The effect of bead size was investigated by comparing two sizes of beads (of 250 μm and 500 μm diameter). The optimal milling time and duration of each burst was determined.

2.2.5.4 Freeze-thawing

Wet cells were incubated at -180 °C in liquid nitrogen for 3 h and then thawed at room temperature. The cells were then resuspended in the appropriate volume of buffer to a biomass concentration of 100 mg/2.5 mL.

2.2.5.5 Detergent treatment

To a suspension of cells in phosphate buffer, 1% w/v of sodium deoxycholate was added, and the mixture incubated at room temperature for 1 h. The concentration of protein in the supernatant of the suspension before and after the treatment was determined. The enzyme activity of the cell suspension was evaluated before and after detergent treatment (See sections 2.2.6.3 and 2.2.6.4).

2.2.6 Assaying for enzyme activity and protein concentration

2.2.6.1 Biocatalytic reaction method

RUKM3s cells were resuspended in cold potassium phosphate buffer (0.1M, pH 8.0) to give a final concentration of 0.1 g cells/2.5mL and homogenised to give a crude enzyme extract. The reaction mixture (5mL), containing 2.5 mL of resuspended cells (equivalent to 100 mg wet cells) or crude extract and 2.5 mL of substrate (100mM hydantoin or 50 mM NCG) was incubated for 3-6 h (40 °C, 200 rpm). After incubation, the reaction mixtures centrifuged in 1.5 mL eppendorf tubes (at 13000 *g* for 5 min) and the supernatant analysed for the concentration of *N*-carbamylamino acid and amino acid products by colorimetric and chromatographic assays (See sections 2.2.6.3, 2.2.6.4 and 2.2.6.5). Hydantoinase activity was calculated on the basis of total

amount (μmoles) of the 5-monosubstituted hydantoin converted to the *N*-carbamyl intermediate plus amino acid per mL of reaction mixture over a 3 h reaction period. NCAAH activity was calculated on the basis of the amount (μmoles) of amino acid produced per mL of reaction mixture over a 3 h reaction period. All reactions were conducted in triplicate in bijou bottles as shown in the Table 2.2.

Table 2.2 Set-up of biocatalytic assay

Components	**Phosphate Blank**	**Substrate Blank**	**Cell Blank**	**Samples (triplicate)**
Phosphate Buffer (0.1M; pH 8.0)	5mL	2.5mL	2.5mL	-
Substrate (eg. 100 mM Hydantoin or 50 mM NCG) in phosphate buffer	-	2.5mL	-	2.5mL
Cells (100 mg/2.5mL) in phosphate buffer	-	-	2.5mL	2.5mL

2.2.6.2 Bradford's assay for protein (Bradford, 1976)

Bradford's reagent was bought from Sigma or prepared[1] in the laboratory. Protein concentration was determined in supernatants of crude extract. To each 100 μL of samples or standards, 3 mL Bradford's reagent was added. After 2 min the absorbance was read at OD_{595}. Bovine serum albumin (BSA) was used to prepare a series of standards with protein concentrations of 0-250 μg/mL as shown in Table 2.3 below.

[1] prepared by dissolving 100 mg Coomassie Brilliant Blue in 50 mL of 95% ethanol, mixing with 100 mL of 85% phosphoric acid and making up to 1 L with distilled water (Walker, 1996)

Table 2.3 BSA Standards for protein assay

BSA (0.5 mg/mL)	NaCl (0.15M)	Std conc.(μg/mL)
0	100	0
10	90	50
20	80	100
30	70	150
40	60	200
50	50	250

2.2.6.3 Ehrlich's assay for N-carbamyl amino acid (Yamada et al., 1978)

The hydantoinase activity was evaluated in terms of μmoles/mL NCG plus glycine produced by 0.1 g cells (or crude extract) suspended in 2.5mL phosphate buffer (0.1M, pH 8) incubated with 2.5 mL hydantoin (100mM, pH 8) over 3 h (40 °C, 200 rpm). The hydantoinase activity is reported as μmole product (NCG plus glycine) per mL of reaction mixture using hydantoin as substrate under these conditions. The amount of product can also be reported as the percentage of theoretical yield, where the theoretical yield for 1 mole hydantoin is 1 mole NCG (or NCG plus glycine). These were the standard conditions for the assay of NCG.

The Ehrlich's assay was used to determine the hydantoinase activity. In the assay, 1 mL aliquots of the supernatant were transferred to test-tubes containing 0.5 mL of 12 % trichloroacetic acid to stop the reaction. The mixture was vortexed briefly and 3 mL of distilled, deionised water added. Ehrlich's reagent[2] (0.5 mL) was added, the test-tubes were allowed to stand at room temperature for 20 min and the absorbance was read at 420nm on a UV-Visible spectrophotometer (Shimadzu UV-20). The concentration of *N*-carbamyl amino acid was determined from a standard curve based on NCG. A solution of 50 mM NCG was used to make 0 – 25 mM NCG standards in a 1 mL final volume as shown in Table 2.4.

[2] Prepared by dissolving 10 %(w/v) *p*-dimethylaminobenzaldehyde in 6M HCl

Table 2.4 Preparation of NCG standards

Standard (mM)	50 mM NCG (µL)	0.1 M Buffer (µL)
0	0	1000
5	100	900
10	200	800
15	300	700
20	400	600
25	500	500

2.2.6.4 Ninhydrin assay for amino acids (Plummer, 1987)

The NCAAH activity was evaluated in terms of µmoles/mL glycine produced by 0.1 g cells (or crude extract) suspended in 2.5mL phosphate buffer (0.1M, pH 8) incubated with 2.5 mL NCG (50mM, pH 8) over 3 h (40 °C, 200 rpm). The NCAAH activity is reported as µmole product (glycine) per mL of reaction mixture using NCG as substrate under these conditions. The amount of product can also be reported as the percentage of theoretical yield, where the theoretical yield for 1 mole NCG is 1 mole glycine. These were the standard conditions for the assay of glycine.

The ninhydrin assay was used to measure the NCAAH activity. NCAAH activity = amount (mM) of amino acid produced over 3 h. In the assay, 0.980 mL aliquots of potassium phosphate buffer (0.1 M, pH 8.0) were added to test-tubes. To each tube 20 µL of each supernatant was added, followed by 1 mL of ninhydrin reagent[3]. The test-tubes were then placed in a boiling water bath for 15 min and allowed to cool to room temperature (approximately 10 min). To each test-tube, 3 mL ethanol (50%) was then added and the reaction mixtures left at room temperature for 10 min. The absorbance was then read at the 570 nm. The concentration of amino acids was determined from a curve of 0-0.5 mM glycine standards. A solution of 1 mM glycine was used to make 0–0.5 mM glycine standards in a 1 mL final volume as shown in Table 2.5 below.

[3] 0.8g Ninhydrin + 0.12g hydrindantin in 30 mL 2-methoxyethanol. Add 10 mL of 4 M acetic acid (pH 5.5).

Table 2.5 Preparation of glycine standards

Standard (mM)	1 mM Glycine (μL)	0.1 M Buffer (μL)
0	0	1000
0.1	100	900
0.2	200	800
0.3	300	700
0.4	400	600
0.5	500	500

2.2.6.5 Chromatographic assays

The use of Reverse Phase HPLC with a UV detector to measure the yield of amino acids and *N*-carbamyl amino acids from the bioconversion of 5-monosubstituted hydantoins is limited to aromatic substituted substrates and products. To enable quantification by UV detection, samples with non-aromatic substituted groups require pre-column derivatisation before they can be detected, but hydantoin derivatives with an aromatic ring can be quantitatively assayed by HPLC. Samples of *p*-hydroxyphenylhydantoin (HPH) and *N*-carbamylhydroxyphenylglycine (NC-HPG) were obtained from Bio/Chemtek, CSIR, South Africa for use as substrates and standards.

Standard solutions were prepared by dissolving appropriate amounts of substrate in a minimum amount of sodium hydroxide (or dimethyl sulphoxide) and then diluted with distilled-deionised water to give the desired concentration. Samples and standards were passed through a Waters Spherisorb ODS1 C18 HPLC column in 9% methanol flowing at a rate of 0.7 mL/min and detected at a wavelength of 280nm in a LabChrom series 7000 HPLC system.

2.3 RESULTS AND DISCUSSION

2.3.1 Identification by 16s rRNA sequence analysis and description by Scanning Electron Microscopy (SEM)

The strain RU-KM3s was previously identified as *Pseudomonas putida* by 16S rRNA analysis (RU Hydantoinase Research Group, *Annual Report* 1998). The SEM image of RUKM3s cells (Fig. 2.2) shows that the cells are rod shaped, approximately 0.5 μm in diameter and 1-2 μm in length. This description is consistent with the description of *Pseudomonas* spp. in literature as rod-shaped, gram-negative, polar-flagellated, non-sporulating, aerobic bacteria (Palleroni, 1984). *Pseudomonas* spp. are very common in soil and water where they are saprophytes.

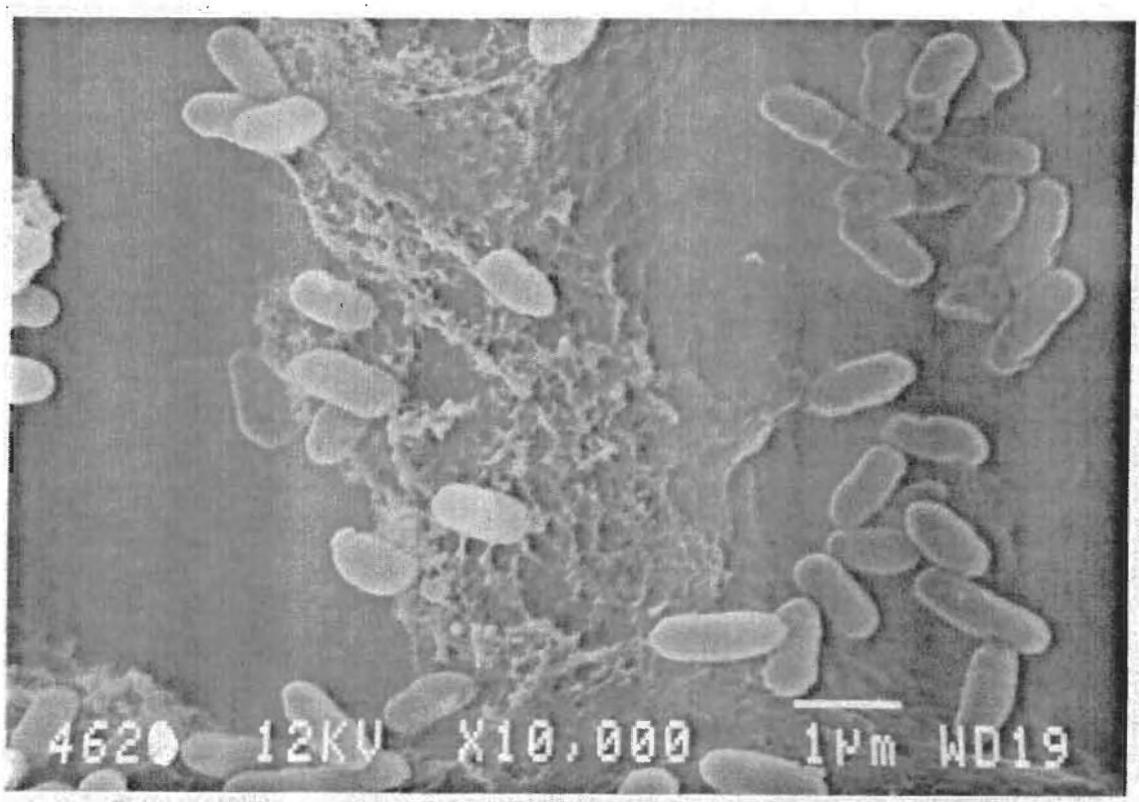


Fig. 2.2 SEM of RUKM3s cells

2.3.2 Optimisation of biomass production

2.3.2.1 Effect of different carbon sources on biomass production

Biomass yield depends on, among other factors, the nature of the carbon source in the growth medium. Yield can be increased by choice of the best carbon source. *Pseudomonas* spp. are known to be nutritionally versatile, with rather simple requirements. Mineral media supplemented with a single organic compound of low molecular weight as the sole carbon and energy source allows growth (Demain and Solomon, 1985). RUKM3s cells were grown in nutrient broth, and in media containing different carbon sources to evaluate the effect on biomass yield. The yields (expressed as concentration, g/L) of biomass from nutrient broth, and from hydantoin minimal media (HMM) supplemented with glucose, glycerol and mannitol showed that growth in NB took less than 24 h to reach stationary phase, while very low growth was recorded in HMM media during the same period (Fig 2.3 and 2.4). This is consistent with the findings of other researchers on the growth characteristics of RUKM3s (Skepu, 2000; Buchanan *et al.*, 2001).

Significant biomass production was observed in HMM cultures only after incubation beyond 24 h, reaching stationary phase after 3-4 days (Fig. 2.4). Different carbon sources in HMM were used in an attempt to reduce the lag phase of growth in the minimal media and achieve high concentrations within a shorter period. However, the use of these different carbon sources did not seem to achieve this. There were no significant differences in the final biomass concentrations obtained from supplementation of HMM with glucose, glycerol or mannitol. Therefore HNB was a better medium for biomass production than the HMM formulations. In later studies,PP2 medium was found to be better than HNB.

In theory, the metabolic availability of the energy in the three carbon sources could be expected to cause a difference. The aldohexose glucose is broken down more easily than the alcohols glycerol and mannitol; and the trihydric glycerol is utilised more easily than higher molecular weight, polyhydric alcohol, mannitol (Demain and Solomon, 1985). Growth of *Pseudomonas putida* strains in minimal media used by other research groups has taken comparable periods to reach stationary phase. Morin *et al.* reported cultivating a *P. putida* strain that took 40 h to reach

stationary phase in H3 medium containing yeast extract, glycerol and potassium phosphate (Morin *et al.*, 1990).

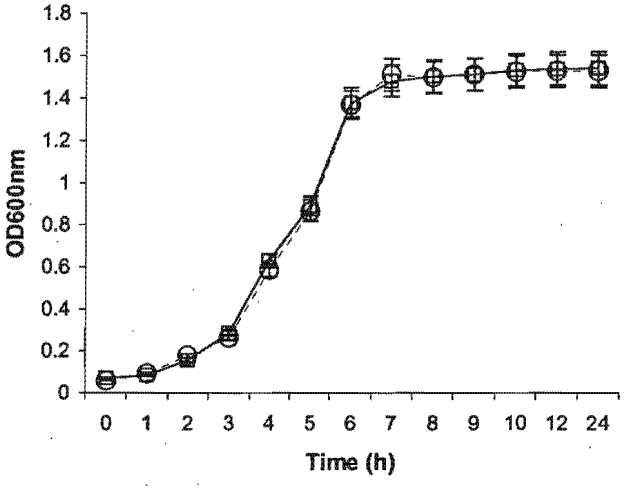


Fig. 2.3 Change in the biomass concentration of RUKM3s cells shown as cell density (OD_{600}) during growth in nutrient broth (□) and in nutrient broth containing 0.1 % hydantoin (○), over 24 h

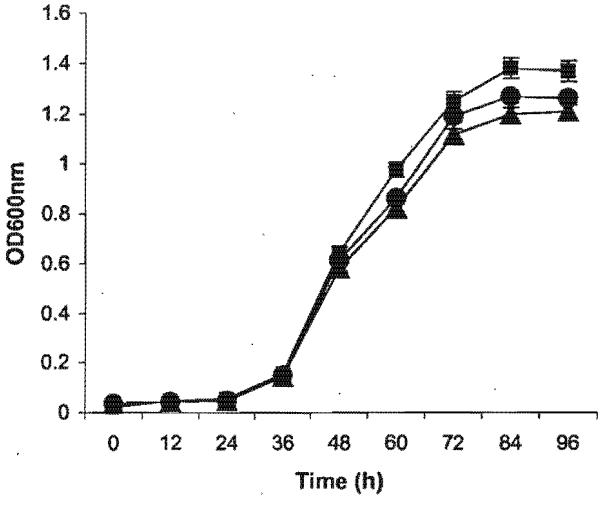


Fig. 2.4 Change in the biomass concentration of RUKM3s cells shown as cell density (OD_{600}) during growth in HMM supplemented with glucose (■), glycerol (●) and mannitol (▲), over 96 h

2.3.2.2 Effect of hydantoin on biomass production

Pseudomonas spp. cannot fix nitrogen. Ammonium salts, nitrate and other nitrogen-rich compounds can be used by most species of *Pseudomonas* as nitrogen sources (Demain and Solomon, 1985). Hydantoin, which is the primary substrate for amino acid production, is also known to induce the synthesis of hydantoin hydrolysing enzymes in RUKM3s (Skepu, 2000). Since hydantoin is also a good nitrogen source and nitrogen is essential for cell growth, hydantoin may affect biomass production. It was therefore important to investigate the effect of inducer-level concentrations of hydantoin on biomass concentration. RUKM3s cells were grown in NB containing different concentrations of hydantoin and the biomass concentration monitored to determine the effect of hydantoin. The results showed that addition of inducer-level concentrations of hydantoin caused no significant difference in the concentrations of biomass (Fig 2.5).

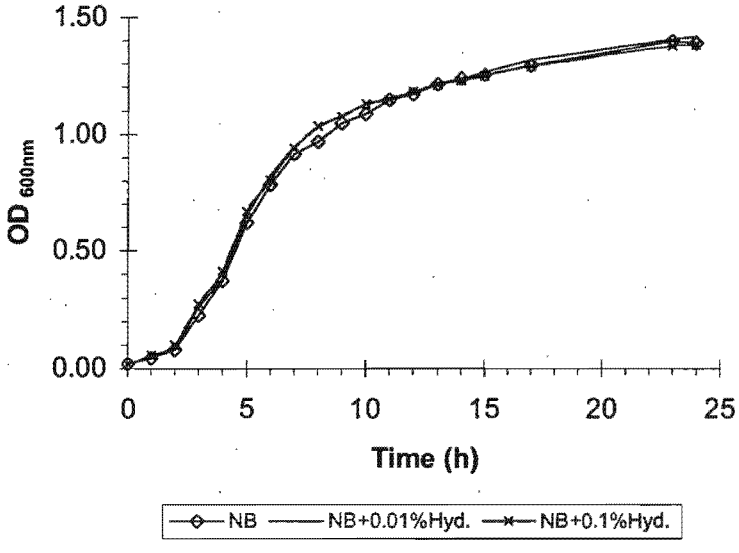


Fig. 2.5 Change in the biomass concentration of RUKM3s cells shown as cell density (OD_{600nm}) during growth in nutrient broth supplemented with 0, 0.01 and 0.1% hydantoin.

2.3.2.3 Optimum temperature for biomass production of RUKM3s

Experiments were set up to determine the optimal temperature for the production of RUKM3s cells in a fermentor. Five cultures were grown in separate fermentors set to 25, 28, 30, 32 and 33 °C. The results of biomass production in grams dry cell weight (DCW) per litre of medium (Fig. 2.6) showed that RUKM3s gave the highest concentration of biomass at 28 °C, followed by 30, 32, 33 and 25 °C. The optimal temperature for cultivation of RUKM3s biomass was therefore chosen as 28 °C. This temperature is consistent with that determined by other research groups for some hydantoinase-producing *Pseudomonas* spp. (Kim *et al.*, 1994; Morin *et al.*, 1990).

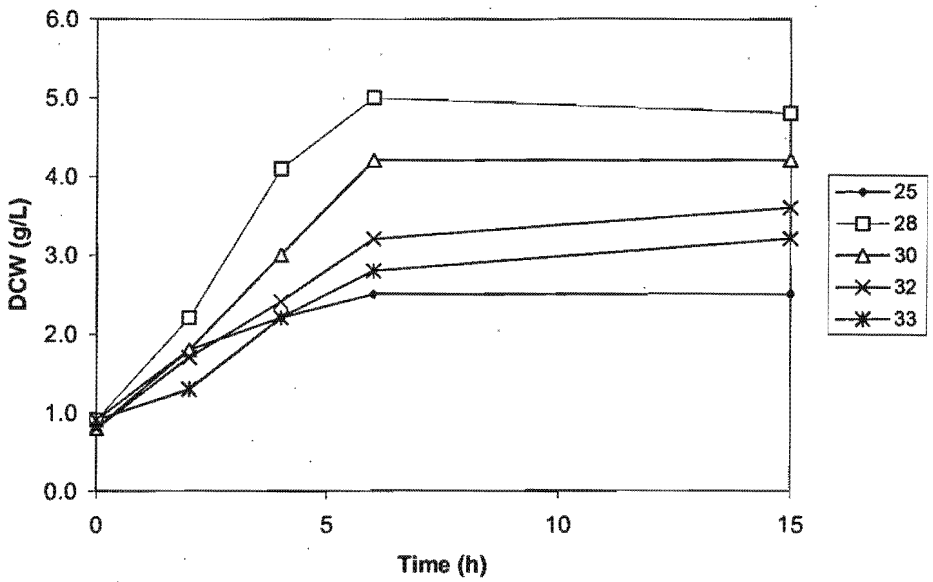


Fig. 2.6 Effects of growth of RUKM3s at different temperatures (25- 33 °C) on the biomass in dry cell weight (DCW) over time

2.3.2.4 Optimum pH for biomass production of RUKM3s

Experiments were set up to determine the optimal pH for the production of RUKM3s cells in a fermentor. Four cultures were grown in separate fermentors set to pH 6.5, 7, 7.5 and 8. The growth of RUKM3s cells at different pHs (Fig. 2.7) gave the highest concentration of biomass at pH 7 and 7.5, followed by 8 and 6.5. The optimal pH for cultivation of RUKM3s biomass was selected as 7. Similar optimal pH levels for cultivation of *P. putida* have been reported (Morin *et al.*, 1990). *Pseudomonas* spp. do not grow at pH 4.5 or lower (Demain and Solomon, 1985).

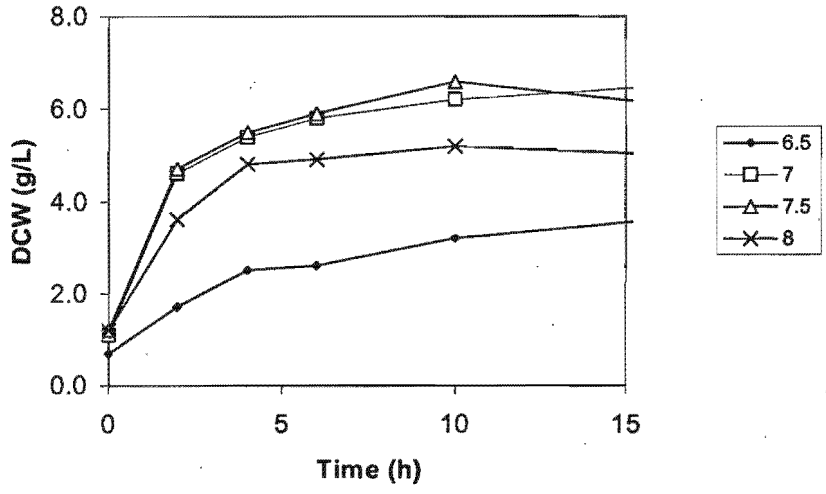


Fig. 2.7 Change in DCW with time showing effects of growth of RUKM3s at different pH values (6.5-8)

2.3.2.5 Effect of dissolved oxygen on biomass production of RUM3s

The growth of RUKM3s cells at 28° C for different levels of relative DO_2 (Fig. 2.8) gave the lowest concentration of biomass (in the first 10 h of growth) at 10% relative DO_2, and the highest at 40%, even though not significantly higher than that of 25%. The possibility of DO_2 limitation at any of these levels is low. The slight decrease in biomass concentration observed for 50% DO_2 may have been due to the adverse effects of increased agitation/aeration needed to maintain his high DO_2 level. Therefore biomass cultivation in subsequent experiments was conducted at a DO_2 level of 40%, not because it is optimal, but jus to assure adequate aeration without adverse effects of increased agitation such. Literature reports on the specific oxygen requirements for the cultivation of biomass of *Pseudomonas* spp. are difficult to find. However, most of the species are known to be aerobes. Typically, the metabolism of the species is described as respiratory, with oxygen as the final electron acceptor (Demain and Solomon, 1985).

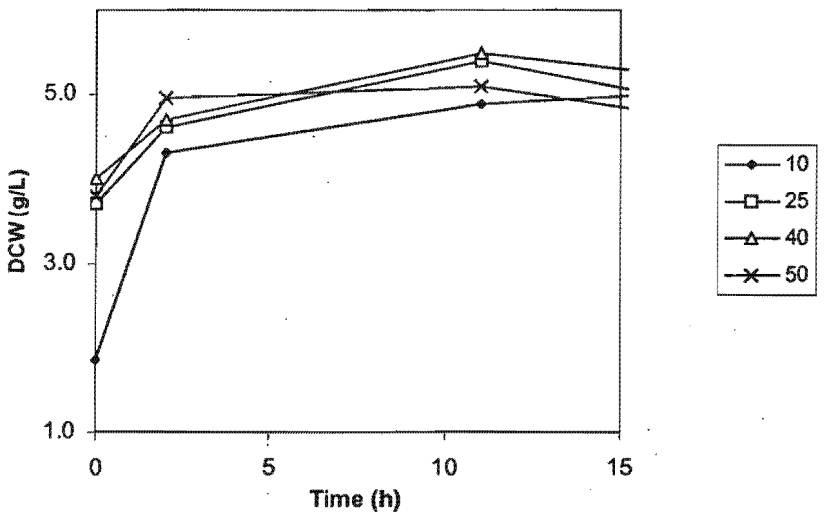


Fig. 2.8 Change in DCW with time, showing effects of growth of RUKM3s at different % DO_2 levels

2.3.2.6 Off-gas analysis during growth of RUKM3s

The progress of a fermentation process involving cultivation of biomass can be monitored by online analysis of the gas mixture in the fermentor for carbon dioxide and oxygen concentrations. The change in the concentration over time represents the rates of carbon dioxide emission (CER) and oxygen utilisation (OUR) respectively. The gas mixture from the exhaust of a fermentor (off-gas) containing RUKM3s cells growing in PP2 medium at 28 °C and pH 7 was passed through an automated gas analyser. The OUR and CER, in millimoles of carbon dioxide or oxygen per litre of exhaust mixture per hour, were recorded and plotted against time (Fig. 2.9). The results indicate that there was an initial increase of both OUR and CER in the early stages of growth up to 6 h. From 7 h onwards there was a decline in both OUR and CER. Stationary phase was reached at 8 h. These observed trends are also noted in the growth curves of RUKM3s in PP2 and HNB media recorded in Fig. 2.10.

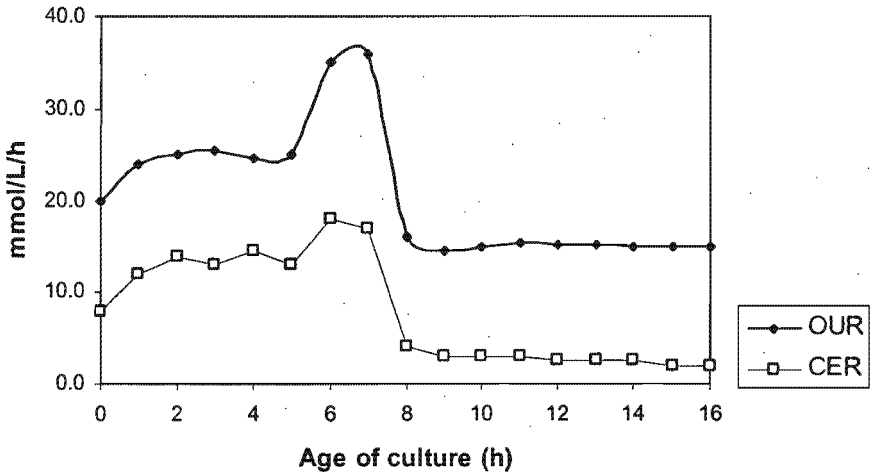


Fig. 2.9 Changes in the rates of oxygen utilisation (OUR) and carbon dioxide emission (CER) in a fermentor during growth of RUKM3s cells

2.3.3 Assessment of biomass concentration, enzyme activity and storage stability of RUKM3s cells and crude extract

2.3.3.1 Biomass production for RUKM3s grown under optimised conditions

RUKM3s cells were grown in nutrient broth with 0.1% hydantoin (HNB) or in PP2 medium, at 28 °C, pH 7 and 40 % DO. The biomass concentration at harvest expressed as dry cell weight were 3.3 g/L and 11.6 g/L for nutrient broth and PP2 medium respectively. The growth curves in the two media are shown in Fig. 2.10. The results show that PP2 medium gave more biomass, and therefore met the nutritional requirements of RUKM3s better than HNB. This may be explained by the fact that that PP2 is a more complex media than HNB. PP2 contains the normal ingredients of HNB as well as an additional carbon source (sunflower oil), and citric acid, magnesium sulphate and potassium dihydrogen phosphate. Comparable increases in biomass concentration when the medium PP2 was used have been recorded by other researchers. Foster (2003) recorded a greater than double increase in biomass concentration for *Agrobacterium tumefaciens* grown in PP2, compared to growth in HMM.

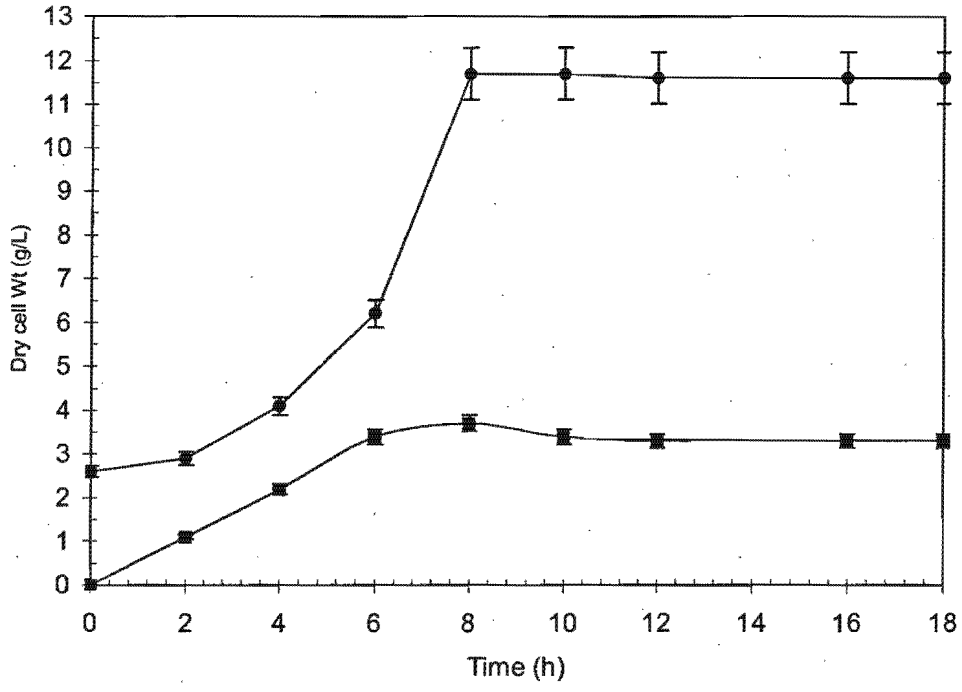


Fig. 2.10 Dry cell weight of RUKM3s cells during growth in nutrient broth (■) and in PP2 medium (●) under optimal conditions.

2.3.3.2 Enzyme activity relative to biomass during growth of RUKM3s

The hydantoinase and NCAAH activities in growing RUKM3s cells were monitored over time during growth at 28 °C, pH 7 and 40% DO. The activities were measured under the standard conditions outlined in 2.2.6.3 and 2.2.6.4 for hydantoinase and NCAAH respectively. The enzyme activity relative to biomass production is shown in Figures 2.11 and 2.12. Hydantoinase activity was highest after about 16 h of growth, in the stationary phase, and NCAAH activity was highest after 10 h of growth, in early stationary phase. These results confirm the findings of Buchanan *et al.*, (2001) and Skepu (2000) that the optimal time in the growth of RUKM3s to harvest cells with highest enzyme activity is in stationary phase at 18-24 h. These growth and harvest times are comparable to those reported by other researchers for related strains. Gokhale *et al.* (1996) screened 125 *Pseudomonas* strains from their culture collection for production of hydantoinase activity using D,L-phenylhydantoin as a substrate and found that for the best hydantoinase producers, the enzymatic reactions were performed with 18-20 h culture grown in nutrient broth and 5-phenylhydantoin as the substrate.

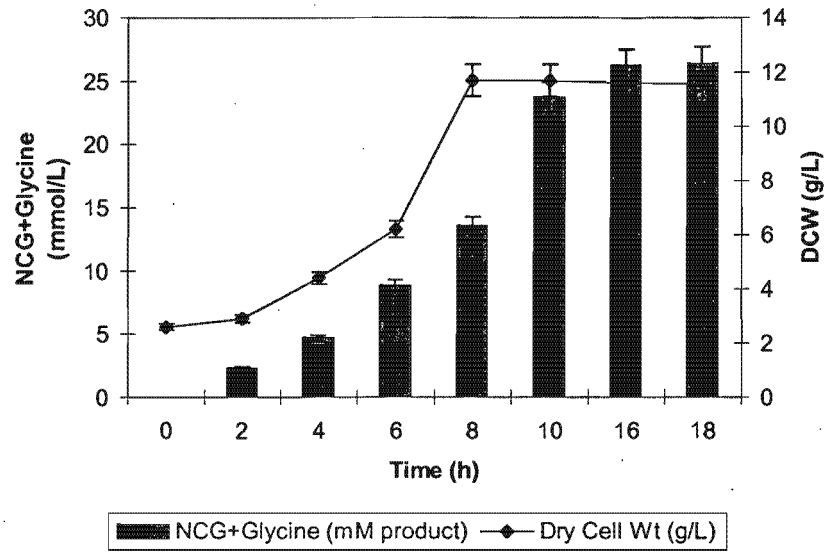


Fig. 2.11 Profile of hydantoinase activity in RUKM3s during growth.

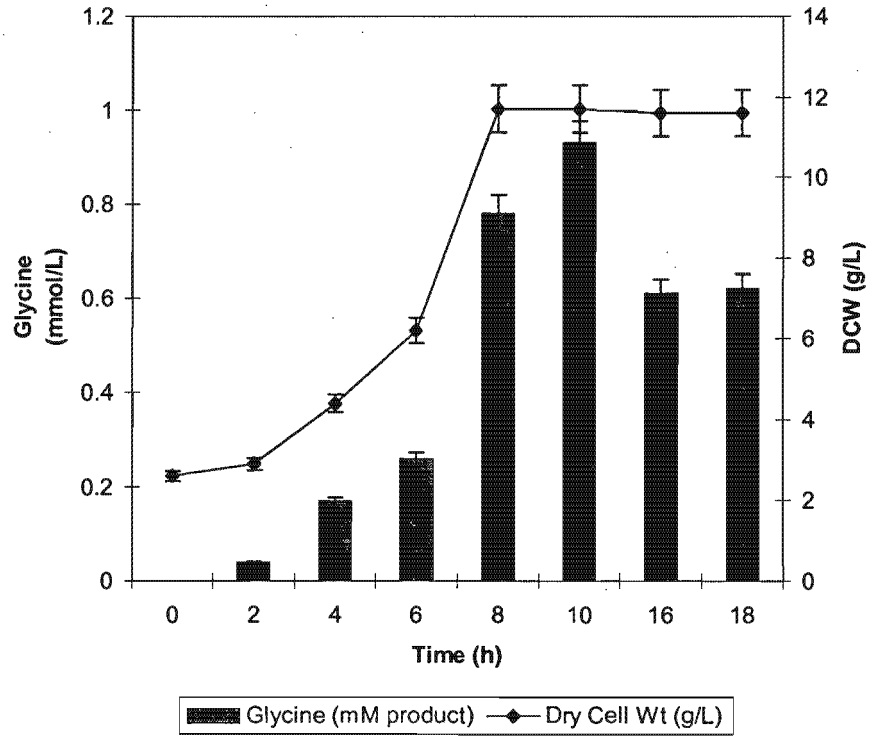


Fig. 2.12 Profile of NCAAH activity in RUKM3s during growth

2.3.3.3 Storage of biomass and effect of freeze-drying of resting cells of RUKM3s

The percentages of original activity retained after freeze-drying were found to be 81 % and 39 % for hydantoinase and NCAAH respectively. The results (Fig.2.13 and 2.14) showed that lyophilisation had a more negative effect on NCAAH than on the hydantoinase activity of the crude extract. When freeze-dried RUKM3s cells previously stored at -20 °C for 3 weeks were resuspended in HNB medium, the cells started growing, which showed that the viability of the resting cells was retained for at least three weeks. The storage stability of hydantoin hydrolysing enzymes has been reported by other research groups. Campbell (1960) found that the storage stability of NCAAH in cells stored at -20 °C was at least 3 months. Brooks *et al.,* (1983) reported the storage stability of purified hydantoinase from bovine liver as several days stored at room temperature or 4 °C, and several months stored at -20 °C.

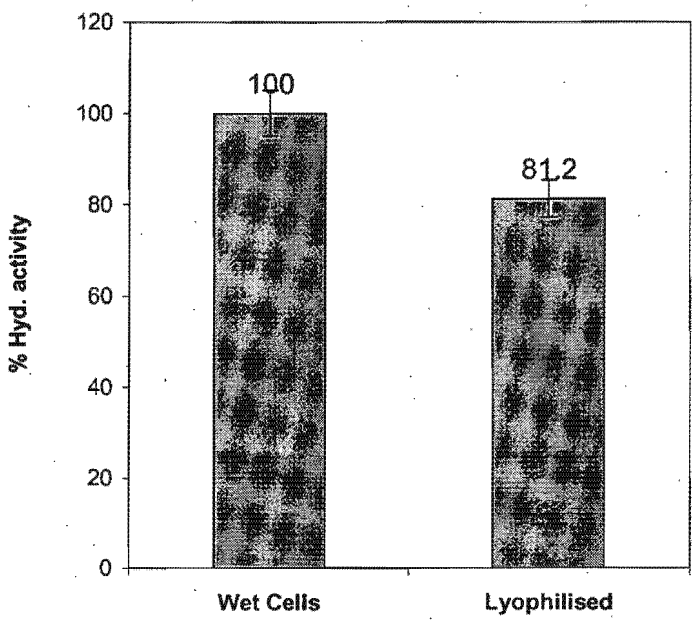


Fig. 2.13 Comparison of % hydantoinase activity of RUKM3s cells before, and after freeze-drying and storage for 3 weeks

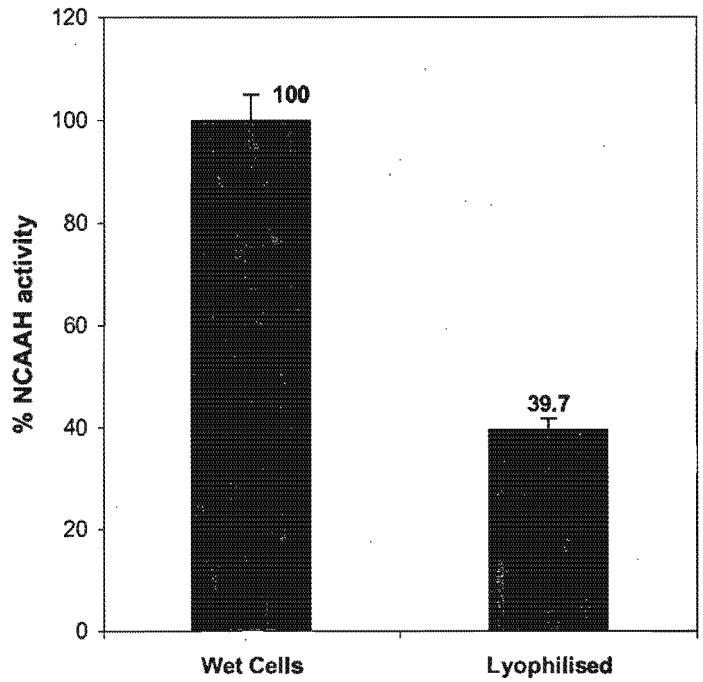


Fig. 2.14 Comparison of % NCAAH activity of RUKM3s cells before, and after freeze-drying and storage for 3 weeks

2.3.4 Evaluation of techniques of cell disruption

The hydantoin hydrolysing enzymes of RUKM3s are intracellularly expressed. They are intracellular and possibly membrane bound because even after homogenisation, more enzyme activity is recorded found in the pellet than in the supernatant. In order to use the enzymes in biocatalytic reactions it is important to disrupt the cells. Various techniques of cell disruption were used to homogenise the cellular material into a crude extract.

2.3.4.1 Effect of French-pressing

Table 2.6 shows the effects of French-pressing on the enzyme activity of RUKM3s whole cells, and the amount of activity in the supernatant after centrifugation of the French pressed extract. There was no increase in the amount of hydantoinase activity in the supernatant due to French pressing. For both whole cells and crude extract, relatively little of hydantoinase was detected in the cell-free supernatant. This may indicate that the enzyme was insoluble or membrane bound,

and the association was not affected by French-pressing. The NCAAH activity in the crude extract was slightly higher than in the resting cells. However, very little NCAAH was noted in the cell-free supernatant, indicating that the NCAAH may also be membrane bound or insoluble. The above effects may also indicate that in order for French-pressing to be effective, a pressure higher than 15 MPa or more than one pass through the French-Press may be required. However, this may have a negative effect on the activity of the enzymes extracted.

Table2.6 Effects of French-pressing on enzyme activity

Enzyme activity over 3 h	**Resting cells**		**French-pressed**	
	Whole	Supernatant	Crude extract	Supernatant
Hydantoinase (μmol. product/mL)	32 ± 10	4 ± 1	32 ± 14	2.5 ± 0.2
NCAAH (μmol. glycine/mL)	3.6 ± 0.5	0.7 ± 0.4	4 ± 1	0.66 ± 0.06

2.3.4.2 Effect of sonication

RUKM3s cells suspended in buffer were sonicated over a 10 min period. The amount of protein in the supernatant, and the levels of enzyme activity in both the supernatant and the crude extract were monitored at 2 min intervals over the 10 min.

After a slight initial decline at 2 min the total hydantoinase activity (Fig. 2.15) became higher in the sonicated crude extract than in the pre-sonication period up to 6 min when it declined again. The activity in the supernatant was highest (50% of the total) after 6 min. This is useful for providing good quantities of enzyme for analytical work. The highest amount of hydantoinase activity in the uncentrifuged crude extract was also obtained at 6 min. At 10 min, the amount of hydantoinase activity in the supernatant was lower than in the pellet, which may indicate that free hydantoinase was more susceptible to deactivation than the membrane-bound enzyme.

NCAAH activity was negatively affected by sonication, with almost 50% of the pre-sonication levels being lost after 2 min (Fig. 2.16). However, more NCAAH activity was observed in the

supernatant after sonication than before, which is useful for analytical purposes where the enzyme is needed for characterisation, even in small quantities. No NCAAH was recorded in the pellet after 10min of sonication. This may indicate that all the NCAAH had been released from the cell membranes or that the bound enzyme had been deactivated.

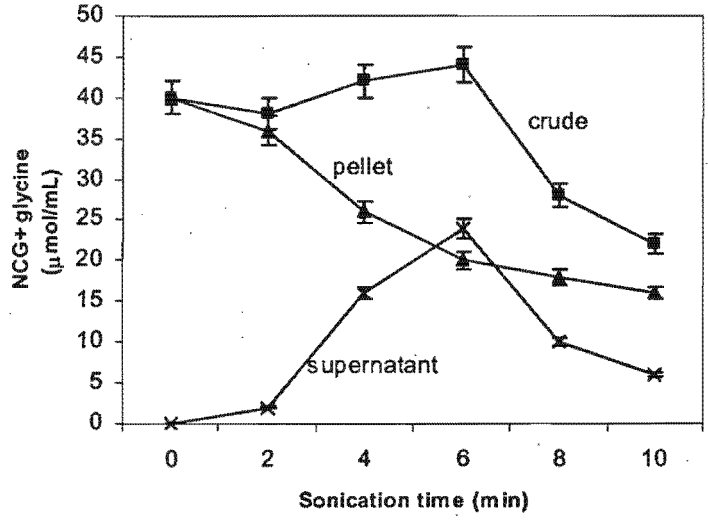


Fig 2.15 Effect of sonication time on hydantoinase activity of supernatant, pellet and crude extract of RUKM3s showing product (NCG plus Glycine over 3 h, as µmol/mL)

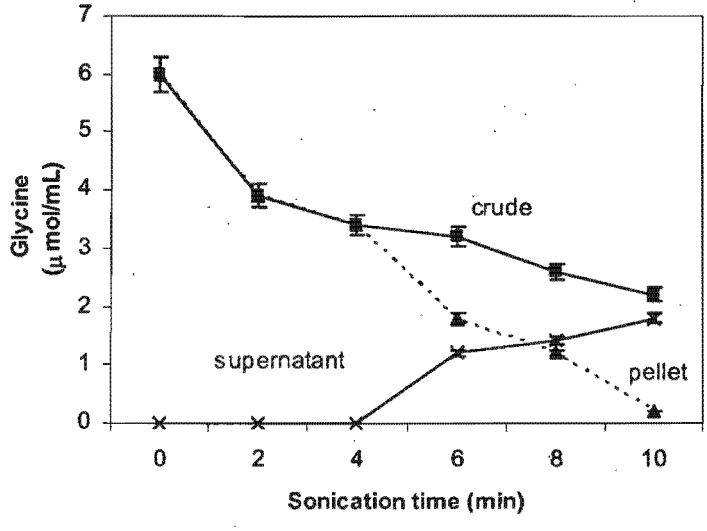


Fig. 2.16 Effect of sonication time on NCAAH activity of supernatant, pellet and crude extract of RUKM3s

The measurement of protein released into solution (Fig. 2.17) indicated that sonication released protein into solution gradually over the first 6 min. Thereafter, there was no more significant increase in the amount of protein. The increase in protein and the decline in enzyme activity towards 10 min show that sonication had a deactivating effect on both enzymes, and that the effect was more pronounced for NCAAH than for hydantoinase.

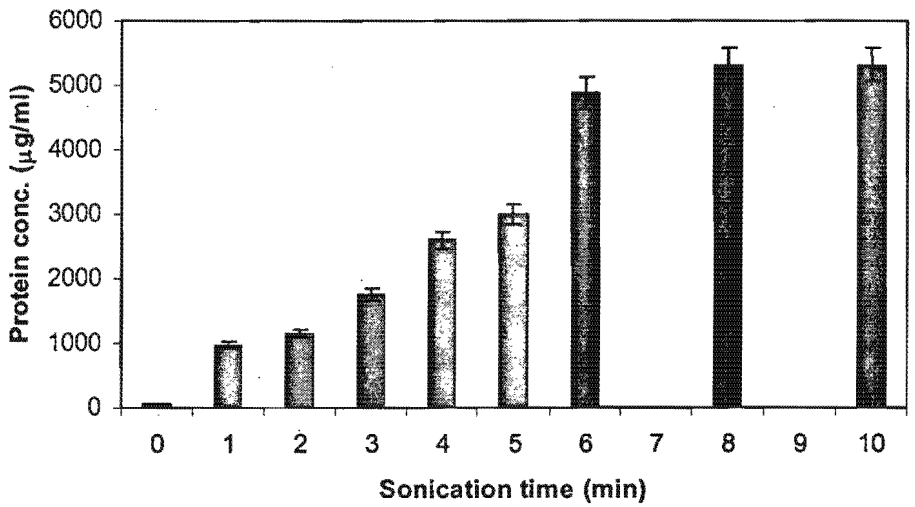


Fig 2.17 Effect of sonication time on protein concentration in supernatant of RUKM3s crude extract

2.3.4.3 Glass-bead milling (0.5 mm; 20% v/v; 1,2 & 3 minutes)

Glass bead-milling of RUKM3s cells suspended in buffer increased the measurable hydantoinase activity (Fig. 2.18) in the supernatant to more than double the level before sonication after 1 minute, but reduced the NCAAH activity (Fig. 2.19) by almost half in the same period. The free hydantoinase was more stable during the three minutes of bead-milling than the free NCAAH, which was completely deactivated.

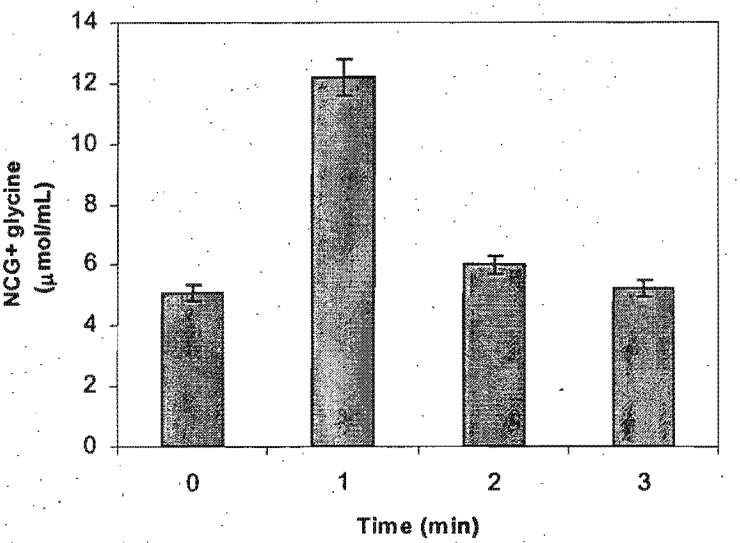


Fig. 2.18 Effects of glass bead-milling time on hydantoinase activity in the supernatant of RUKM3s

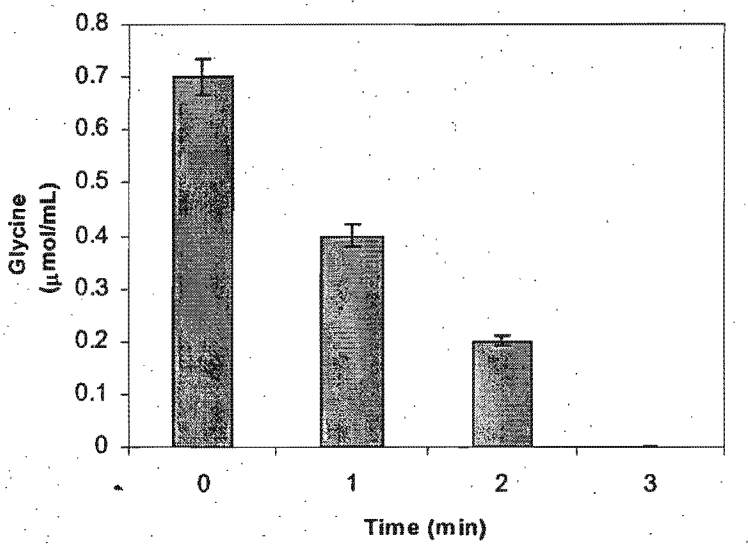


Fig. 2.19 Effects of glass bead-milling time on NCAAH activity in the supernatant of RUKM3s

2.3.4.4 Freeze-thawing (3 h, -180 °C)

Freeze-thawing (Table 2.7) caused the NCAAH activity of the crude extract to decrease, and only increased the hydantoinase activity by a small margin. So, it is not a suitable method for extraction of active protein.

Table 2.7 Effect of freeze-thawing on enzyme activity over 3 h

	NCAAH Glycine (µmol/mL)	Hydantoinase NCG + Glycine (µmol/mL)
Resting cells	3.1	33.2
Freeze-thawed extract	0	34.78

2.3.4.5 Summary of the results of celldisruption treatments

Results of assays of enzyme activity and solubilised protein concentration in RUKM3s subjected to various cell disruption treatments are shown in Table 2.10. It is apparent that sonication was the best among the methods tested in terms of the amount of protein extracted, and the amount of hydantoinase activity observed in the cell-free supernatant after treatment. However, French pressing was the better method for the extraction of high NCAAH activity from a smaller concentration of extracted protein. This may indicate that the high pressure used in French pressing de-activates NCAAH less than sonication. The enzyme activity of protein obtained from detergent treatment could not be evaluated for activity because the detergent interfered with the enzyme activity assays.

For industrial purposes, the ultimate scale of production and purification should be kept in mind during design and in the choice of methods of cell disruption. Sonication of batch quantities is an appropriate method for disrupting up to 500 g of cells, but may be time-consuming for large scale industrial production. For larger quantities of cells, it is best to use a 'flow-through' sonication probe to allow faster processing of larger scale volumes (Foster *et al.*, 1995). Most

methods of cell disruption produce heat and cause denaturation of protein. Glass bead milling is known to produce more heat than other methods, causing deactivation of most heat-sensitive enzymes (Reed and Nagodawithana, 1995). Cell disruption using pressure is the most widely used method. In addition to the French Press, various models are available for a wide scale of processing from 300 to 6000 grams of wet cells. Other methods that have been used employ chemical and biological agents such as alkalis, detergents (SDS, Sodium deoxycholate) and enzymes such as lysozyme and deoxyribonucleases. The limited use of the latter methods is mainly due to cost considerations, and the introduction of additional contaminants that may produce purification problems downstream (Foster *et al.*, 1995).

Table 2.10 Comparison of enzyme activities and protein concentration after cell disruption by different methods.

Cell disruption method	Hydantoinase (μmol/mL)	NCAAH (μmol/mL)	Protein in cell-free supernatant (μg/ml)
Resting Cells	33.2	3.1	51.2
French-pressing (crude extract)	32.0	4.0	
French-pressing (supernatant)	2.5	0.7	1256.5
Sonication (crude extract)	39.5	4.2	
Sonication (supernatant)	13.4	0.3	2617.9
Glass bead milling	28.0	1.3	174.4
Freeze-thawing	34.78	0	1712.0
Detergent (Na-deoxycholate)	-	-	2541.6

2.4 CONCLUSIONS

Observation of the morphology of the strain RUKM3s on SEM scans showed that its appearance conformed to the general description of *Pseudomonas putida* as found in literature. *Pseudomonas* spp. are rod-shaped, gram-negative polar-flagellated, non-sporulating aerobic bacteria (Palleroni, 1984). The identity of the strain was also confirmed by 16sRNA analysis (RU Hydantoinase Research Group, *Annual Report* 1998).

In the investigation of growth media, RUKM3s grew faster in nutrient broth than in hydantoin minimal medium. However, given a longer time of growth, HMM eventually produced the same amount of biomass as nutrient broth. HMM supplemented with glucose produced a higher concentration of biomass than mannitol and glycerol. The presence of hydantoin as an inducer in nutrient broth did not affect the biomass concentration. However, growth in PP2 medium provided higher concentration of biomass than growth in nutrient broth. Comparison of growth in NB and in PP2 showed that the biomass concentration from PP2 was 11.6 g /L while that in NB was 3.3 g/L on dry cell weight basis.

The optimal fermentation conditions for high concentration of RUKM3s biomass were found to be 28°C, pH 7 and a relative DO_2 concentration of above 25%. Analysis of OUR and CER indicated that from 7 h of growth onwards there was a decline in both OUR and CER, which would imply that the exponential phase of growth was complete and the stationary phase was approached from approximately 7 h of fermentation. Stationary phase was reached at 8 h. These observed trends were also noted in the growth curves of RUKM3s in PP2 and HNB. Hydantoinase activity was highest at approximately 16 h of growth and NCAAH activity was highest at approximately 10 h of growth. Growth of the strain in nutrient broth and PP2 medium reached stationary phase after 16-24 h.

An investigation of the storage stability of the hydantoin hydrolysing enzymes of RUKM3s crude extract showed that the percentages of original activity retained after freeze-drying were 81 and 39 % for hydantoinase and NCAAH respectively. The enzyme activity of RUKM3s resting cells was not affected negatively by storage of wet cells at -20 °C. Resting cells remained viable after freeze-drying and storage at -20 °C over a period of at least three weeks.

The biocatalytic product yields from resting cells were lower than those from crude extract. Sonication was found to be the best method of protein extraction in terms of the amount of protein released and the amount of hydantoinase activity recorded in the supernatant after treatment. French-pressing was found to be the best method for extraction of protein with high NCAAH activity. Further work on protein extraction should investigate the use of protease inhibitors during the extraction, so as to determine the effect of proteases on the hydantoin hydrolysing enzymes.

The biomass concentration of RUKM3s in PP2 medium (11.6 g/L) is comparable to that of other hydantoinase producing strains. In experiments using *Agrobacterium tumefaciens* RU-OR PN1, Foster (2002) obtained a biomass concentration of 15 g/L in PP2 medium. The hydantoinase product yield of RUKM3s resting cells (33.2 µmol/mL) was relatively high compared with that of RUOR-PN1, while the NCAAH product of RUKM3s resting cells (3.1 µmol/mL) was lower than that of RUOR-PN1 (16.5 µmol/mL). This difference in the yields of the two strains was later used to advantage in development of a dual column bioreactor (See chapters 5 and 6)

CHAPTER 3

OPTIMISATION OF ENZYME SYNTHESIS AND BIOCATALYTIC PRODUCTION OF AMINO ACIDS

3.1 INTRODUCTION

In work reported in chapter 2, conditions were optimised for the highest concentration of biomass and the most efficient extraction of protein from RUKM3s cells. In work reported in chapter 3, the aim was to investigate the optimum conditions for the synthesis of enzymes, their expression and the production of amino acids. The enzyme activities of RUKM3s cells grown under various conditions of pH, temperature, DO_2, substrate induction, metal ions and culture age were evaluated.

The rates of enzyme-catalysed reactions depend on a number of factors, including the concentration and nature of substrate, pH and temperature of the environment, presence or absence of some chemical components. Experiments were conducted to investigate the effects of variations in pH and temperature on the amounts of amino acid and *N*-carbamyl amino acid produced. Hence, the optimal conditions for the biocatalytic reactions for production of amino acids from 5-monosubstituted hydantoins were determined.

The ability of the hydantoin-hydrolysing enzymes of RUKM3s to produce amino acids from 5-monosubstituted hydantoins other than hydantoin itself was investigated. The aim was to determine the yields, and the effects of size of steric substituents on the amount of product formed. The substrates used were methylhydantoin, isopropylhydantoin, isobutylhydantoin, nor-butylhydantoin, *tert*-butylhydantoin and hydroxyphenylhydantoin.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Sulphates of manganese, magnesium, cobalt, copper, iron and zinc were obtained from Merck Chemicals (South Africa). Hydantoin was purchased from Sigma-Aldrich. *N*-carbamylglycine was purchased from Sigma Chemical Company (St Louis, MO., USA). Methylhydantoin,

isopropylhydantoin, isobutylhydantoin, nor-butylhydantoin, *tert*-butylhydantoin and hydroxyphenylhydantoin were previously synthesized in the lab by the Bucherer-Bergs method (Skepu, 2000; Bucherer and Stein, 1934).

3.2.2 Optimization of growth conditions for enzyme production

3.2.2.1 Effect of induction by substrate on enzyme activity

RUKM3s cells were grown in NB supplemented with hydantoin or NCG or a mixture of both in concentrations of 0.1% for each additive. Erhlich's and Ninhydrin assays were used to measure hydantoinase and NCAAH activity (See Sections 2.2.6.3 and 2.2.6.4).

3.2.2.2 Effect of divalent metal ions on enzyme activity

RUKM3s cells were grown in HNB supplemented to concentrations of 2.5 and 5.0 mM with the sulphates of manganese, magnesium, cobalt, copper, iron and zinc. The enzyme activities of the biomass harvested were assayed by the Ninhydrin and Erhlich's assays.

3.2.2.3 Optimization of growth temperature, pH and DO_2 for high enzyme activity

The effects on the enzyme activity of biomass due to temperature, pH and DO_2 of the growth medium were investigated. Biomass obtained during experiments for the optimization of yield was harvested and assayed for hydantoinase and NCAAH activity.

3.2.3 Optimization of conditions for biocatalytic production of amino acids

3.2.3.1 Profile of enzyme activity in the presence of substrate

This experiment was designed to show a profile of substrate utilisation by the hydantoin hydrolysing enzymes of RUKM3s. A series of bijou bottles representing 10 sets of reactions containing 2.5 mL portions of 100 mM hydantoin and 50 mM NCG mixed with 2.5 mL of RUKM3s extract (100mg/mL) were incubated for periods ranging from 1 to 10 h. Every hour, the reaction in one set of the reaction mixtures was stopped, centrifuged and the supernatant

assayed for yields of amino acid and *N*-carbamylglycine by the Ninhydrin and Erhlich's assays respectively.

3.2.3.2 Effect of substrate concentration on enzyme activity during biocatalysis

2.5 mL portions of RUKM3s crude extract (100 mg/mL) were incubated with 2.5 mL 10, 30 and 50 mM hydantoin and 5, 15 and 25 mM NCG for a period of 3 h. The effective concentrations of substrate in the reaction mixtures were 5, 15, and 25 mM hydantoin, and 2.5, 7.5 and 12.5 mM NCG. Samples were collected at 30-min intervals and assayed for yields of amino acid and *N*-carbamylglycine by the Ninhydrin and Erhlich's assays respectively.

3.2.3.3 Effect of pH on enzyme activity during substrate incubation

2.5 mL portions of RUKM3s crude extract (100 mg/mL) were incubated for 3 h with 2.5 mL of 100 mM hydantoin and 50 mM NCG at pH values from 5 to 10 set with phosphate buffer. Samples were collected at 30-min intervals and assayed for yields of amino acid and *N*-carbamylglycine by the Ninhydrin and Erhlich's assays respectively.

3.2.3.4 Effect of Temperature on enzyme activity during substrate incubation

Triplicate 2.5 mL portions of RUKM3s crude extract were incubated for 3 h with 2.5 mL of 100 mM hydantoin and 50 mM NCG for 3 h at 20, 30, 40, 50 and 60 °C. Samples (1.1 mL) were collected at 30-min intervals and assayed for yields of amino acid and *N*-carbamylglycine by the Ninhydrin and Erhlich's assays respectively.

3.2.4 Production of amino acids from various 5-substituted hydantoins

The ability of RUKM3s cells to produce *N*-carbamoyl amino acid and amino acids from various 5-substituted hydantoin substrates was tested. Triplicate 2.5 mL portions of each substrate were incubated with 2.5 ml RUKM3s crude extract over 3 h at 40° C. The reaction mixtures were then centrifuged and the supernatants assayed for yields of amino acid and *N*-carbamylglycine by the Ninhydrin and Erhlich's assays respectively. The substrates used were hydantoin, methylhydantoin, *iso*-propylhydantoin, *iso*-butylhydantoin, *nor*-butylhydantoin, *tert*-butylhydantoin and hydroxyphenylhydantoin.

3.3 RESULTS AND DISCUSSION

3.3.1 Optimal conditions for enzyme synthesis during growth

3.3.1.1 Substrate induction of enzyme activity

Previous research has shown that the hydantoin hydrolysing enzymes of RUKM3s are inducible (Hartley *et al.*, 1998; Phehane, 1998; Skepu, 2000; Buchanan *et al.*, 2001). In other research Syldatk *et a.l* (1987) investigated the inducibility of a hydantoinase from an *Arthrobacter* spp and found that the enzyme was induced by hydantoin and other 5-monosubstituted hydantoins to various degrees. In the present work, experiments were devised to investigate the induction of hydantoinase and NCAAH of RUKM3s by their substrates. It was found that addition of 0.1% hydantoin to the growth medium had a positive inductive effect on the synthesis of hydantoinase, or expression of hydantoinase activity (Fig. 3.1). The presence of hydantoin increased the hydantoinase activity of resting cells almost twenty-fold. NCG had a small inductive effect. Since NCG is a product of hydantoinase activity, its presence in growth medium could even repress the synthesis of hydantoinase by the cells.

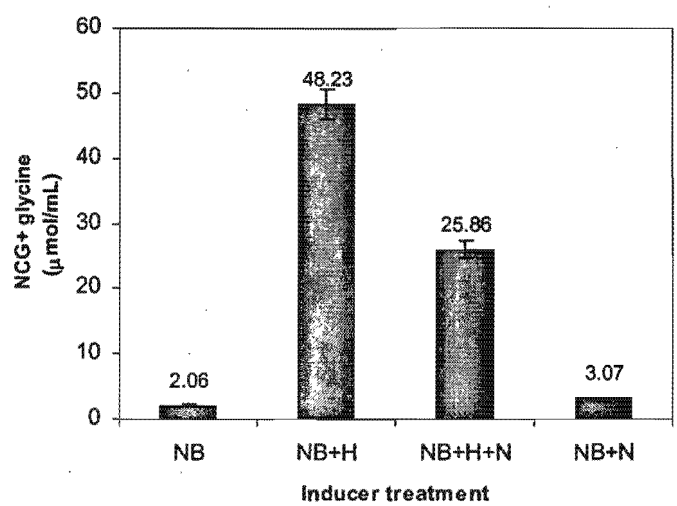


Fig. 3.1 Induction of hydantoinase activity of RUKM3s biomass grown in NB in the presence of 0.1% hydantoin (H) or *N*-carbamyl glycine (N) showing concentration of product (NCG + Glycine).

The addition of inducer level concentrations of hydantoin to the growth medium also had a positive inductive effect on the synthesis of NCAAH (Fig. 3.2), while the presence of 0.1% NCG in the growth medium had an inhibitory effect on NCAAH synthesis. Since NCG is the substrate for NCAAH, it could be expected to induce the enzyme. The observed inhibition may imply that the concentration of 0.1% NCG used may have been in excess of the required amount for induction. In future experiments, it could be useful to vary the concentrations of both hydantoin and NCG added to the medium and determine the effects on activity. Other researchers (Syldatk *et al.,* 1987) have used a number of different compounds in various concentrations before choosing the appropriate inducer. Compounds that have been used include hydantoin, 5-methylhydantoin, 5-hydroxymethylhydantoin, dihydrouracil, indolylmethylhydantoin, hydroxybenzylhydantoin, hydantoin acetic acid, and hydantoin propionic acid. Syldatk *et al.* (1987) also found that resting cells grown without inducer had as low as 1/20th of the activity with inducer. In the present work the presence of hydantoin in the medium during cell growth induced the NCAAH activity of the cells and increased it ten-fold.

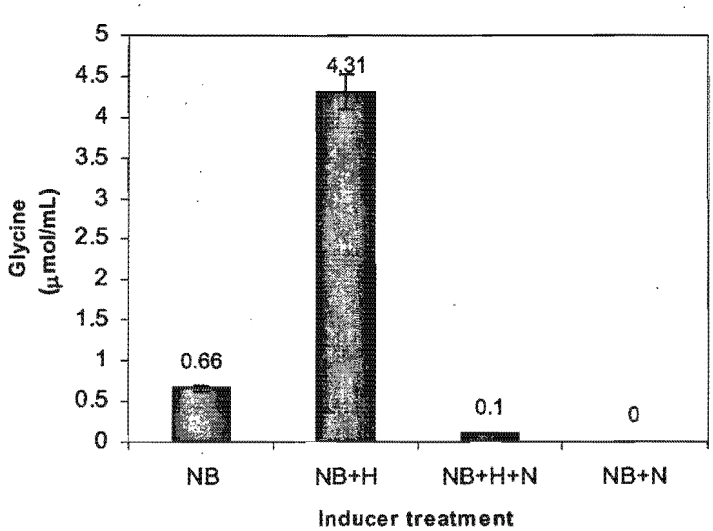


Fig. 3.2 Induction of NCAAH activity of RUKM3s biomass grown in NB in the presence of hydantoin (H) or *N*-carbamyl glycine (N) showing concentration of product (glycine).

3.3.1.2 Effect of divalent metal ions on enzyme activity

The metallo-dependence of hydantoin hydrolysing enzymes has been reported by various research groups. Investigations by some of the research groups have indicated that the synthesis of hydantoinase is dependent on Mn^{2+} ion which is required as a cofactor (Cotoras and Wagner, 1984; Syldatk *et al.*, 1986). Gross *et al.* (1990) found that the hydantoinase from a strain of *Arthrobacter sp.* was induced by the addition of 0.5 mM Mn^{2+} ions to the growth medium. This effect of Mn^{2+} ions was also observed by Ishikawa *et al.* (1996) who found that an *N*-carbamyl-L-amino acid amidohydrolase purified from cells of a recombinant strain of *E. coli* required Mn^{2+} ions (above 1 mM) for optimal activity. However, metal ions can also be inhibitory to activity (Graf *et al.*, 1997; Kim *et al.*, 1986).

In common with reported hydantoin-hydrolysing enzymes, the enzymes of RUKM3s are also metallo-dependent. The effects of direct addition of metals (2.5 mM) on hydantoinase and NCAAH activity are shown in Fig. 3.3 and 3.4 respectively. Manganese increased the hydantoinase product yield over 3 h from 22.1 μmol/mL in the control to 26.4 μmol/mL, and increased the NCAAH product yield from 0.2 μmol/mL in the control, to 0.5 μmol/mL. Magnesium decreased the hydantoinase product yield to 8.6 μmol/ml, and the NCAAH product yield to 0.05 μmol/mL. Zinc reduced the hydantoinase product yield to 0.2 μmol/mL and the NCAAH product yield to zero. Copper, cobalt and iron also had inhibitory effects on the two enzymes, reducing the enzyme activities to zero. These results are consistent with the findings of Graf *et al.* (1997) who found that Cu^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} inhibited hydantoinase activity; and Kim *et al.*, (1986) who found that an *N*-carbamoylase purified from *Pseudomonas putida* 77 was strongly inhibited by Cu^{2+}, Hg^{2+}, and Ag^{2+} ions. The effect of zinc was rather surprising because other reports have indicated that zinc seemed to play an essential role for the catalytic activity as well as the stabilisation of the active quaternary structure of the hydantoinase (May *et al.*, 1998). The use of different concentrations of the metal could establish the optimal concentrations and determine when the metal ion concentrations become inhibitory to enzyme activity.

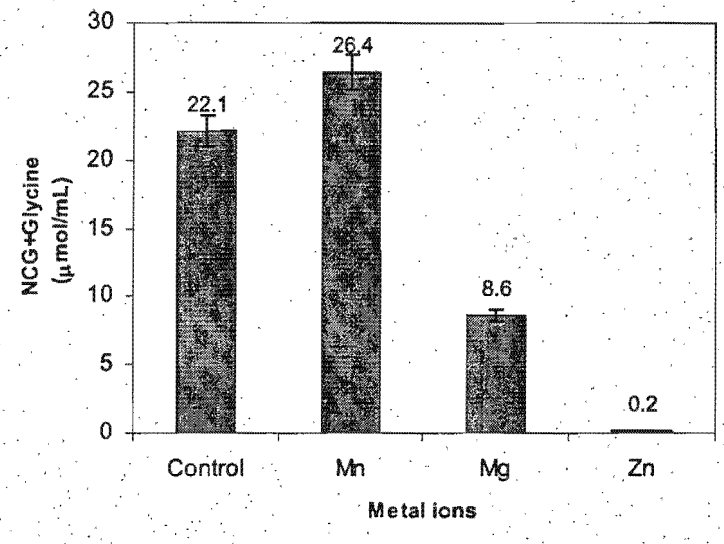


Fig. 3.3 Effect of metal ions on hydantoinase product yield of RUKM3s cells

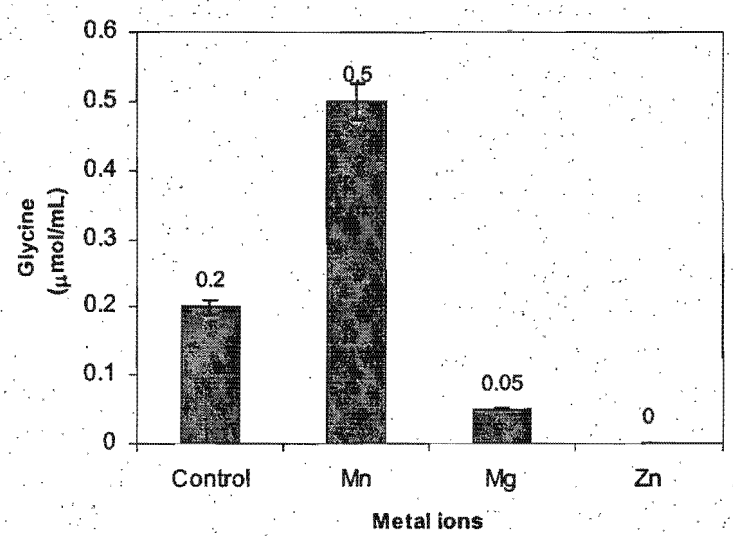


Fig. 3.4 Effect of metal ions on NCAAH product yield of RUKM3s cells

3.3.1.3 Effect of growth temperature on enzyme activity

The results of the determination of the optimal temperature for growth of biomass with high levels of hydantoinase activity indicated that biomass grown at 28 °C had a slightly higher level of hydantoinase than that grown at the other temperatures tested. The expression of NCAAH activity did not show a notable dependence on growth temperature (Fig. 3.5). For both enzymes, it may therefore be concluded that room temperature (25-30 °C) was optimal for enzyme synthesis during growth of RUKM3s cells. This is consistent with growth temperatures optimal for enzyme synthesis reported in literature. Most reports indicate that the optimal growth temperature for hydantoin hydrolysing strains is around 30 °C (Takahashi *et al.*, 1978; Morin *et al.*, 1990).

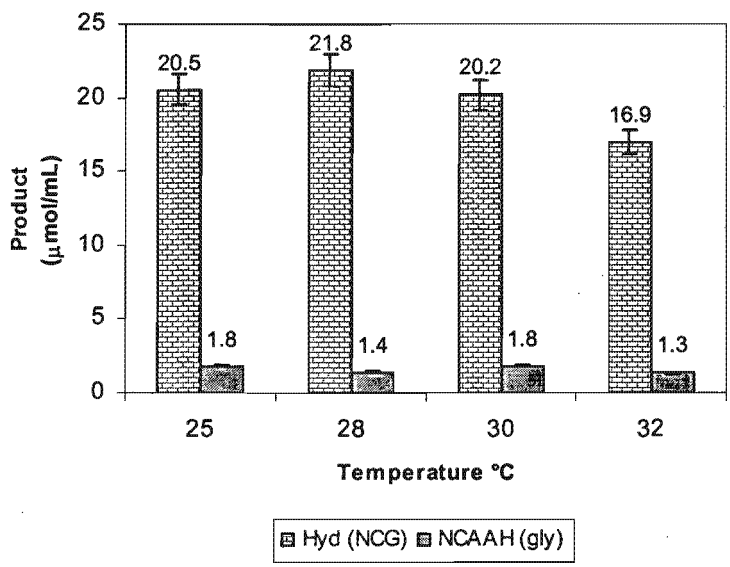


Fig. 3.5 Effect of growth temperature on enzyme activity of RUKM3s cells showing amount of products (NCG and Glycine) after 3 h incubation

3.3.1.4 Effect of growth medium pH on enzyme activity

The results of the determination of the optimal pH for growth of biomass with high levels of hydantoinase activity indicated that biomass grown at pH 7.5 (Fig. 3.6) had a significantly higher level of hydantoinase than at pHs 6.5, 7 and 8. The NCAAH activity was highest at pH 8. The

2.3 RESULTS AND DISCUSSION

2.3.1 Identification by 16s rRNA sequence analysis and description by Scanning Electron Microscopy (SEM)

The strain RU-KM3s was previously identified as *Pseudomonas putida* by 16S rRNA analysis (RU Hydantoinase Research Group, *Annual Report* 1998). The SEM image of RUKM3s cells (Fig. 2.2) shows that the cells are rod shaped, approximately 0.5 μm in diameter and 1-2 μm in length. This description is consistent with the description of *Pseudomonas* spp. in literature as rod-shaped, gram-negative, polar-flagellated, non-sporulating, aerobic bacteria (Palleroni, 1984). *Pseudomonas* spp. are very common in soil and water where they are saprophytes.

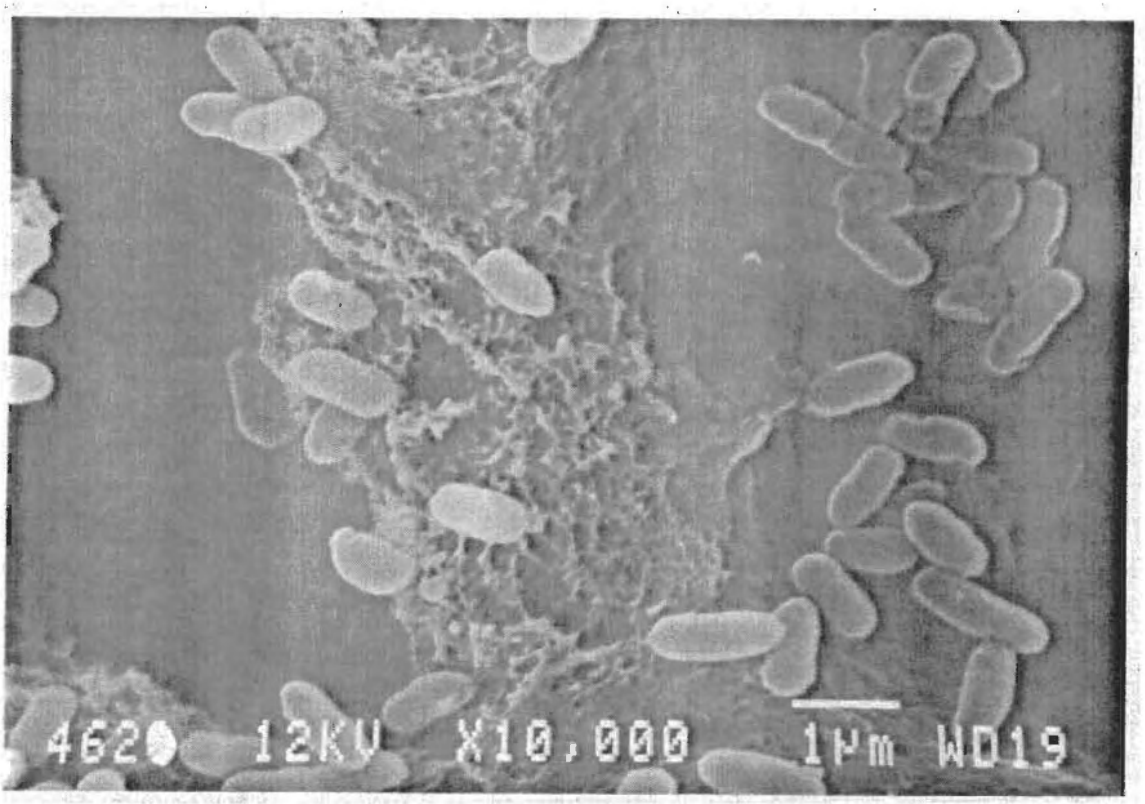


Fig. 2.2 SEM of RUKM3s cells

reported optimal growth pH for most *Pseudomonas* spp that show hydantoin hydrolysing activity is pH 5-7 (Demain and Solomon, 1985; Kim *et al.*, 1994; Morin *et al.*, 1990). The values reported here are about 1 pH unit lower than the pH optima of the enzymes themselves (section 3.3.2.3).

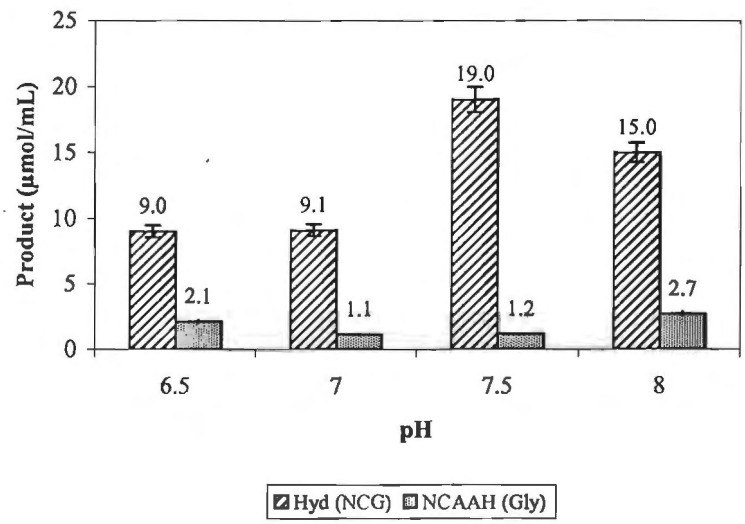


Fig. 3.6 Effect of growth pH on enzyme activity of RUKM3s cells

3.3.1.5 Effect of DO_2 on enzyme activity during growth of RUKM3s cells

Enzyme activity in biomass grown at different levels of DO_2 (Fig. 3.7) did not demonstrate a clear DO_2-dependent trend. The highest levels of hydantoinase were noted at 10% and at 50% DO_2, and lower levels at 25 and 40% DO_2. The NCAAH activity was highest at 40-50 % DO_2. There are no reports on determinations of the dependence of hydantoinase and NCAAH enzyme activities on dissolved oxygen levels. This is probably due to the fact that most of strains are grown under fully aerobic conditions and high biomass levels are usually obtained when there is no oxygen deficiency for aerobic microorganisms (Demain and Solomon, 1985).

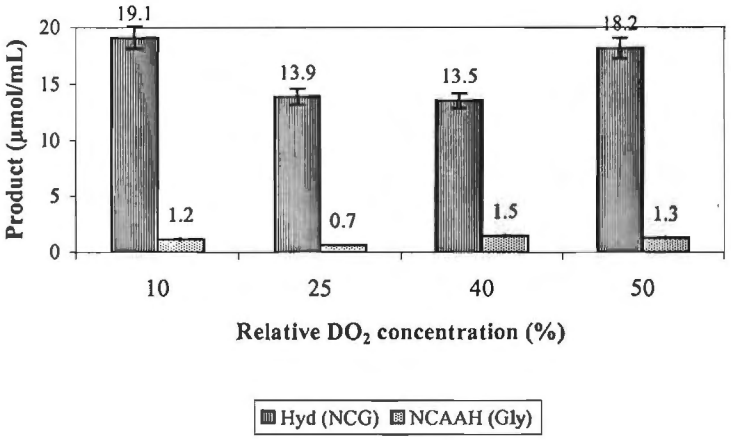


Fig. 3.7 Effect of relative dissolved oxygen on enzyme activity during growth of RUKM3s cells

3.3.2 Optimal conditions for biocatalytic production of amino acids

3.3.2.1 Effect of substrate concentration on of enzyme activity

The results of an investigation into the dependence of hydantoinase activity of RUKM3s on substrate concentration (Fig 3.8) showed that the rate of product formation increased with initial substrate concentration for hydantoin concentrations of 5, 15 and 25 mM. However, the apparent difference in initial rates between 15 and 25 mM was less than that between 5 and 15 mM. The product yield from the 5 mM hydantoin, obtained after 3 h of reaction was 72% of theoretical yield (3.6 mM). The product yield from 15 mM hydantoin, obtained after 3 h of reaction was 95% of theoretical yield (14.3 mM). Product yield from 25 mM hydantoin was 90% of theoretical yield (22.5 mM) and was still increasing at 3 h when the reactions were stopped.

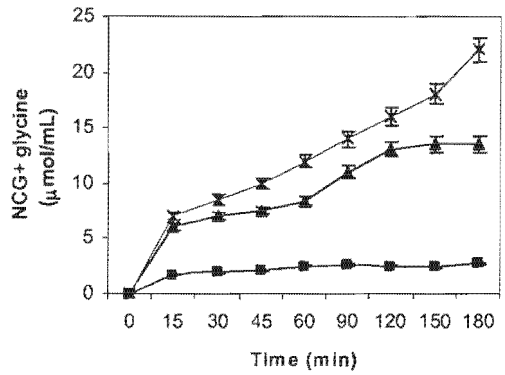


Fig. 3.8 Effect of substrate concentration on hydantoinase product yield (NCG + Glycine) of RUKM3s extract from 5 (■), 15 (▲) and 25 (×) mM hydantoin.

The results of the investigation of the dependence of NCAAH activity on substrate concentration (Fig. 3.9) show that the rate of product formation increased with initial substrate concentration for NCG concentrations of 2.5, 7.5 and 12.5 mM. The product yield obtained from 2.5 mM NCG was 24% (0.6 mM) of the theoretical yield at 3 h. The yield recorded for 7.5 mM substrate was 14 % (1.1 mM) of the theoretical yield at 3 h, and that yield recorded for 12.5 mM substrate was 18 % (2.3 mM) of the theoretical yield at 3 h. The reactions with 7.5 and 12.5 mM substrate were still in progress when the experiment ended at 3 h.

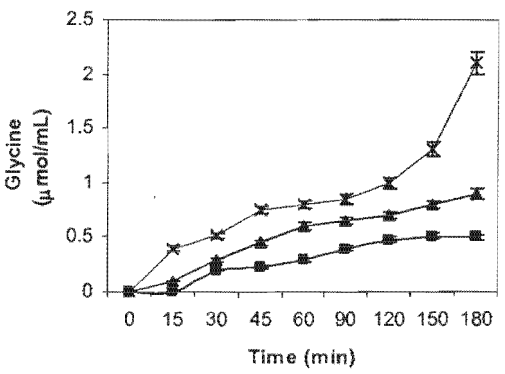


Fig. 3.9 Effect of initial substrate concentration on NCAAH product yield (glycine) of RUKM3s extract from 2.5 (■), 7.5 (▲) and 12.5 (x) mM NCG.

3.3.2.2 Effect of pH on enzyme activity

Hydantoin hydrolysing enzymes from *Pseudomonas* spp have been described with optimum reaction pH at various points from pH 5 to 11 (Morin *et al.*, 1990). It was therefore necessary to determine the optimum pH for the biocatalytic reactions of RUKM3s. The effect of pH on amino acid production was tested by evaluating the hydantoinase and the NCAAH activity of RUKM3s extract during biocatalytic reaction at different pH from 5 to 10 in phosphate buffer. The results of this evaluation are presented in Figs. 3.10 and 3.11 for hydantoinase and NCAAH respectively. The highest hydantoinase activity was recorded at pH 9 to 10 and that of the NCAAH at pH 9. These results are consistent with those previously reported for RUKM3s (Buchanan *et al.*, 2001).

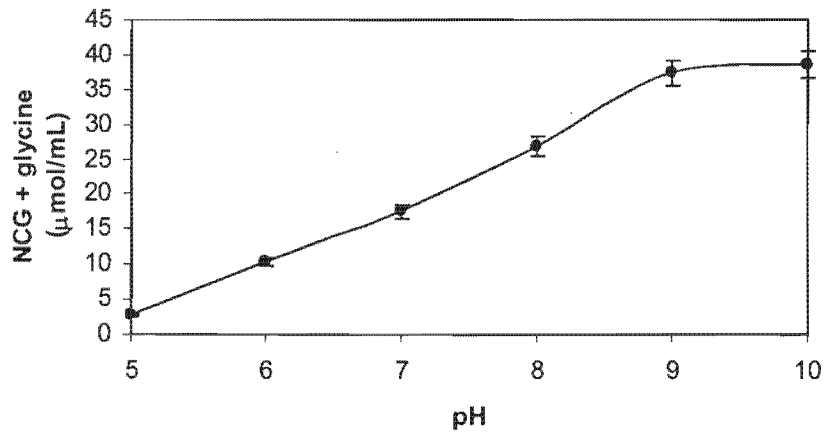


Fig. 3.10 Profile of RUKM3s hydantoinase product yield (NCG + glycine) from 50 mM hydantoin at different pH values.

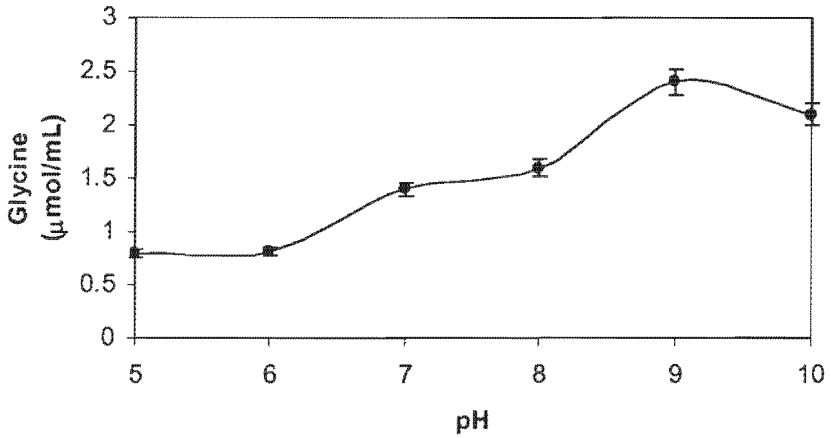


Fig. 3.11 Profile of NCAAH product yield (glycine) from 25 mM NCG as substrate at different pH values.

3.3.2.3 Effect of Temperature on enzyme activity

The temperature optima reported for biocatalytic reactions of hydantoinases are as varied as the optimum pH. Hydantoinases and *N*-carbamoylases from *Pseudomonas* spp have been described in literature with optimal reaction temperatures of 30-55 °C (Morin *et al.*, 1990). The effect of temperature on amino acid production was tested for RUKM3s extract by evaluating the NCAAH and the hydantoinase activity during biocatalytic reactions carried out at temperatures of 20, 30, 40, 50 and 60 °C. The results of this evaluation are presented in Fig. 3.12 and 3.13 for hydantoinase and NCAAH respectively. The optimum temperature for product formation by hydantoinase and NCAAH of RUKM3s extract was 40°C. These results are consistent with those previously reported for RUKM3s (Buchanan *et al.*, 2001).

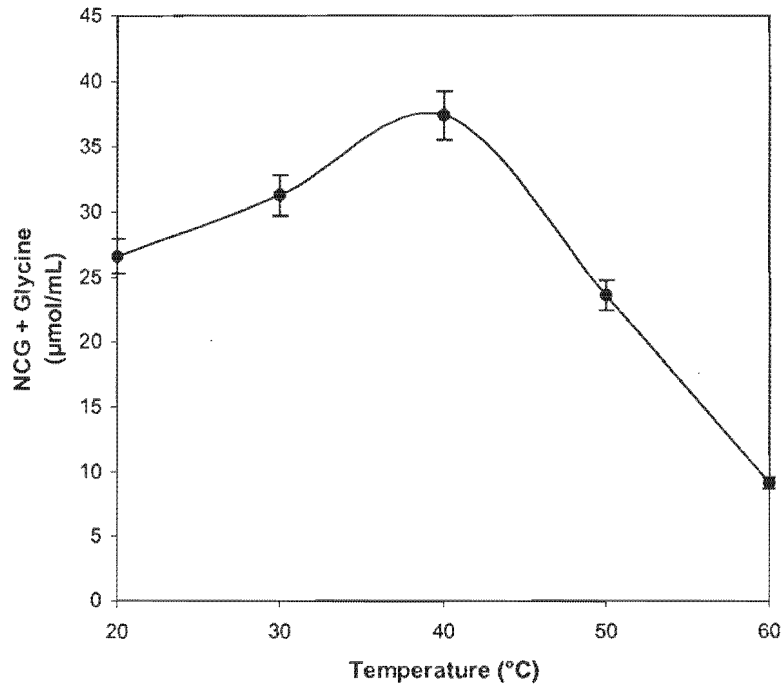


Fig. 3.12 Profile of RUKM3s hydantoinase product yield (NCG + Glycine) from 50 mM hydantoin at different temperatures

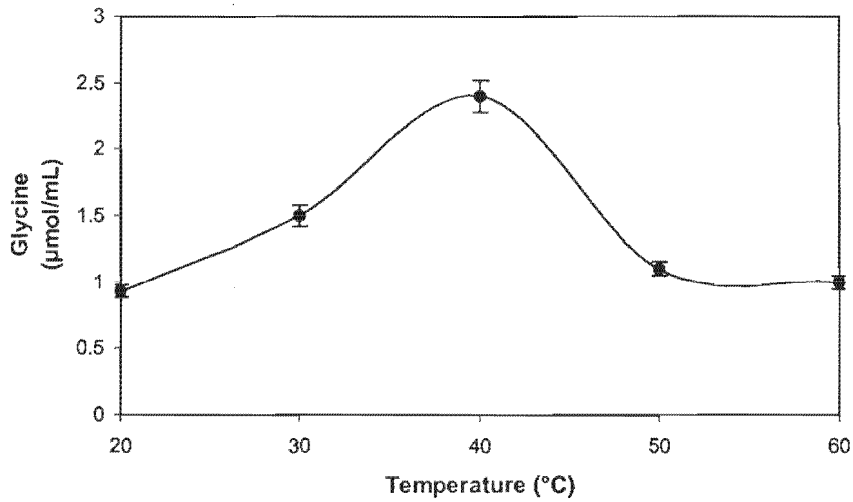


Fig. 3.13 Profile of RUKM3s NCAAH product yield (Glycine) from 25 mM NCG at different temperatures

3.3.3 Yield of amino acids from various substrates

The hydrolytic conversion of 5-monosubstituted hydantoins is likely to be affected by various factors including the steric size of side groups in the substrate molecules. In previous work on RUKM3s, it was found that product yields were substrate-dependent (Skepu, 2000). Other researchers have reported that hydantoinases have broad substrate selectivity, and stereoselectivity that seems to depend on the side chain (Syldatk *et al.*, 1987).

In the present work, experiments were devised to determine the relationship between yield and the size of the side groups in 5-monosubstituted hydantoins. The yields of *N*-carbamoyl amino acids and amino acids from various hydantoin substrates were evaluated by using their respective substrates in biocatalytic reactions with RUKM3s extract and assaying for the products as described previously (see section 2.2.6.3 and 2.2.6.4). The activity of the NCAAH was found to be very low, and close to zero yields of amino acid were obtained for the substrates tested other than hydantoin. However, it was found that appreciable yields of the intermediate products of alanine, valine, leucine, *nor*-leucine and *p*-HPG were achieved. The amount of product decreased with increases in the size of the side groups of the 5-monosubstituted hydantoin substrates. The yields are reported in Fig. 3.14 for hydantoin, methylhydantoin (MH), isopropylhydantoin (IPH), isobutylhydantoin (IBH), *nor*-butylhydantoin (*n*BH) and hydroxyphenylhydantoin (HPH). The general structure of a 5-monosubstituted hydantoin was presented in Fig. 1.2. The structural formula of the R-substituents of the 5-monosubstituted hydantoin substrates used are presented in Table 3.1.

In this experiment, the yields obtained from various 5-monosubstituted hydantoins relative to hydantoin (100 %), were methylhydantoin (56.7 %), *n*-bultyhydantoin (53.5 %), iso-butylhydantoin (35.9 %), *p*-hydroxyphenylhydantoin (35.9 %) and isopropylhydantoin (25.5 %). The results confirm that the product yield decreases with the size of the side groups. Substrates with small and straight-chain side groups yielded more product than the sterically bulky substrates with branched-chain side groups.

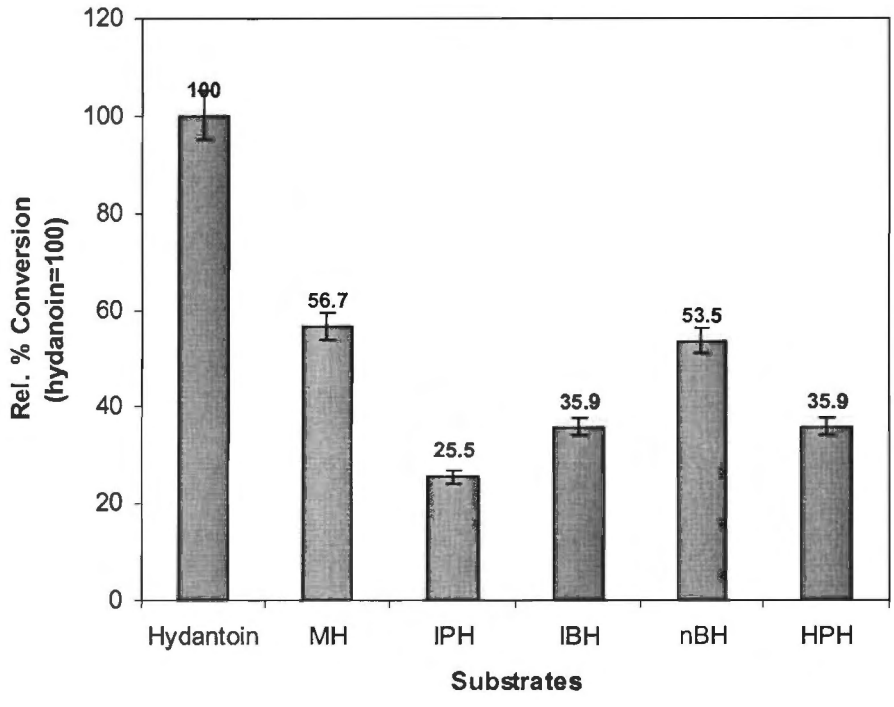
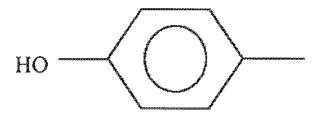


Fig. 3.14 Yield of *N*-carbamylamino acids from various 5-monosubstituted hydantoin substrates relative to hydantoin (100%)

Table 3.1 Structural formula of R-group of 5-monosubstituted hydantoin substrates

Name	Structure of R-group
Hydantoin	H-
Methyl hydantoin	CH_3-
Iso-propylhydantoin	(CH3)2CH-
Iso-butylhydantoin	(CH3)2CH-CH2-
Nor-butylhydantoin	CH3-CH2-CH2-CH2-

Hydroxyphenylhydantoin	

3.4 CONCLUSIONS

The synthesis of hydantoinase and NCAAH is induced by the presence of hydantoin in the growth medium. However, the presence of NCG in the growth medium resulted in lower NCAAH activity, and had only a very small inductive effect on hydantoinase. The addition of manganese sulphate to the growth medium resulted in higher enzyme activity. Magnesium and zinc added to biocatalytic reaction mixtures inhibited the activities of both enzymes. Further work should determine the effect of different concentrations of various metal ions to determine the optimum concentrations for induction of activity and thresholds for inhibition. This is important to improve productivity, as well as achieve optimal, economical concentrations of metal in the industrial process for production of amino acids.

In investigation of growth conditions, it was found that RUKM3s biomass grown at 28 °C had the highest hydantoinase activity, but NCAAH production did not show a dependence on the growth temperature. The biomass grown at pH 7.5 had the highest hydantoinase activity, and as in the case of temperature, the dependence of NCAAH on the pH of medium during growth was not significant. The levels of enzyme activity measured did not show dependence on the DO_2 levels during growth. To our knowledge, the effect of dissolved oxygen saturation has not been reported on with respect to hydantoin hydrolysing enzymes. However, the possible influence of oxygen on the amount of enzyme synthesized could be investigated further because this may have important implication for development of large scale processes. Conversely, high concentration of biomass, usually favoured by high levels of oxygen saturation, may be achieved at the expense of metabolite formation (Demain and Solomon, 1985).

The optimal temperature for the biocatalytic reactions for both hydantoinase and NCAAH of RUKM3s was 40 °C. The optimal pH for NCAAH activity during the reactions was found to be 9, and that for hydantoinase was 9 to10 under the reaction conditions used here. The amount of product from NCAAH was highest after 5 h of reaction, and that from hydantoinase was highest

from 3 h of incubation. This confirmed earlier findings for RUKM3s (Buchanan et al., 2001), and is consistent with reported temperature and pH optima for hydantoin hydrolysing enzymes from *Pseudomonas* spp (Morin *et al.*, 1990; Syldatk *et al.*, 1978).

In these experiments the objective was to determine the broad operating conditions of the system. The experiments were not carried out in the context of classical enzyme kinetics since the study sought to develop an industrial process and the enzymes used were part of an impure or crude extract. A detailed kinetic study of the enzyme would require a pure enzyme. Further work could be done to determine the threshold concentrations of substrate and product, as well as the possible incidence of product inhibition, which can affect the maximum yield obtainable.

The hydantoinase enzymes of RUKM3s were able to convert substrates other than hydantoin to amino acids. The system is therefore potentially a good catalyst for amino acid production in a general process adaptable to the various substrates. In experiments to produce amino acids from various 5-monosubstituted hydantoin substrates, yields of *N*-carbamyl amino acids as a percentage relative to the value obtained for hydantoins's conversion to *N*-carbamylglycine (100%), were by *N*-carbamylalanine (56.7 %), *N*-carbamyl-*nor*-leucine (53.5 %), *N*-carbamyl-*iso*-leucine (35.9 %), *N*-carbamyl hydroxyphenylglycine (35.9 %) and *N*-carbamylvaline (25.5%). It was found that the amount of product yield decreased with increase in the steric size of the side groups on the hydantoin substrates. In this regard, steric size was found to be more influential than molecular weight.

The work reported in this chapter established the most suitable conditions for application of RUKM3s crude extract as a biocatalyst in the production of amino acids, and has shown that, under these conditions, the hydantoinase was more stable, robust and highly active, while the NCAAH is less stable and less active.

CHAPTER 4

PURIFICATION AND CHARACTERISATION OF HYDANTOIN HYDROLYSING ENZYMES FROM RUKM3s

4.1 INTRODUCTION

The novelty of a new biocatalytic process derives in part from the unique characteristics of the enzymes(s) involved. In order to establish the novelty of the biocatalyst from the hydantoin hydrolysing enzymes of RUKM3s, experiments were designed to purify and characterise the hydantoinase and the NCAAH from the strain. Enzyme purification protocols generally commence with precipitation steps to separate the desired enzyme from solution. Precipitated protein pellets are resuspended in buffer, and assayed for the desired enzyme activity. Fractions containing the desired enzyme are then subjected to further steps that isolate the enzyme from non-active protein. Isolation can be achieved by separation in aqueous two-phase systems or by elution through gel filtration chromatographic columns. After isolation, fractions containing the desired enzyme activity can be pooled, concentrated by dialysis and freeze-dried into an enzyme powder.

The most common precipitation methods use salts such as ammonium sulphate, non-ionic polymers such as polyethylene glycol (PEG) and dextran, and organic solvents such as acetone (McPherson, 1982). The underlying principle is that proteins precipitate from solution at different concentrations of the precipitant depending on their hydrophobicity or hydrophilicity. Precipitation is based on the interaction of the precipitant with water rather than with the protein. When the salt, polymer or organic solvent molecules make water less available from around the proteins, the protein molecules form aggregates that precipitate.

Precipitation of proteins by *salts* depends on differential precipitation as a function of the salt concentration. Individual proteins display specific solubilities at varying levels of salt concentration. They can therefore be selectively precipitated without denaturation and can be removed by centrifugation or filtration. The points at which individual proteins precipitate, the precipitation points or solubility minima, depend on the pH, temperature, protein concentration and other ambient conditions. *Non-ionic polymers* such as PEG can also be used to precipitate proteins. The most commonly used are PEG 6000-8000 or 20000 MW. Most proteins precipitate

below a concentration of 20% (w/v) PEG. Solvents such as water and dimethylsulphoxide (DMSO) with large dielectric constants can stabilise the interaction between themselves and protein molecules in favour of the dissolution of protein. Organic solvents such as acetone and methanol, with small dielectric constants, discourage the dispersion of protein molecules in media. The solubility of proteins can be lowered, and their precipitation induced, by lowering the effective dielectric constant of media. Thus, this can be achieved by adding a water-soluble solvent such as acetone to an aqueous solution of protein.

Aqueous two-phase systems (ATPS) are also useful for separation of proteins. Dextran, PEG, and ammonium phosphate are all soluble in water, but when an aqueous solution of PEG and Dextran, or of PEG and ammonium phosphate is prepared, two phases appear in the solution. PEG is less polar than both dextran and phosphate, so most proteins will partition into the PEG phase. For mixtures of proteins, factors that determine which phase each protein migrates to, and the extents to which partitioning into the two phases occurs are length of polymer, size of protein, salt, temperature, added hydrophobic modifier and pH (Johansson, 1985).

Proteins that have been separated by precipitation and partitioning in ATPS may still contain some undesired impurities, such as other proteins or high levels of salt/polymer, or may be very dilute, requiring concentration. Some of these problems are overcome by membrane dialysis. Further concentration of the protein can be achieved by freeze-drying the dialysate into a powder of pure or partially pure protein. This partially purified protein can be used in bioconversions, be subjected to further analysis and characterisation, or be purified further through gel permeation, ion-exchangers, and affinity adsorbents.

The purity of enzymes is commonly determined by electrophoresis on polyacrylamide gels, where proteins are separated on the basis of their size and net charge under denaturing or non-denaturing conditions. On non-denaturing gel, pure proteins appear as single bands whose molecular weight and relative intensities can be determined. Under denaturing conditions, SDS-PAGE provides information on the number and size of the molecular subunits of proteins. The molecular weight of proteins can also be determined by the use of size exclusion chromatography (SEC), which separates molecules on the basis of their size (Stokes radius). Protein detection in SEC is typically achieved by UV absorbance at 280nm or 254nm (Yau *et al.*, 1979). Chiral substrates can be used to determine the stereospecificity of enzymes.

The objective of the work reported in this chapter was to investigate the suitability of various methods of enzyme isolation, purification and characterisation for use with the hydantoin hydrolysing enzymes of RUKM3s. Hydantoinase and NCAAH were precipitated from supernatant by ammonium sulphate or acetone precipitation. The precipitated pellets were resuspended in buffer and the fractions positive for hydantoinase and NCAAH activity were eluted through gel filtration chromatography columns to isolate the active proteins. The fractions containing the enzymes were pooled together, concentrated and characterised by gel electrophoresis and size exclusion chromatography. The stereoselectivity of the enzymes was determined by chiral chromatographic analysis of their reaction products.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Ammonium sulphate, acetone, ammonium persulphate and glycerol were purchased from SAARCHEM (RSA); HPLC grade methanol was purchased from BDH chemicals (England); polyethylene glycol 6000, glacial acetic acid, *N,N,N',N'*- tetramethylethylene-diamine (TEMED) and bromophenol blue were purchased from Merck (Germany); Tris-hydrochloride, Tris[hydroxymethyl]aminomethane, blue dextran, molecular weight standards and mercaptoethanol were purchased from Sigma (USA); Sephadex G 75, Sephacryl S-100, and Spherisorb 6B were purchased from Pharmacia Biotech (USA); Acrylamide, *N,N'*-methylene-*bis*-Acrylamide and sodium dodecyl sulphate were purchased from Riedel-de Haen (Germany); hydroxyphenyl hydantoin, *N*-carbamyl hydroxyphenylhydantoin and hydroxyphenylglycine were synthesised at the CSIR (South Africa).

4.2.2 Precipitation of protein

4.2.2.1 Ammonium sulphate precipitation (Wang, 2001; Clark and Switzer, 1977)

RUKM3s cells suspended in potassium phosphate buffer (0.1M, pH 8) at 100 mg cells/mL were sonicated for 5 min and the crude extract centrifuged at 8000 rpm for 15 min. A sample of the supernatant was assayed for protein and for enzyme activity. After removing the precipitate, ammonium sulphate was added to the supernatant to 20 % saturation. A precipitate was obtained after incubation at 4 °C for 60 min with stirring, followed by centrifugation at 8000 rpm for 15

min. The supernatant was decanted and additional ammonium sulphate was added to 30 % saturation. The process was repeated for 40, 50, 60 and 70 % and the precipitates retained.

Samples of the supernatants were collected and assayed for protein concentration and for enzyme activity. The precipitates were resuspended in phosphate buffer to 10 mL, assayed for activity. Hydantoin was used as substrate for the colorimetric assay, and hydroxyphenyl hydantoin (HPH) was used as substrate for assays by HPLC. SDS- and native- polyacrylamide gel electrophoresis (PAGE) were carried out using 10 % resolving and 4 % stacking gels (Walker, 1996).

4.2.2.2 Acetone precipitation (Wang, 2001; Clark and Switzer, 1977)

To 10 mL fractions of supernatant from sonicated crude extract, acetone was added to make 20, 40, 50, 60 and 80 (v/v %). The solutions were vortexed and held in an ice bath for 30 minutes. Precipitates were removed by centrifugation at 10 000 g for 15 minutes. The supernatants of the fractions after centrifugation were assayed for enzyme activity and protein concentration. The precipitate was assayed for activity and characterised by SDS-PAGE.

4.2.3 Separation in aqueous two-phase systems (Johansson, 1985)

An aqueous two-phase system (ATPS) consisting of 20% (w/w) PEG 6000 and 10% (w/w) ammonium sulphate in potassium phosphate buffer (0.1 M, pH 8) was prepared. Cell suspensions (50 mL, 100 mg/mL) were prepared in the ATPS solution. These were then sonicated for 5 minutes, and the phases allowed to partition in a separating funnel. The phases were then drained out of the funnel separately, the fractions dialyzed to remove ammonium sulphate and PEG, then assayed for activity.

4.2.4 Separation by gel filtration and perfusive chromatography

A 250 mL crude extract of RUKM3s cells (100 mg/mL) in Tris buffer (pH 8, 20 mM) was prepared by sonication for 5 min. The extract was centrifuged at 8000 rpm for 10 min to remove cell debris. The supernatant was then micro-filtered through a 0.45 μm Whatman® membrane. The supernatant was chilled on ice and precipitated with 60 % acetone. The precipitate was redissolved in *Tris*-HCl buffer (pH 8.0, 20 mM).

Redissolved protein from fractional precipitation with acetone was loaded on reversed phase HPLC (POROS R1 and R2) and ion exchange 20R2 4.6 D/100 mm columns in a BIOCAD perfusive chromatography system (PerSeptive Biosystems, Framingham, MA, USA). A sample of the protein precipitated with 60% acetone, and known to contain both hydantoinase and NCAAH activity, was also eluted through a Sephacryl S-100 HR column in the same instrument.

In additional experiments, gel filtration matrices were prepared by suspending 5g of Sephadex G 75, Sephacryl S-100, and Spherisorb 6B (Pharmacia Biotech) in 200 ml of water. The columns were pre-equilibrated with Tris-HCl buffer (20 mM, pH 8). Measured volumes (0.1-1 mL) of protein solution (500mg/mL) were applied to the columns. Tris-HCl buffer (20 mM, pH 8) was then passed through the column at a flow rate of 0.4 mL/min and 1.5 mL fractions were collected as the sample components eluted. Bradford's assay was used to measure the concentration of eluted protein in the fractions. Ninhydrin and Erhlich's assays were used to measure the enzyme activity in the fractions. Fractions showing activities were pooled, dialyzed against 0.01 M Tris-HCl buffer (pH 8.0), concentrated by dialysis against a 20% solution of PEG, freeze-dried and stored at -20 °C until needed for characterization by gel electrophoresis.

4.2.5 Characterisation of partially purified protein by ND- and SDS- PAGE (Determination of purity by gel electrophoresis)

Fractions from gel permeation chromatography prepared as described in 4.2.4, were characterized by gel electrophoresis (see APPENDIX E for detailed protocol). ND-PAGE was used to determine the purity of the protein samples and SDS-PAGE was used to determine the number and size of the subunits (Walker, 1996).

4.2.6 Characterisation by size exclusion chromatography

Freeze-dried samples of protein powder prepared as described in section 4.2.4 were resuspended in *Tris*-HCl buffer (20 mM, pH 8.0) and applied to a Waters Ultrahydrogel size exclusion chromatography column to determine the molecular weights. The mobile phase was potassium phosphate buffer (100 mM, pH 8) at a flow rate of 0.6 mL/min. Blue dextran (MW 2 000,000 Da) was used to determine the void volume of the column, and glycine was used to determine the total volume. The elution times of known molecular weight standards were measured, and a plot

of the distribution coefficient, Kd, against the log MW was used to estimate the molecular weights of the sample components on the basis of their calculated Kd's (Walker, 1996).

4.2.7 Determination of enantioselectivity of RUKM3s hydantoinases

To determine the enatioselectivity of the hydantoin-hyrdolysing enzymes, 2.5 mL of crude extract of RUKM3s (100 mg/mL) were used in biocatalytic reactions with 2.5 mL portions (25 mM) of L-methylhydantoin, D-methylhydantoin and *N*-carbamyl-D,L-alanine. The products of the biocatalytic reaction were analysed for chirality. Samples were collected from the reaction mixture, centrifuged, filtered and eluted through a Nucleodex beta-PM, 200 mm X 4 mm ID chiral HPLC column (Macherey-Nagel, Germany). L-alanine was used as a standard. The samples were eluted with 20% methanol in phosphate buffer (40mM, pH 3.7) at a flow rate of 0.2 mL/min with detection at 210 nm.

4.3 RESULTS AND DISCUSSION

4.3.1 Assessment of techniques of protein precipitation used to purify RUKM3s enzymes

Different techniques of protein precipitation are reported in literature. The suitability of a specific technique for precipitation of a particular set of proteins is dependent on factors such as the source, type and characteristics of the binding forces at the primary, secondary and tertiary structures of the protein. The suitability of precipitation by salt and organic solvent, and at the isoelectric point, was investigated for RUKM3s enzymes.

4.3.1.1 Ammonium sulphate precipitation

The change in protein concentration in the supernatant and in the precipitate, and the activity of the enzymes in the precipitated fractions, were monitored during ammonium sulphate precipitation of the protein in the cell-free supernatant of RUKM3s extract. As expected, a gradual decrease in the protein concentration in the supernatant was observed with addition of increasing concentrations of ammonium sulphate (Fig. 4.1).

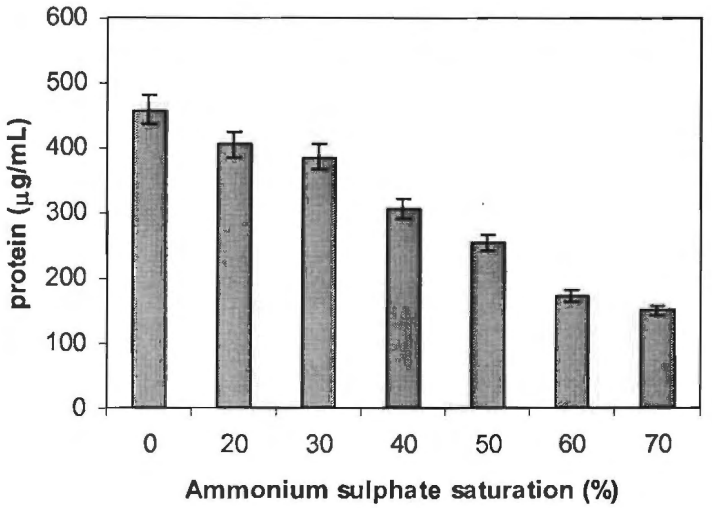


Fig. 4.1 Change in protein concentration in supernatant of RUKM3s crude extract with increasing ammonium sulphate saturation (%)

The relative activities of hydantoinase in the pellets and the supernatants were assayed by the Erhlich's assay and recorded for different salt concentrations, expressed relative to the initial activity in the supernatant (Fig. 4.2). A salt concentration of 40% ammonium sulphate saturation precipitated all the hydantoinase activity from the supernatant.

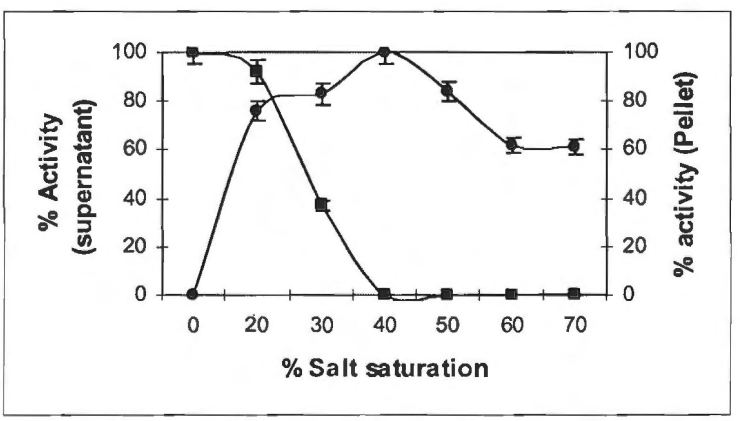


Fig. 4.2 Changes in the hydantoinase activity of the supernatant (■) and the resuspended pellet (●) as % of original activity of the supernatant recorded for different levels of % ammonium sulphate saturation

Ammonium sulphate interferes with the ninhydrin assay for NCAAH activity. A method using chromatographic analysis had to be devised to evaluate yields from the enzyme activities. However, hydantoin and its products are not suitable for measurement by HPLC using UV detection. Derivatives with phenyl groups that can absorb UV light need to be used. Therefore, the NCAAH and hydantoinase activities were assayed by reverse phase HPLC using hydroxyphenylhydantoin (HPH) as substrate, and *N*-carbamyl hydroxyphenylglycine (NC-HPG) and hydroxyphenylglycine (HPG) as standards for the products. The activities of hydantoinase and NCAAH of RUKM3s are lower with substituted hydantoins than in hydantoin itself, but still high enough to provide a reasonable comparison between the activities of the precipitated fractions. The results showed that the total conversion of HPH was highest in the sample containing precipitate obtained at 40% ammonium sulphate saturation (Fig. 4.3). The same precipitate also produced the highest yield of NC-HPG product (Fig. 4.4) and as expected the highest yield of HPG (Fig. 4.5). These results indicated that both the hydantoinase and NCAAH levels were highest in the protein fraction precipitated at 40% ammonium sulphate saturation.

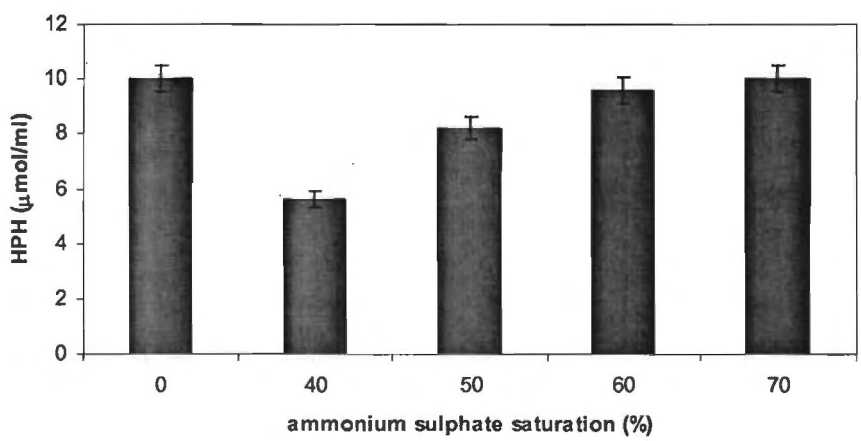


Fig. 4.3 Amount of HPH remaining after biocatalytic reaction of protein fractions precipitated by ammonium sulphate from supernatant of RUKM3s crude extract, measured by HPLC.

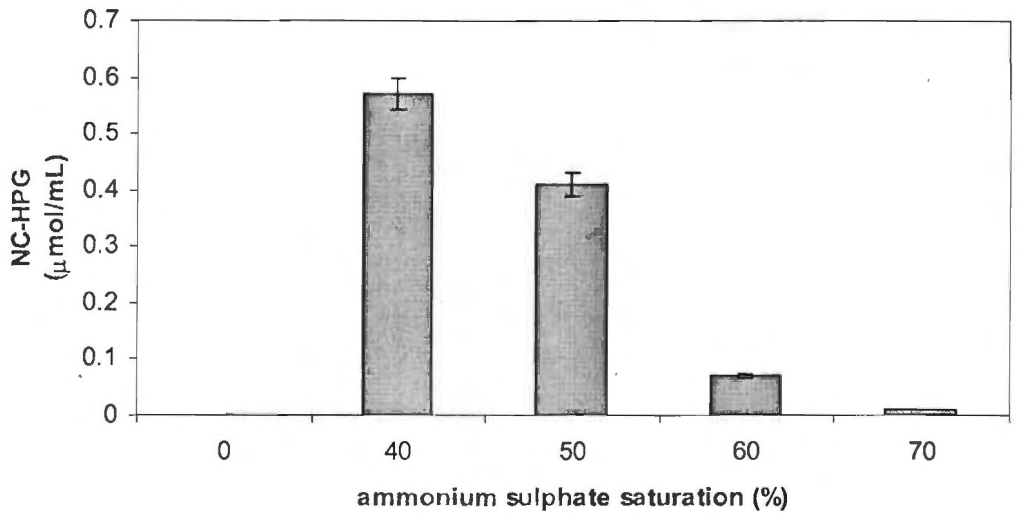


Fig. 4.4 Amount of NC-HPG produced from biocatalytic reaction of HPH with protein fractions precipitated by ammonium sulphate from supernatant of RUKM3s crude extract, measured by HPLC.

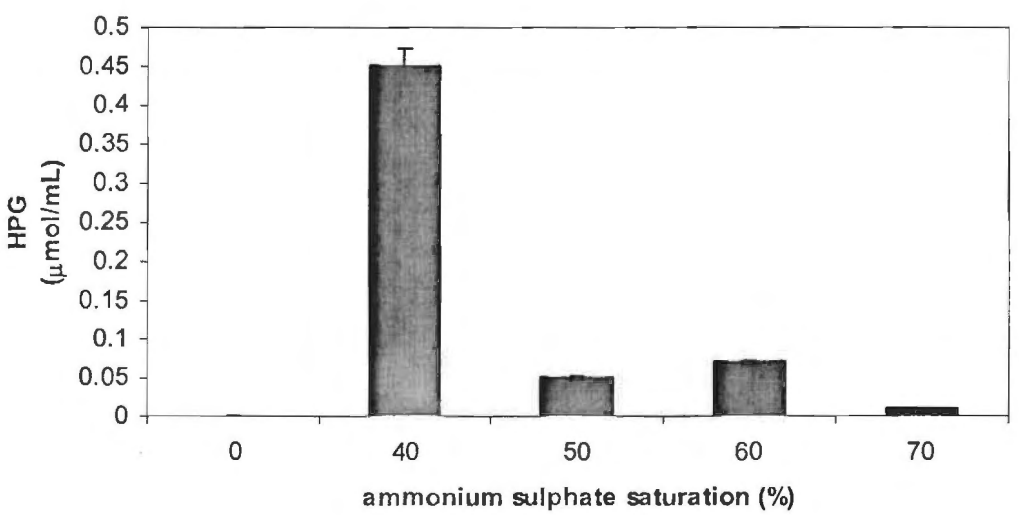


Fig. 4.5 Amount of HPG produced during biocatalytic reaction of HPH with protein fractions precipitated by ammonium sulphate from supernatant of RUKM3s crude extract, measured by HPLC.

The protein pellets precipitated by ammonium sulphate at different levels of saturation were analysed for the molecular weight and the subunit structure by ND and SDS- PAGE. The results

of ND-PAGE performed on the protein fractions precipitated at 20, 30, 40, 50, 60 and 70 % ammonium sulphate saturation are shown on Fig. 4.6. The hydantoinase and NCAAH active protein from the fraction precipitated by 40% salt saturation showed the strongest band at approximately 200 kDa. The NCAAH active fraction precipitated by 60% salt saturation gave the strongest bands at approximately 60 and 30 kDa. Assuming that these were the NCAAH bands, it is likely that the band at 60 kDa was a dimer and the band at 30 kDa would be the subunits. Most NCAAHs are reported in literature to be homo-dimeric (Expasy, 2000). Confirmation of the true identity of the bands would require a determination of the enzyme activities of the protein in each band.

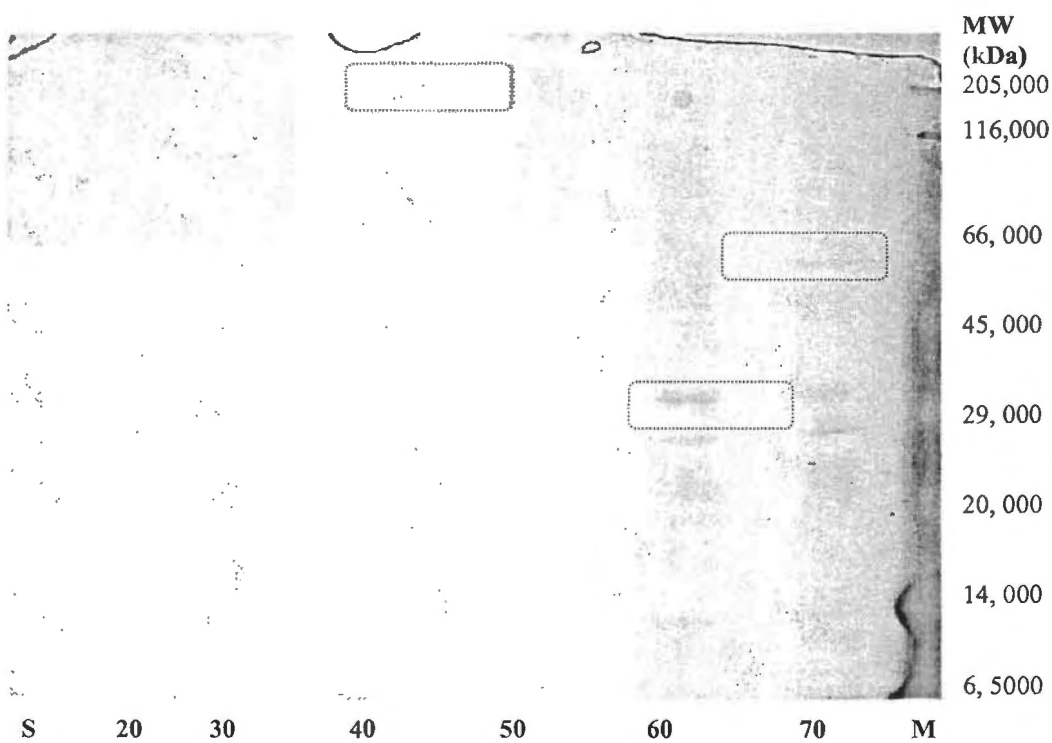


Fig. 4.6 Native-PAGE showing bands from protein fractions precipitated by ammonium sulphate (20-70% saturation) from supernatant (S) and markers (M).

SDS-PAGE (Fig. 4.7) showed that the hydantoinase and NCAAH- active protein precipitated at 40% salt saturation had very distinct bands at about 70 and 35 kDa. Assuming that the band noted at approximately 200 kDa on the ND-PAGE (Fig. 4.6) was the hydantoinase, it is likely that the bands at 70 and 35 were its subunits. Therefore, the hydantoinase of RUKM3s is likely

to be a hetero-tetramer with two subunits of approximately 70, and two of approximately 35 kDa. This is in agreement with reports by others, where most hydantoinases reported in literature are tetramers (Expasy, 2000). After SDS-PAGE, the NCAAH active protein precipitated at 60% salt saturation showed the strongest band at approximately 30 kDa and a fainter band at approximately 60 kDa, which may be residual native NCAAH. The deduction made in the earlier discussion of the ND-PAGE results, that the NCAAH is dimeric with an estimated molecular weight of about 60 kDa and two subunits of about 30 kDa is therefore supported. The additional band at about 40 kDa in the 60% fraction may be due to another non-active protein in the precipitate.

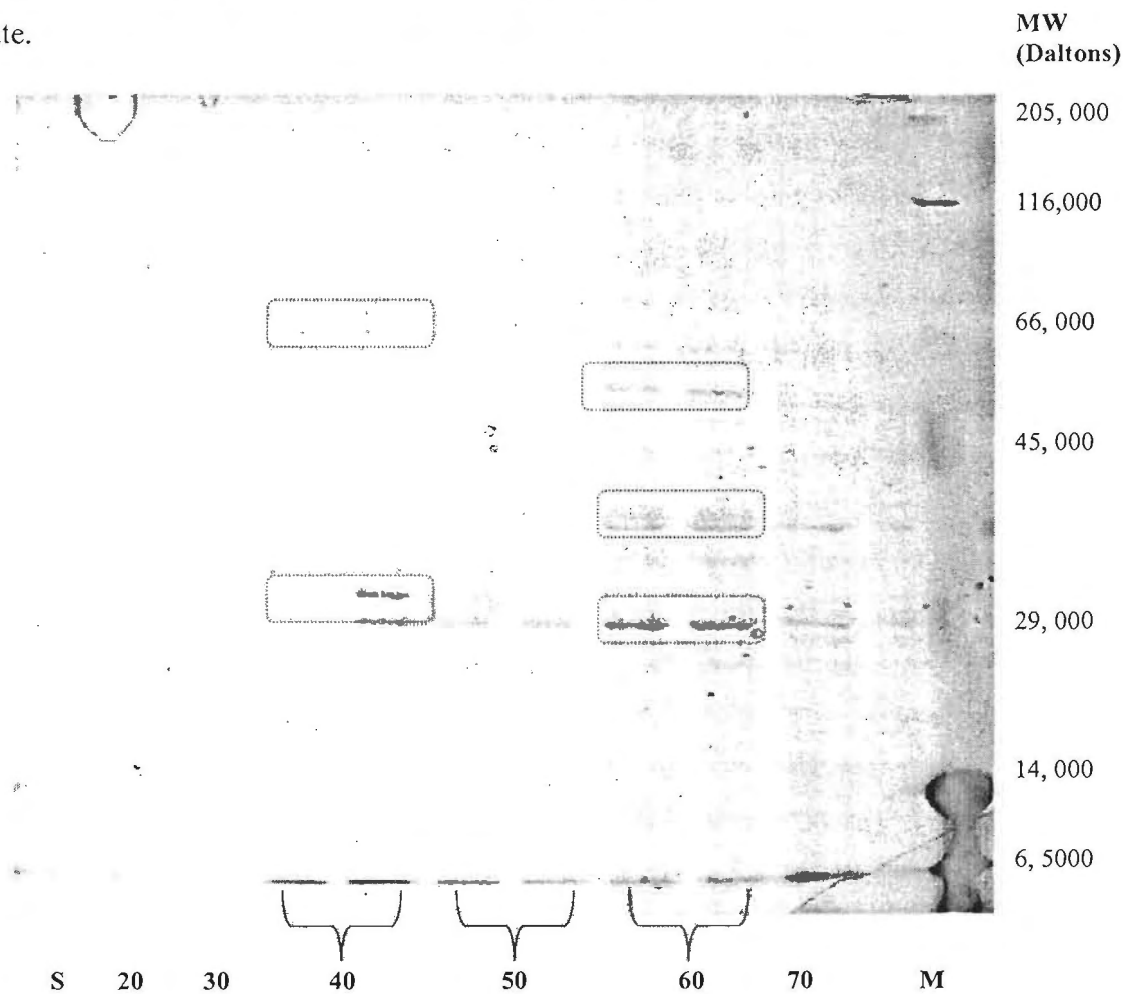


Fig. 4.7 SDS-PAGE showing bands from protein fractions precipitated by ammonium sulphate (20-70% saturation) from supernatant (lane S) and MW markers (lane M).

4.3.1.2 Acetone Precipitation

Protein in the supernatant of RUKM3s crude extract was precipitated by cumulative addition of 20, 40, 50, 60 and 80 % (v/v) acetone. The change in protein concentration during this addition was evaluated by Bradford's assay and the results are shown in Fig. 4.8. The effects of the precipitation on the hydantoinase and NCAAH activities in the supernatant and the precipitated protein fractions were also evaluated by the Ehrlich's and Ninhydrin assays. The results showed that the amount of product from hydantoinase activity was highest at 60% acetone (Fig. 4.9), and most of the NCAAH (Fig. 4.10) activity was precipitated at 20% acetone (Fig. 4.10). To isolate the NCAAH from other proteins in the supernatant of the crude extract of RUKM3s, it is therefore sufficient to use 20 % acetone

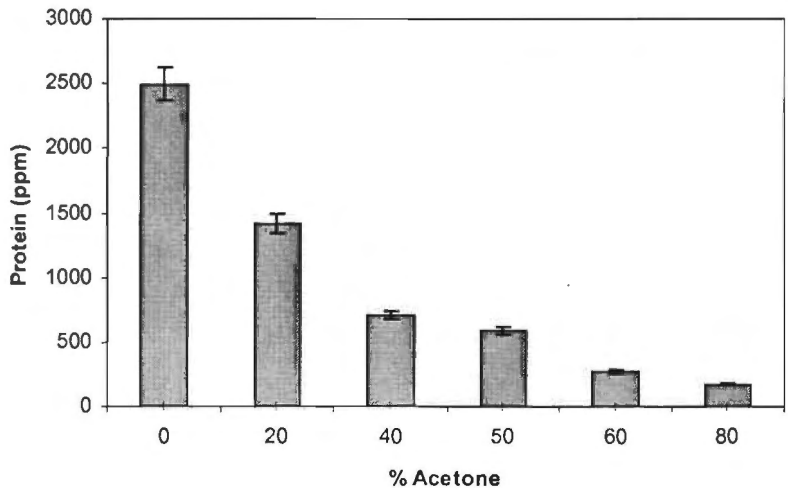


Fig. 4.8 Change in protein concentration in supernatant of RUKM3s crude extract solution due to addition of increasing concentrations of acetone % (v/v)

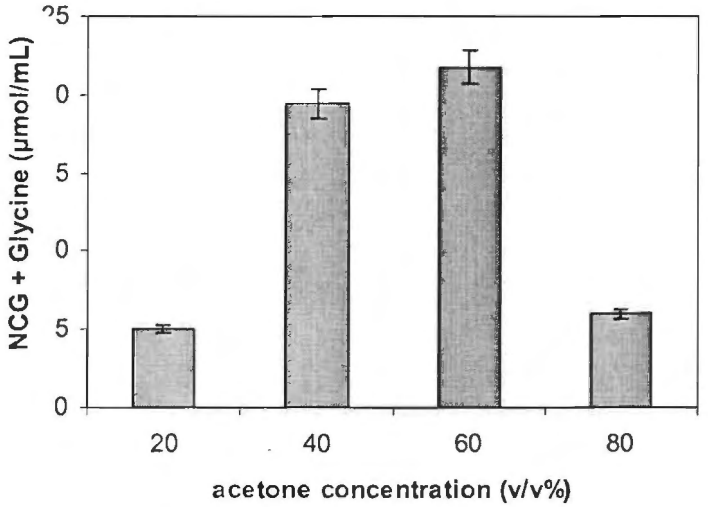


Fig. 4.9 Product of hydantoinase activity of protein fractions precipitated by different concentrations of acetone

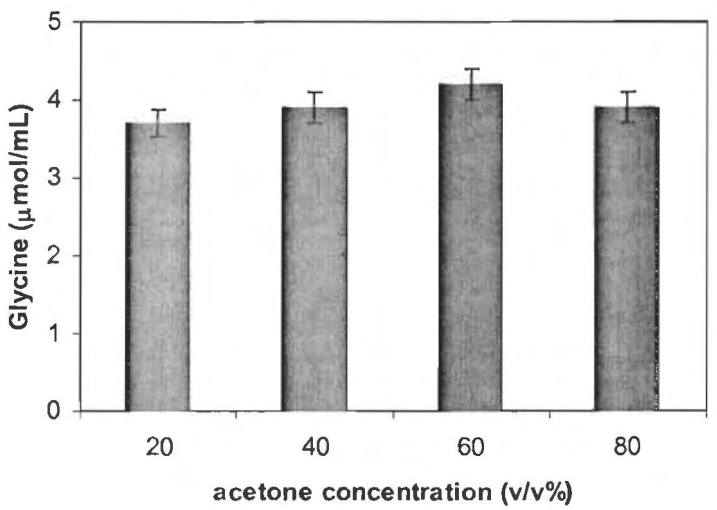


Fig. 4.10 Product of NCAAH activity of protein fractions precipitated by different concentrations of acetone

Addition of 40 % acetone caused the hydantoinase activity of the supernatant (Fig 4.11) to decrease. The amount of product (NCG plus Glycine) from hydrolysis of hydantoin by the

supernatant over 3 h incubation decreased from approximately 37 μmol/mL to 28 μmol/mL when 40% acetone was added, and then to 5 μmol/mL when 60% acetone had been added. Therefore, 40-60 % acetone could be used to precipitate a protein fraction containing partially purified hydantoinase.

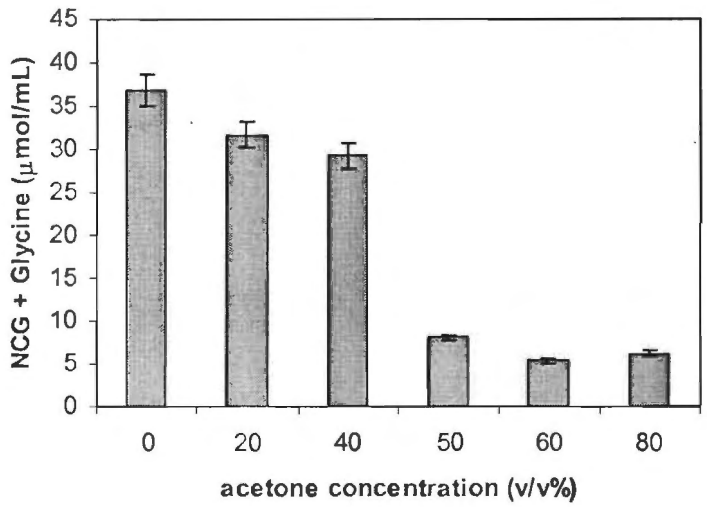


Fig. 4.11 Change in hydantoinase activity in supernatant of RUKM3s crude extract at different concentrations of acetone shown as amount of total product (μmol NCG plus glycine per mL of reaction mixture)

Addition of 20 % acetone reduced the NCAAH activity of the supernatant (Fig. 4.12) from approximately 5 μmol/mL to 0.9 μmol/mL. Therefore, most of the NCAAH was precipitated with the protein fraction from 20 % acetone, and this acetone concentration is suitable for obtaining partially purified NCAAH.

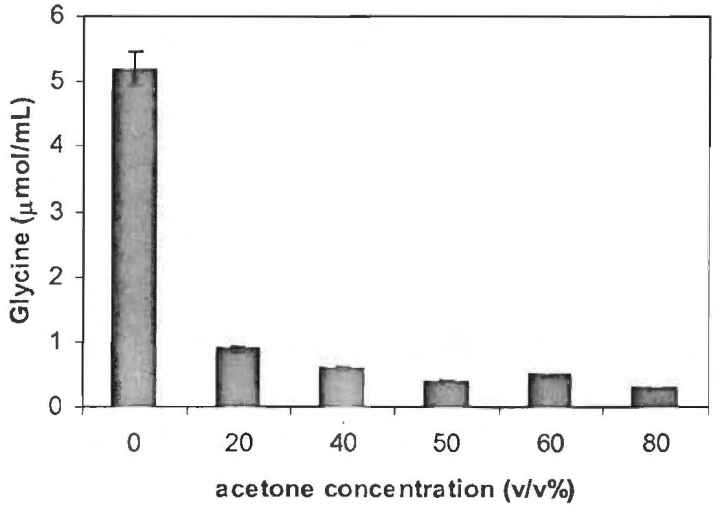


Fig. 4.12 Change in NCAAH activity of the supernatant of RUKM3s crude extract at different concentrations of acetone shown as amount of product (µmol glycine per mL of reaction mixture)

SDS-PAGE of the protein samples from 20-80% acetone fractionation (Fig 4.13) showed that the fraction collected from 20 % acetone had one major band at approximately 30 kDa, which is the same level as the molecular weight of the subunits suspected to be NCAAH in Fig. 4.7 (showing SDS-PAGE of protein precipitated at 60% ammonium sulphate saturation). The precipitation products of 40-60% acetone showed bands on SDS-PAGE at approximately 70 and 35 kDa which correspond to the protein suggested to be hydantoinase from SDS-PAGE in section 4.3.1.1. These results show that the acetone method was as good as the ammonium sulphate method for the precipitation of hydantoinase and NCAAH from supernatants of RUKM3s extract. Acetone is more volatile than ammonium sulphate and therefore easier to separate from precipitated protein. However, precipitate from the acetone method needs a dialysis step to remove extraneous low-molecular weight protein, and that from ammonium sulphate needs dialysis to remove both the ammonium sulphate and extraneous low-molecular weight protein.

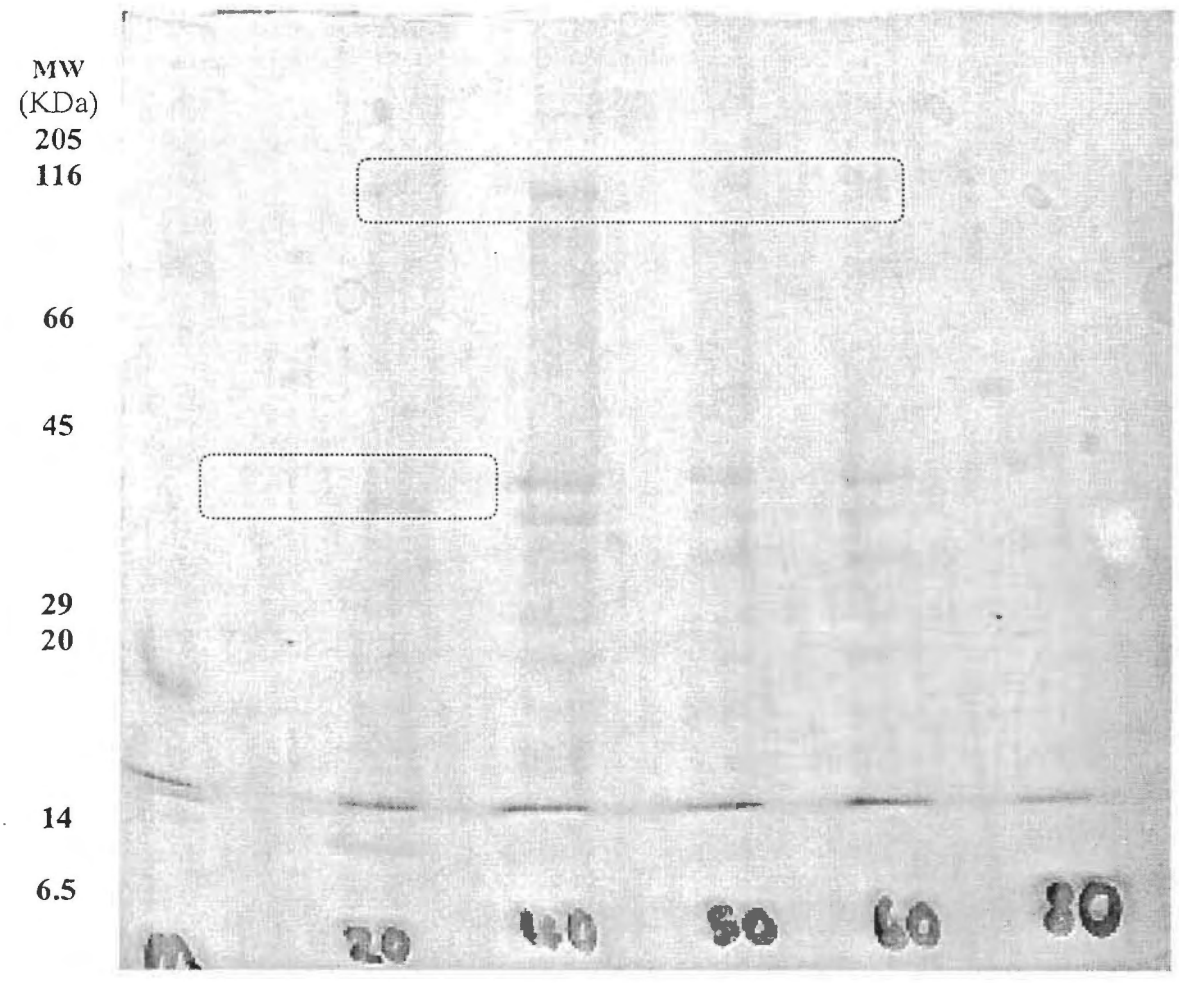


Fig. 4.13 SDS PAGE showing bands from protein precipitated at 20-80 % (v/v) acetone concentrations

4.3.2 Separation in aqueous two-phase systems

The results of assays of protein concentration in the fractions of sonicated RU-KM3s cells separated using ATPS are presented in Table 4.1. Almost all the NCAAH activity was retained in the PEG phase of the system, while about a third of the hydantoinase activity was detectable in the aqueous extract. This means that if the PEG extract is repeatedly partitioned using this system, more hydantoinase could be extracted into the aqueous phase, and the two enzymes ultimately separated.

Table 4.1 Protein concentration and product yield over 3 h from ATPS fractions

Extract	Hydantoinase (μmol/mL)	NCAAH (μmol/mL)	Protein (μg/ml)
Sonicated cells	13.1	0.8	
Supernatant	4.7	0.7	1615
Polyethylene Glycol	5.6	0.6	826
Ammonium sulphate	2.5	0	995

4.3.3 Products of gel filtration and perfusive chromatography

Several attempts were made to separate the hydantoin-hydrolysing enzymes of RUKM3s with various chromatographic techniques. It was found that the enzymes were not retained during reversed phase HPLC (POROS R1 and R2) or by ion exchange 20R2 4.6 D/100 mm columns. Different formulations of mobile phase in both isocratic and gradient modes were used without success. Positive results were obtained with gravity flow gel filtration chromatography, although the sample still eluted with poor resolution. Separation of the enzyme activities was finally achieved with gel filtration through a Sephacryl S-100 HR column in a BIOCAD perfusive chromatography system.

4.3.3.1 Gravity-flow Gel filtration chromatography

When Sepharose 6B was used in a gravity-flow column, it was found that the proteins eluted as single, broad peak (Fig. 4.14). Enzyme assays on the eluted fractions showed that both the hydantoinase and NCAAH activity were present in the same broad peak, and thus the column had poor resolving power for the two enzymes (Fig. 4.15 and 4.16). Due to the low concentrations of the proteins and the hydantoin-hydrolysing enzymes in the fractions obtained from the gel filtration columns, the quantitative analysis presented in Fig. 4.14 to 4.16 is simply reported as relative absorbance of the individual fraction, instead of μg/mL for protein and μmol/mL/h for activities. Further gel filtration to separate the two activities was performed on the samples using perfusive chromatography on Sephacryl S-100 HR (Fig. 4.17)

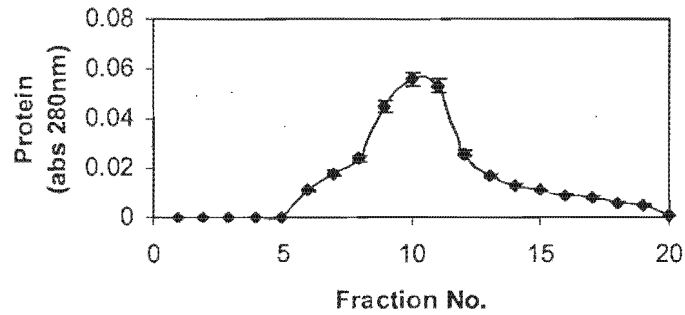


Fig. 4.14 Relative protein concentration of sepharose 6B filtration products

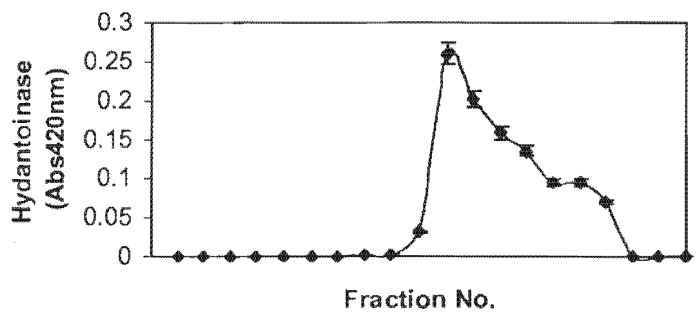


Fig. 4.15 Relative hydantoinase activity of Sepharose 6B filtration products

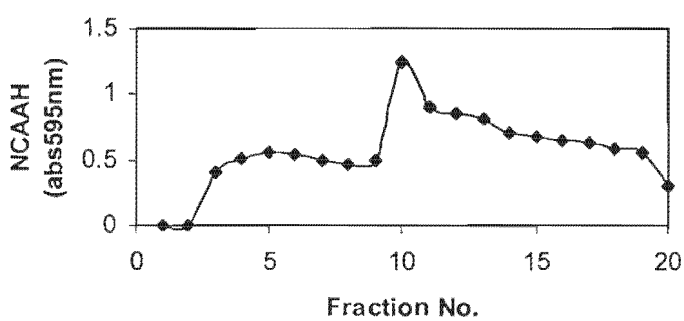


Fig. 4.16 Relative NCAAH activity of Sepharose 6B filtration products

4.3.3.2 High-throughput perfusive chromatography

A sample of protein precipitated with 60% acetone and known to contain both hydantoinase and NCAAH activity was eluted through a Sephacryl S-100 HR column in a BIOCAD perfusive chromatography system. A chromatogram (Fig. 4.17) with two distinct peaks was recorded at retention times of 60 and 90 minutes. Gel filtration fractions collected from each of the two peaks were pooled and assayed for activity. It was found that the first peak was positive for hydantoinase activity, while the second one was negative for both hydantoinase and NCAAH. The chromatogram recorded was superimposed on a chromatogram of molecular weight markers eluted under the same conditions. It was found that the first peak had a molecular weight of approximately 220 kDa and the second was approximately 30 kDa. Thus the first peak was likely to be the native hydantoinase, and the second may represent subunits of dissociated NCAAH. This further supports the proposition that the native hydantoinase and the NCAAH from RUKM3s have molecular weights of approximately 200 and 60 kDa respectively.

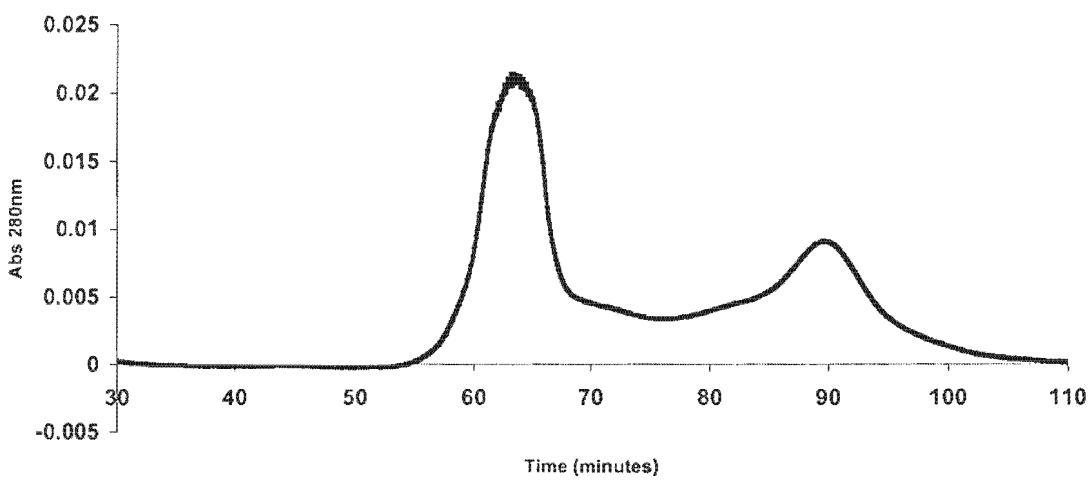


Fig. 4.17 Chromatogram showing the elution times of hydantoinase (60 min) and NCAAH (90 min) through a sephacryl S 100HR column

4.3.4 Determination of purity by gel electrophoresis

Gel filtration products from the two fractions obtained after perfusive chromatography (Fig. 4.17) were concentrated by dialysis against 20 % PEG and electrophoresed on non-denaturing gel to determine their relative purity and estimated molecular weights. Fig. 4.18 shows the ND-PAGE with protein from the first and second peaks on lanes A and B respectively and molecular weight markers on lane M. The dominant band from the first fraction was observed to correspond to approximately 200 kDa, with fainter peaks at about 66 and 45 kDa, suggesting either that the fraction was not completely pure, or that the concentration step may have caused partial dissociation of the hydantoinase into its subunits. Lane B showed faint peaks at approximately 60 and 30 kDa, which may be those of the NCAAH and its subunits. Although the NCAAH showed no activity in the eluted filtration product, it is possible that its subunits may have re-associated to form the native molecule during the concentration steps when buffering salts were removed by dialysis.

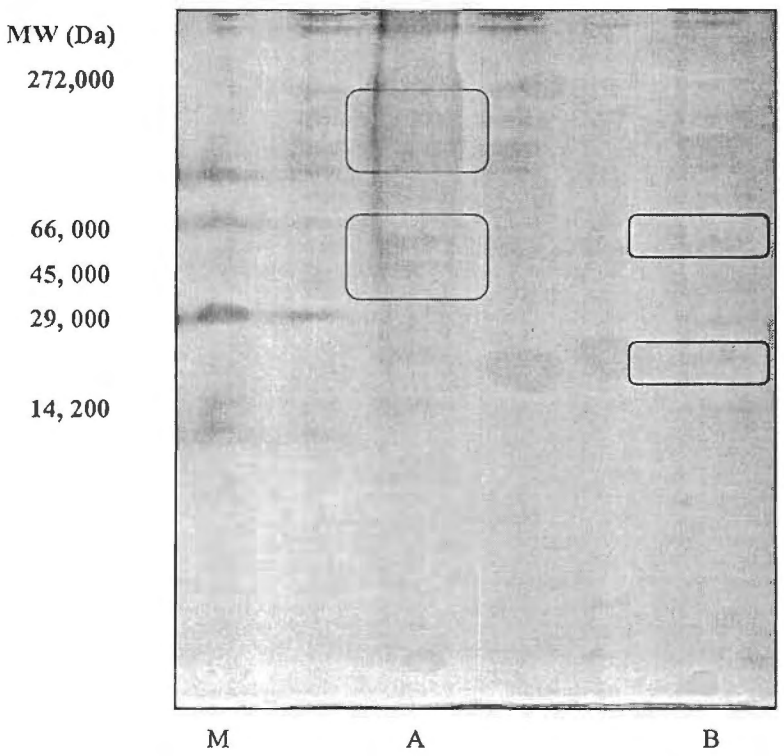


Fig. 4.18 ND-PAGE of Sephacryl S 100HR gel filtration products showing MW markers (M), suspected hydantoinase (lane A) and suspected NCAAH (lane B)

4.3.5 Characterization of purified protein by size exclusion chromatography (SEC)

A method for the characterisation of proteins of RUKM3s was developed. Conditions were optimised for buffer type, buffer strength, pH, flow rate and UV detection wavelength.

4.3.5.1 Optimisation of protocol for size exclusion chromatography

In experiments conducted to determine the optimal conditions for SEC of proteins it was found that the best resolution of peaks was obtained with a mobile phase of potassium phosphate buffer (pH 7, 100 mM) at a flow rate of 0.6 ml/min with a UV detection wavelength of 280 nm, and using a Waters Ultrahydrogel column.

4.3.5.2 Characterisation of protein by Size Exclusion Chromatography

To estimate molecular weights of the unknown proteins in acetone precipitated protein samples, a standard curve (Fig. 4.19) was prepared using molecular weight standards (Table 4.2). Retention times of the standards and the samples were obtained and used to calculate elution volumes and hence distribution coefficients. A plot of the distribution coefficients against log molecular weights of standards was used to estimate the molecular weights of the unknowns. The main peaks from the pooled samples were found to have molecular weights of approximately 60 and 200 kDa (Table 4.3), and thus these are the molecular weights of the NCAAH and the hydantoinase of RUKM3s.

Table 4.2 Distribution Coefficients, Kd's of molecular weight standards.

Standard	Retention Time (min)	Elution Volume,Ve (mL)	MW (Da)	Kd	Log MW
Glycine	17.84	10.7*	75	1.0	1.9
α-Lactalbumin	15.72	9.4	14,200	0.64	4.2
Carbonic Anhydride	15.46	9.3	29,000	0.61	4.5
Chicken Egg Albumin	15.00	9.0	45,000	0.53	4.6
Bovine Serum Albumin	14.69	8.8	66,000	0.47	5.1
Urease	13.97	8.3	272,000	0.33	5.7
Blue Dextran	11.89	7.1**	2,000,000	0	6.3

* Total Volume, Vt

** Void Volume,Vo

Kd = (Ve-Vo)/(Vt-Vo)

Ve = (Retention time, min)*(Flow rate, ml/min)

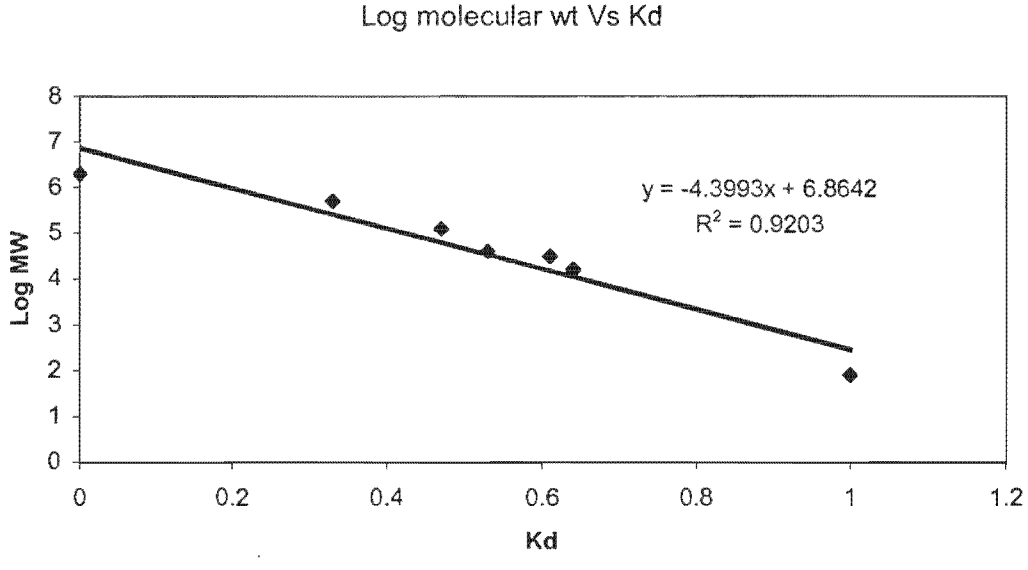


Fig 4.19 Standard curve of log molecular weight against distribution coefficients used for estimation of molecular weights in size exclusion chromatography

Table 4.3 SEC estimation of molecular weight of protein in acetone precipitated fractions showing hydantoinase (P4) and NCAAH (P5-7) activities

Sample	RT (min)	Ve (mL)	Kd	Est. MW
P4	13.03	7.8	0.19	200,000
	14.93	9.0	0.53	60,000
P5-7	14.87	8.9	0.5	60,000

4.3.6 Enantioselectivity of hydantoin hydrolysing enzymes of RUKM3s

The enantioselectivity of the hydantoin hydrolysing enzymes of RUKM3s was determined by analysis of biocatalytic products from reactions of crude extract with chiral substrates. The retention times of the substrates L-methylhydantoin and D-methylhydantoin, and the products *N*-carbamoyl-D,L-alanine and L-alanine were recorded on chiral HPLC as shown in table 4.4. A standard for D-alanine was not available for this work. RUKM3s crude extract was incubated with L-methylhydantoin, D-methylhydantoin, and *N*-carbamoyl-D,L-alanine at 40 °C and pH 7 for 3 h. After the biocatalytic reactions, 1.5 mL of each reaction mixture was centrifuged and the supernatant analysed by chiral HPLC. The percentage of each of the substrates and products found in the reaction mixtures after incubation are as shown in Table 4.5.

Table 4.4: Retention times of enantiomers of methylhydantoin and their products on chiral HPLC

Standard compound	Retention time (minutes)
L-Methylhydantoin	10.93
D-methylhydantoin	10.99
***N*-carbamoyl-D,L-alanine**	8.59 and 11.0
L-alanine	8.28

Table 4.5: Products of bioconversion of chiral substrates

Substrate	% Composition of reaction products		
	Unreacted L/D-Methylhydantoin	N-carbamoyl-D,L-alanine	L-alanine
L-Methylhydantoin	50	1	49
D-Methylhydantoin	2	70	28
***N*-carbamoyl-D,L-alanine**	_	13	87

From the results, the crude extract was shown to have an L-selective NCAAH and a non-selective or D-selective hydantoinase activity. Most (98%) of the D-methylhydantoin was converted to the intermediate, of which 28% is further converted to L-alanine. Only 50 % of the L-methylhydantoin was converted to the intermediate, of which 49% is further converted to L-alanine. The *N*-carbamyl-D,L-alanine standard had peak retention times at 8.59 and 11.0 min, presumably representing the D and L enantiomers of this substrate. Products from the bioconversion of *N*-carbamyl-D,L-alanine showed two peaks, one that coincided with L-alanine constituting 87 % of product, and another that coincided with one of the peaks of the *N*-carbamyl-D,L-alanine comprising 13%. One can deduce that the crude extract had high L-selective NCAAH activity, and that some *N*-carbamyl-D-alanine may have been converted to *N*-carbamyl-L-alanine, possibly by a racemase because the yield of L-alanine from the substrate (*N*-carbamyl-D,L-alanine) is more than 50%. Therefore, the present determination of the chirality of the enzymes of RUKM3s supports earlier findings (Buchanan *et al.*, 2001) that the RUKM3s system possibly has a non-selective or D-selective hydantoinase, and an L-selective NCAAH. The present work also suggests the possibility of the existence of a racemase in the system. However, no significant peak was noted that suggested the production of this enantiomer from either D-methylhydantoin or *N*-carbamyl-D-alanine. The absence of such a peak means that there is a strong likelihood that D-selective NCAAH activity is absent in the RUKM3s system.

The characterisation of the hydantoinase and NCAAH enzymes reported in the present work is consistent with earlier studies of amidohydrolases by other research groups. A summary of characteristics of hydantoin hydrolyzing enzymes reported in literature is presented in Table 4.6. From the reported characterisations, it is worth noting that while most reported hydantoinases are homotetramers, there are few reports of heterotetramers. Therefore, the characterisation of the hydantoinase of RUKM3s as a heterotetramer with two sets of subunits with molecular weights 70 and 35 kDa is possibly a novel finding for *Pseudomonas* species.

Table 4.6 Summary of reported characteristics of hydantoin hydrolysing enzymes

Enzyme	Source	Molecular weight (kDa)	Subunits # and mw (kDa)	Purification method	Reference
dihydropyrimidinase	calf liver	217	4 x 54 (homotetramer)	Acetone, heat, ammonium sulphate fractionation, gel chromatography	Kauz and Shnackerz, 1989
dihydropyrimidinase	rat liver	215	4 x 54 (homotetramer)	heat, ammonium sulphate fractionation, and gel chromatography	Kikugawa *et al.*, 1994
carbamoylase	*Agrobacterium* sp	Not specified	2 x 38 (dimer)	Not specified	Louwrier and Knowles, 1996
hydantoinase	*Pseudomonas fluorescens*	230	4 x 60 (homotetramer)	HI chromatography, gel filtration, preparative gel electrophoresis	Morin *et al.*, 1986
carbamoylase	Recombinant *E. coli.*	Not specified	2 x 36 (dimer)	Not specified	Buson *et al.*, 1996
hydantoinase	*B. stearothermophillus*	226	4 x 54 (homotetramer)	ammonium sulphate, anion exchange chromatography, heat, HI chromatography and preparative gel electrophoresis.	Lee *et al.*, 1995
hydantoinase	*Arthrobacter aurescens*	220	2 x 16 and 2 x 89 (heterotetramer)	Not specified	Graf *et al.*, 1997
carbamoylase	*Pseudomonas* sp	Not specified	2 x 35 (dimer)	Not specified	Ikenaka *et al.*, 1998

4.4 CONCLUSIONS

Ammonium sulphate precipitation of the enzymes in the supernatant of sonicated crude extract of RUKM3s allowed the isolation of the hydantoinase and NCAAH. However, in their native forms, both enzymes co-precipitated in the pellet obtained from addition of 40% saturated ammonium sulphate. Ammonium sulphate interfered with the ninhydrin assay for NCAAH activity. HPLC analysis using an aromatic substrate showed that the protein pellet from 40% saturated ammonium sulphate had both NCAAH and hydantoinase activity, and some NCAAH activity is detectable in the fraction from 60% saturated ammonium sulphate. The co-precipitation of enzymes during ammonium sulphate precipitation is not a unique problem. Other researchers have used the method as an initial step and followed it with other separation techniques such as gel filtration chromatography (Kauz and Shnackerz, 1989; Kikugawa *et al.*, 1994), hydrophobic interaction and anion exchange chromatography (Morin *et al.*, 1986; Lee *et al.*, 1995).

Acetone precipitation allowed better separation of hydantoinase and NCAAH. Most of the NCAAH was precipitated by 20% acetone, and hydantoinase was precipitated by 60 % acetone. Acetone precipitation has been used in conjunction with other separation techniques to achieve better purification. Kauz and Shnackerz (1989) used acetone precipitation followed by gel filtration chromatography to purify a hydantoinase from calf liver.

Isoelectric precipitation of the proteins indicated that the isoelectric point of hydantoinase was at pH 6, while that of NCAAH was at pH 8. Precipitation based on pH was more efficient than salt and organic solvent precipitation because the activities of the precipitated proteins were distinctly separate, whereas co-precipitation is a major problem with the salt and acetone precipitations.

To improve the purity of protein precipitated by acetone, the precipitate from addition of 60% acetone was further purified by perfusive chromatography using Sephacryl S-100 HR column. The method permitted easier separation of the hydantoin-hydrolysing enzymes of RUKM3s co-precipitated at 60% acetone, eluting them in two separate fractions at 60 and 90 minutes. This system allowed collection of fractions containing the active enzymes, which could be concentrated by dialysis and characterised further. The hydantoinase was still active after elution,

while the NCAAH was denatured. The NCAAH subunits seemed to re-associate into the native enzyme molecule during dialysis. SDS-PAGE analysis and size exclusion chromatography indicate that the NCAAH of RUKM3s is a homo-dimeric molecule of molecular weight approximately 60 kDa, with two subunits of approximately 30 kDa each. The hydantoinase is likely to be a hetero-tetramer weighing approximately 200 kDa, with two subunits of approximately 35 each and a second pair of approximately 70 kDa each. This is consistent with the reports on other hydantoinases. To the best of our knowledge, there are no previous reports of separative purification of hydantoinase and NCAAH by high-throughput perfusive chromatography, per se. In addition, this work is the first to report on the characterisation of hydantoinase and NCAAH by size exclusion chromatography.

The determination of the chiral selectivity of the enzymes of RUKM3s indicated that the system has a non-selective or D-selective hydantoinase activity and an L-selective *N*-carbamoylase activity. There is a possibility that a racemase is present in the system, which converted *N*-carbamyl-D-alanine to *N*-carbamyl-L-alanine. The presence of racemases in microbial systems bearing hydantoin-hydrolysing activity has been reported on by many research groups (Rozzell and Wagner, 1992).

CHAPTER 5

DEVELOPMENT AND EVALUATION OF OPERATIONAL PARAMETERS OF AN IMMOBILISED RUKM3s BIOCATALYST

5.1 INTRODUCTION

5.1.1 Stabilisation of biocatalysts by immobilisation

In previous chapters conditions were optimised for biomass cultivation, enzyme synthesis, and biocatalytic activity, and techniques were evaluated for protein extraction, enzyme purification and characterisation. The use of hydantoin-hydrolysing enzymes in industrial processes is limited by the relative instability of the enzymes, especially the *N*-carbamoylase (Hartley *et al.*, 1998). The investigation of methods for the stabilisation of both the hydantoinase and the NCAAH for application in industrial processes is therefore important. The immobilisation of whole cells and crude extracts having hydantoinase and NCAAH activity has been reported for supports including polyacrylamide (Meyer and Runser, 1993) and, activated charcoal (Yamada *et al.*, 1980), DEAE – cellulose (Lee *et al.*, 1996) and calcium alginate beads (Chevalier *et al.*, 1989; Foster *et al.*, 2003). These reports indicated varying effects on the operational and storage stability of the enzymes. In this chapter, the suitability of some of the reported methods for immobilisation of the hydantoin-hydrolysing enzymes of RUKM3s was investigated.

The most important criteria in the choice and industrial application of enzymes are the price and stability of the biocatalyst (Martin *et al.*, 2003). The use of immobilised enzymes, pioneered in the 1960s, is one of the most useful approaches to stabilisation of biocatalysts (Katchalski-Katzir and Kraemer, 2000). Immobilised enzymes were defined by Katchalski-Katzir in 1971 as 'enzymes physically confined or localized in a defined space with retention of their catalytic activities, and which can be used repeatedly and continuously.' Production processes using immobilised enzymes offer several advantages including the reuse of expensive enzymes (Plou *et al.*, 2002), simplifying product purification, and providing opportunities for scale-up (Abdel-Naby, 1999). Tosa *et al.* at the Tanabe Seiyaku Institute in Japan reported the first industrial application of immobilised enzymes in 1967. These workers developed columns of immobilised *Aspergillus*

oryzae aminoacylase for the resolution of synthetic, racemic D,L-amino acids into the corresponding optically active enantiomers.

In earlier studies (chapter 2) it was demonstrated that the hydantoinase and NCAAH activities of *Pseudomonas putida*, RUKM3s were higher in crude extract than in whole cells. In this chapter, the crude extract was immobilised by adsorption, cross-linking, entrapment and microencapsulation (Fig. 5.1). The effect of immobilisation on the hydantoinase and NCAAH activities was evaluated by assay of the activity of equivalent amounts of enzyme in the immobilised and free states. The amount of protein bound (binding yield), activity yields and effects of each of the matrices were evaluated. The immobilised enzymes were used in biocatalytic reactions to produce amino acids, and their operational and storage stabilities were evaluated.

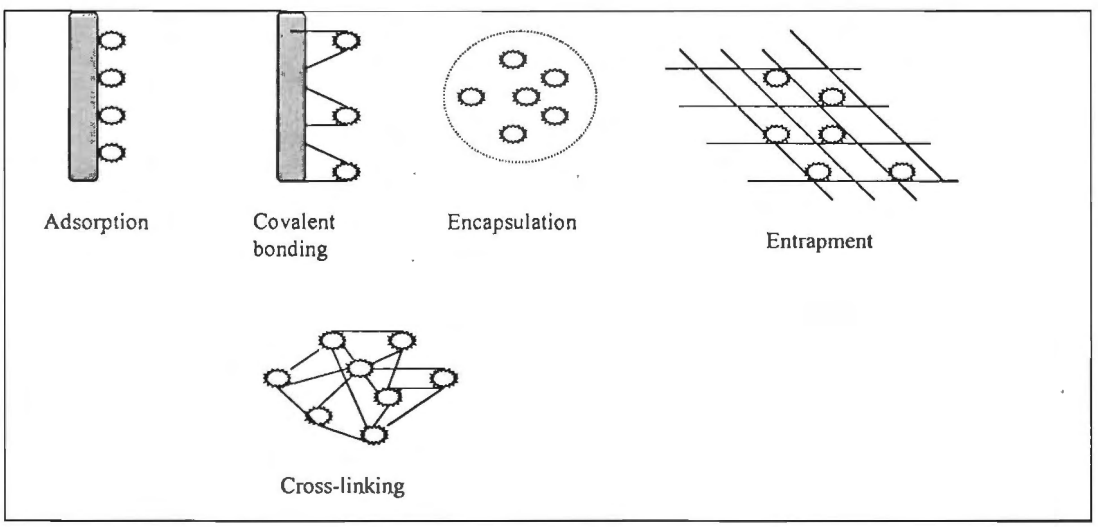


Fig. 5.1 Methods of immobilisation of enzymes and cells

5.1.2 Immobilisation Strategies

Important considerations in the selection of suitable methods of immobilisation include stability and retention of enzymatic activity; and the binding capacity of the support material, which is a function of charge density, functional groups, porosity and hydrophobicity of the support surface.

Other considerations are factors such as the nature of ligands at the active site of the enzyme, and the surface chemistry, which is determined by the types of amino acid residues. Immobilisation of enzymes usually causes lower activity and higher stability, but it may also cause increases in activity (Foster *et al.*, 2003) due to more favourable micro-environmental conditions.

5.1.2.1 Surface Immobilisation

Binding to surfaces is the simplest form of immobilisation. This relies on covalent binding, van der Waals or dispersion forces to adsorb bio-molecules to surfaces, and has the advantage that in many cases, the activity of the enzymes is not negatively affected. A major problem with surface immobilisation is desorption due to hydrodynamic forces when used in bioreactors. Adsorption can be stabilised by the addition of cross-linking agents such as glutaraldehyde, but an inappropriate agent, or a suitable one in the wrong concentration, may denature enzymes. In covalent bonding, enzymes are bound to support materials via functional groups such as amino, carboxyl, hydroxyl, and sulfhydryl groups. Such bonding groups must not be in the active sites of the enzymes.

The crude extract of RUKM3s was immobilised by adsorption to membrane filters in the presence (and absence) of the cross-linking agents glutaraldehyde and EDAC. Experiments were devised to evaluate the amount of free enzyme that could stably bind to the membrane surfaces, and investigate the effects of the concentrations of glutaraldehyde and EDAC.

5.1.2.2 Cross-linking

Cross-linking can be used independently of surface supports. Cross-linking agents such as glutaraldehyde, *bis*-diazobenzidine and 2,2-disulfonic acid can be used to initiate linkages between functional groups in biomolecules. The cross-linked enzyme molecules form aggregates with each other and with the agent, producing cross-linked enzyme aggregates (Cowan, 1996). Cross-linking can be used to link surface-adsorbed or porous matrix-entrapped enzyme molecules with each other. Intermolecular cross-linking is thought to stabilise proteins by reducing irreversible unfolding which inactivates enzymes. In multimeric enzymes, cross-linking prevents

dissociation of subunits (Mozhaev and Martinek, 1982). Disadvantages of cross-linking include changes to active sites and severe diffusion limitations.

Several attempts have been made to stabilise hydantoinases by cross-linking. Mozhaev and Matinek (1982) observed that in its native state, a tetrameric D-hydantoinase from *Bacillus stearothermophilus* SD1 is dissociated into monomers under operational conditions. This results in a detrimental loss of its catalytic activity. As an approach to reduce the dissociation of the subunits and to maintain its catalytic activity, inter-subunit cross-linking was attempted using EDAC. The cross-linking conditions were optimised in terms of stabilisation and catalytic activity of the recovered enzyme. The cross-linked D-hydantoinase showed a four-fold longer half-life under operational conditions and was very stable even at elevated temperatures, whereas the native enzyme was almost completely deactivated. In addition the enzyme was stable during use in organic solvents and under acidic conditions. The cross-linked enzyme was more efficient in the conversion of substrate, possibly due to increased stability. In the present investigation RUKM3s crude extract was immobilised by cross-linking with EDAC and glutaraldehyde.

5.1.2.3 Matrix entrapment and micro-encapsulation

Matrices for immobilisation of cells and enzymes can be particles, polymer gels, membranes or fibres. Fixing the enzymes to solids such as activated carbon, porous ceramics and diatomaceous earth, or mixing enzyme solutions with polymer solutions before the onset of polymerisation are two major methods of immobilisation by entrapment in matrices. When enzymes are entrapped in polymerised gels, the gel can be extruded or a template used to shape the particles from a liquid polymer-enzyme mixture. It has become common to shape polymer-entrapped cells and enzymes into beads. During immobilisation to solid matrices, entrapment can be combined with surface attachment. Entrapment can also be attained in the hollow fibres of semi-permeable membranes. Membranes of nylon, cellulose, polysulphone and polyacylate are commonly used. Micro-encapsulation is a special kind of matrix entrapment that uses polymers to surround a solution of enzyme or cells in a microcapsule. The process of encapsulation is practically a reversal of the bead-entrapment protocol.

Immobilisation by entrapment is associated with problems of enzyme leakage, diffusion limitation, reduced activity and stability and a lack of control over micro-environmental conditions. Reducing the molecular weight cut-off (MWCO) of the membranes or the pore size of solid matrices can solve the problem of enzyme leakage. Reducing the particle size of matrices or capsules can eliminate diffusion limitations. Problems of reduced activity and stability are very common in the microenvironments of immobilisation matrices, but can be minimised by optimising the choice of matrix, chemical ingredients, processing conditions, bead size or capsule size. Diffusion barriers are less significant in microcapsules than in polymer beads (Lee *et al.*, 1995).

Immobilisation of RUKM3s crude extract and whole cells, by entrapment in calcium alginate beads, and by micro-encapsulation within capsules of calcium alginate, was performed. Calcium alginate is formed when sodium alginate $(C_6H_7NaO_6)_n$, reacts with calcium chloride. In the reaction, a divalent Ca^{2+} ion replaces two Na^+ ions and binds adjacent chains of alginate monomers into a three dimensional insoluble matrix. The overall reaction is represented by the equation below:

$$\mathbf{2\ Na\ (Alginate) + CaCl_2(aq) \rightarrow\ Ca(Alginate)_2 + 2\ NaCl\ (aq)}$$

5.1.2.4 Immobilisation by covalent binding

Among immobilisation methods, covalent immobilisation is of major interest because the bonds formed are more stable than those formed by electrostatic interactions or adsorption (Ragnitz *et al.*, 2001). For industrial purposes, it is required that covalently bound biocatalysts have a high stability because the carrier cannot be regenerated. Various matrices can be used for covalent binding of proteins. Among such matrices, Eupergit (in its various forms) provides for interesting study. An example of these is Eupergit® C, which has been widely reported as a carrier suitable for covalent immobilisation of enzymes for industrial application. This carrier was developed in the 1970s by Rohm, Darmstadt, Germany (Kraemer *et al.*, 1985). Eupergit® C consists of macroporous beads with a diameter of 100-250 μm, made by copolymerisation of *N,N'*-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide.

Upon immobilisation, Eupergit® C binds proteins when its oxirane groups react with the amino groups of protein molecules to form covalent bonds, which are stable within a pH range of 1-12. The covalent binding of Eupergit® C to proteins is shown by the reaction in Fig. 5.2.

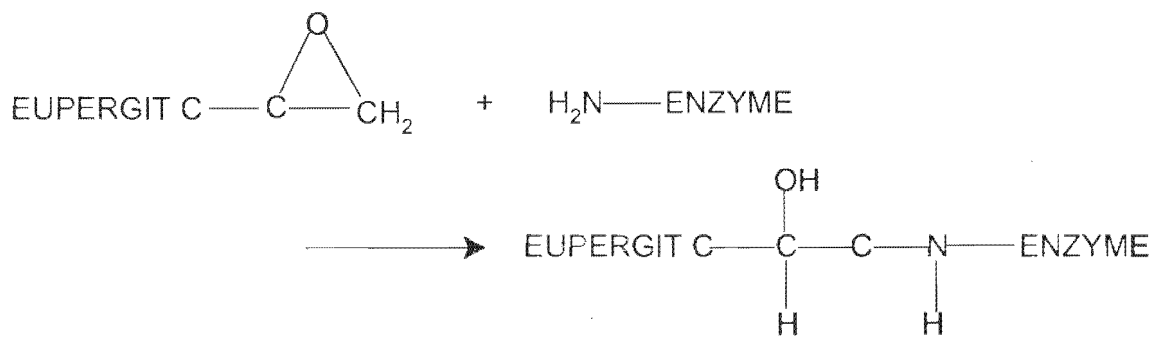


Fig. 5.2 Covalent immobilisation of proteins to Eupergit® C via free amino groups

Eupergit® C is chemically stable and mechanically robust, showing no pH dependent swelling or shrinking over a pH range of 1-14, and no significant attrition after 650 cycles of re-use (Katchalski-Katzir and Kraemer, 2000). The surface of Eupergit® C beads has a high density of oxirane groups (600 μmol/g dry Eupergit® C). This provides for the multi-point-attachment of enzymes during immobilisation, leading to a high operational stability for enzymes bound to Eupergit® C. There have been reports of the operational stability of enzymes being considerably increased upon immobilisation in Eupergit® C (Keller *et al.*, 1987; Albrecht *et al.*, 1993; Wirz, *et al.*, 1993). However, decreased activities due to immobilisation have also been reported. Ragnitz *et al.* (2001) optimised the immobilisation of the hydantoin hydrolysing enzymes of an *Arthrobacter spp* on various modifications of Eupergit® C. They found that while crude hydantoinase could be immobilised successfully with yields of up to 60%, crude L-*N*-carbamoylase resulted in only low yields.

One of the major attractions of immobilisation in Eupergit® C is that the enzymes do not need to be purified, and direct immobilisation of crude cell homogenates can be easily carried out with Eupergit® C. After enzyme immobilisation, the cell debris is simply washed off. Mahmoudian *et al.* (1997) at the Glaxo Wellcome Research in the UK immobilised an aldolase from an over

expressing *E. Coli* on Eupergit® C, and found that it was sufficient to mix the homogenate with the beads for 5 days and then just wash the beads. In work reported in this chapter, RUKM3s crude extract was pre-incubated with Eupergit® C, and the biocatalyst was evaluated in terms of activity yield, binding yield, storage and operational stability.

5.2 MATERIALS AND METHODS

5.2.1 Materials

RUKM3s and RUOR-PN1 crude extracts were obtained from biomass grown in a 5-L bioreactor, harvested by centrifugation, suspended in phosphate buffer to 100 mg/ml and sonicated for 5 min. Nylon, nitrocellulose, polysulphone and cellulose acetate membrane filters manufactured by Millipore Corporation, Bedford, Massachusetts, USA were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Whatman® filter paper was purchased from Whatman International Ltd, Maidstone, England. Glutaraldehyde (Grade 1: 25% aqueous solution), 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC), Sodium alginate, xanthan gum and Polyoxyethylene Sorbitan (Tween 20) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Calcium chloride ($CaCl_2.2H_2O$) was purchased from Merck NT Laboratory supplies, Midrand, RSA. Eupergit® C (Oxirane Acrylic beads) was generously donated by Degussa, Rohm Pharma Polymer, Rohm GmbH & Co., KG Darmstadt, Germany, or purchased from Sigma Chemical Co. Hydantoin was purchased from Sigma-Aldrich Chemie, Steinheim, Germany. Hydantoic acid (*N*-carbamoyl Glycine) was purchased from Sigma Chemical Co., and Glycine AR was purchased from Merck, Darmstadt, Germany. Biocatalytic reactions were carried out in 14 mL bijou bottles.

5.2.2 Immobilisation by surface adsorption and cross-linking

The membrane filters used were nylon, nitrocellulose, polysulphone and cellulose acetate. Pre-weighed sheets of the membrane filters were equilibrated in phosphate buffer overnight. The membranes were placed in a cell-free enzyme solution and agitated gently at 4 °C for 24 h. The protein concentration and enzyme activity in the enzyme solution were measured before and after immobilisation. The weights of the membranes after immobilisation were determined following a period of drying at 4 °C. Each membrane was cut into small pieces of about 15x10 mm and then

placed for 3 h in reaction bottles containing 50 mM hydantoin and 25 mM NCG, and the products NCG and glycine assayed.

The effects of the concentration of cross-linking agents glutaraldehyde and EDAC on enzyme activity were tested by addition of glutaraldehyde or EDAC to crude extract to concentrations of 0, 0.5, 1.0, 1.5 and 2.0 % (w/v). The suspensions were then incubated for 24 h at 4 °C to immobilise the enzymes, and used in biocatalytic reactions with hydantoin or NCG as substrates.

5.2.3 Immobilisation by Entrapment in Calcium alginate beads *(Chang et al., 1998)*

Calcium alginate beads were prepared by reacting sodium alginate with calcium chloride. RUKM3s cells or crude extract were uniformly mixed with 0.6% (w/v) sodium alginate, and dropped into a 1% (w/v) solution of calcium chloride by the aid of a syringe or a pipette tip to produce beads. A cylindrical vessel of 7.5 cm diameter was used, where the depth of the sodium alginate solution was 3 cm and the height of the whirlpool cavity that occurred while stirring was 1 cm. The beads were washed in distilled water and hardened in 1 % (w/v) calcium chloride for 20 min. For immobilisation of cells, the beads were incubated in growth medium in a shaking incubator at 28 °C to condition and concentrate the cells. The growth medium contained 0.5% (w/v) calcium chloride to prevent swelling of the beads.

5.2.4 Immobilisation by micro-encapsulation in calcium alginate *(Park and Chang, 2000)*

The preparation of calcium alginate capsules is a reversal of the method for production of beads. Cells or crude extract were mixed with a 100 mL mixture of 1.3% (w/v) calcium chloride and 0.26 % (w/v) gum xanthan at room temperature. The mixture was then dropped to 0.6% (w/v) sodium alginate containing 0.1 % (v/v) Polyoxyethylene Sorbitan (Tween® 20) while stirring and left for 10 min. The capsules were washed with distilled for 10 min, 1.3 % (w/v) calcium chloride added, and the stirred for 10 minutes for hardening. The capsules were placed in a growth medium containing 0.5 % (w/v) calcium chloride to prevent swelling.

5.2.5 Immobilisation by coupling to Eupergit® C beads

Crude extracts of RUKM3s and RUOR-PN1 cells suspended in potassium phosphate buffer (1 M, pH 8.0) to 100 mg/mL. Eupergit® C (Oxirane Acrylic beads) was added to the suspension to a concentration of 1 g beads per 5 mL suspension, and mixed by gentle shaking in a flask. The flask was then sealed and allowed to stand at 4 °C for 72-96 h without additional shaking. The beads were collected on a Buchner funnel using No. 1 Whatman filter paper, and the remaining solution drained off by vacuum. The beads were washed thoroughly on the filter with 3 x 15 mL portions of potassium phosphate buffer (0.1 M, pH 8.0). The beads were stored at 4 °C until used. The binding yield of the immobilisation matrix was determined by assays of the amount of protein in the filtrate and in the washings, and the activity yield was determined by assays of the enzyme activity of the crude extract and that of the immobilised biocatalyst.

5.2.5.1 Determination of dependence of binding capacity of Eupergit® C on weight of support

To determine the dependence of the binding capacity of the biocatalyst on the weight of carrier or on the ratio of enzyme to support, 5 mL portions of crude enzyme extract of known protein concentration were used for immobilisation to 200, 400, 600, 800, 1000, 1200 and 1400 mg of Eupergit® C. The amount of residual protein in the filtrate and washings after immobilisation was determined, and the bound amount of protein calculated.

5.2.5.2 Determination of dependence of coupling yield on protein concentration

The dependence of coupling yield on the concentration of protein in the enzyme solution was evaluated by immobilising protein from solutions containing 1000, 2000, 3000, 4000 and 5000 µg/ml onto 1 g portions of Eupergit® C. The amount of residual protein in the filtrate and washings after immobilisation was determined, and the bound amount of protein calculated.

5.2.5.3 Determination of pH and temperature optima

The effects of pH and temperature on the immobilised hydantoinase and NCAAH in the biocatalyst were studied. The biocatalyst was used for bioconversion of hydantoin and NCG at

different pH values (6-11). The temperature optimum of the enzymes was determined by using the biocatalyst for bioconversion of the substrates at 20, 30, 40, 50 and 60 °C.

5.2.5.4 Storage and operational stability

The immobilised biocatalyst was stored at 4 °C and assayed for hydantoinase and NCAAH activity on a weekly basis to evaluate storage stability. Operational stability of the biocatalyst was determined by re-use of the biocatalyst in repeat batch experiments at 40 °C using hydantoin and NCG as substrates. At the end of each cycle, samples were taken for enzyme assay. Reaction products and any remaining substrate were then filtered off, the biocatalyst washed with buffer and fresh substrate was added to commence the next biocatalytic cycle. For long term storage, the biocatalyst was frozen at −180 °C in liquid nitrogen and lyophilised at −50 °C in an Instruvac lyophilizer model 5 KL (Air and Vacuum Technologies, South Africa). The enzyme activity of the biocatalyst before and after freeze-drying was assayed to determine the effect of the process.

5.2.5.5 Effects of cross-linking agents on immobilisation

To mixtures of 1 g Eupergit® C and 5 ml enzyme extract, portions of EDAC and glutaraldehyde were added to final concentrations of 0.5 to 2.0% (w/v). After the immobilisation period, the filtrates were assayed for protein and the biocatalysts assayed for enzyme activity to determine the effects of cross-linking on binding and activity yields.

5.3 RESULTS AND DISCUSSION

5.3.1 Immobilisation of RUKM3s crude extract by surface adsorption and cross-linking

Crude extracts of RUKM3s enzymes were immobilised by adsorption to surfaces of cellulose acetate, nitrocellulose, nylon, polypropylene and polysulphone membranes, with the aim of evaluating the protein binding capacity of the membranes and their enzyme activity after immobilisation. Pre-weighed sheets of the membrane filters were equilibrated in phosphate buffer overnight. The membranes were then placed in a cell-free enzyme solution containing 0.5 w/v % glutaraldehyde and agitated gently at 4 °C for 24 h. The impact of cross-linking on the activity of the enzymes adsorbed to the membranes was investigated by comparing the effects of

immobilisation by surface adsorption in the presence and absence of the cross-linking agent using membranes containing equal amounts of adsorbed protein.

5.3.1.1 Protein binding capacity of adsorptive membranes

It was found that among the five membranes tested, nitrocellulose adsorbed the most protein (Table 5.1), followed by nylon, polysulphone and cellulose acetate. Polypropylene adsorbed no protein at all. This may be an indication of the influence of the hydrophobicity of support matrices on the degree of immobilisation. Polypropylene is hydrophobic, floating on the aqueous medium without apparently adsorbing any of it. The presence of glutaraldehyde reduced the amount of protein adsorbed to the membranes, which may have been due to cross-linking of the protein molecules to each other without fixation to the membranes.

Table 5.1 Binding yield of adsorptive membranes and effect of glutaraldehyde on the amount of bound protein, reported as weight protein per weight of membrane (%).

Membrane	(+) or (-) Glutaraldehyde	Protein binding yield (% w/w)
Nitrocellulose	+	14.0
	-	12.0
Nylon	+	11.9
	-	13.4
Polysulphone	-	10.6
		10.0
Cellulose acetate	+	9.9
	-	7.3
Polypropylene	+	0
	-	0

5.3.1.2 Activity yield of adsorptive membranes

The membranes containing immobilised RUKM3s extract were tested in biocatalytic reactions under conditions as outlined in 5.2.2. The product yield of the membrane-immobilised enzymes

was calculated under the standard conditions stated in Sections 2.2.6.3 and 2.2.6.4. Assays of the reactions (Table 5.2) demonstrated that the nylon-adsorbed biocatalyst had the highest hydantoinase activity, followed by nitrocellulose, and polysulphone and cellulose acetate adsorbed the same amounts. The nylon-adsorbed biocatalyst also had the highest NCAAH activity, followed by cellulose acetate, nitrocellulose and polysulphone with the least. However, for industrial purposes, enzymes immobilised by any of the adsorption methods is generally unfavourable because desorption due to hydrodynamic forces occurs when the matrices are used in bioreactors

The effects of glutaraldehyde on the hydantoinase and NCAAH activities of adsorbed protein are also shown in Table 5.2. Nylon, polysulphone and nitrocellulose adsorbed less hydantoinase in the presence of glutaraldehyde. Other than nylon, the membranes adsorbed less NCAAH in the presence of glutaraldehyde than without. When immobilised, the enzymes showed lower activity with cross-linking than without. The implication here is that cross-linking affects the active site of the enzyme. However, the activity of NCAAH immobilised on nylon was higher than in the non-immobilised state, and this could mean that nylon had a stabilising effect on NCAAH activity.

Table 5.2 Product yield of biocatalytic reactions using membrane-immobilised RUKM3s extract

Membrane	(+) or (-) Glutaraldehyde	Hydantoinase yield (μmol/mL)	NCAAH yield (μmol/mL)
Cellulose acetate	+	4.4 ± 0.1	0.6 ± 0.1
	-	3.8 ± 0.1	2.5 ± 0.1
Nylon	+	9.9 ± 0.2	7.1 ± 0.2
	-	11.3 ± 0.2	6.6 ± 0.2
Polysulphone	+	3.2 ± 0.1	0.4 ± 0.1
	-	4.7 ± 0.1	0.8 ± 0.1
Nitrocellulose	+	6.0 ± 0.2	0.6 ± 0.1
	-	6.9 ± 0.2	1.9 ± 0.1

5.3.1.3 Effects of concentration of cross-linking agent on the enzyme activity

The effects of varying concentrations of glutaraldehyde and EDAC on the hydantoinase and NCAAH activity of RUKM3s crude extracts in solution were evaluated. Compared to levels before cross-linking, it was found that glutaraldehyde at the lowest concentration used (0.5 w/v%) reduced the hydantoinase activity of the suspension by 70% (Fig. 5.3), the NCAAH activity by 100% (Fig. 5.4) and the protein concentration of the supernatant by 50% (Fig.5.5). EDAC at the highest concentration used (2 w/v%) reduced hydantoinase activity by 25 % (Fig. 5.6), the NCAAH activity by 40% (Fig. 5.7) and the protein by 20% (Fig. 5.8). Therefore, glutaraldehyde had a more deactivating effect on the enzymes than EDAC.

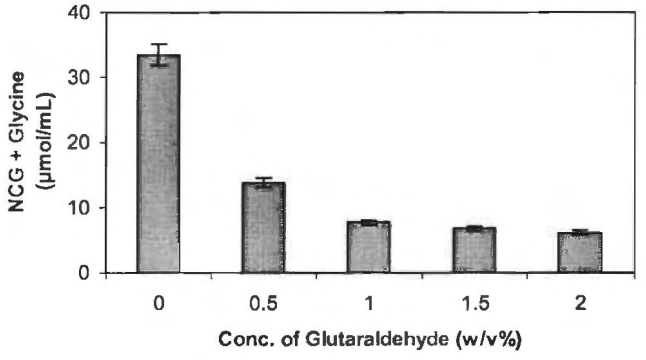


Fig. 5.3 Effect of glutaraldehyde concentration on hydantoinase activity of RUKM3s crude extract

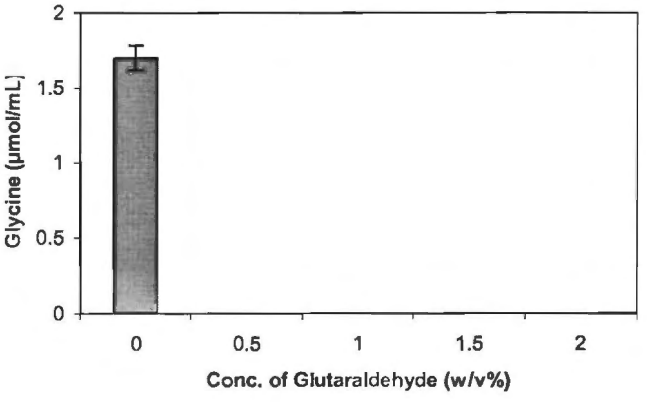


Fig. 5.4 Effect of glutaraldehyde concentration on NCAAH activity of RUKM3s crude extract

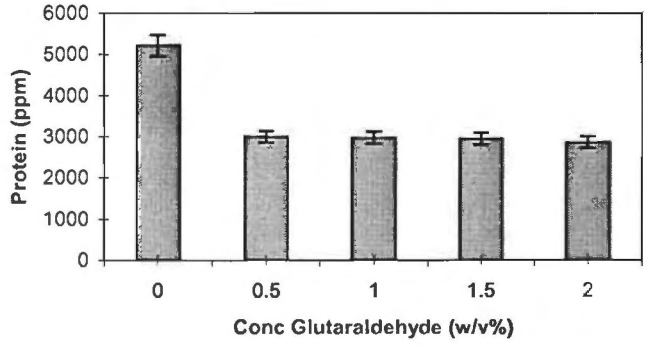


Fig. 5.5 Effect of glutaraldehyde concentration on protein in the supernatant of RUKM3s crude extract

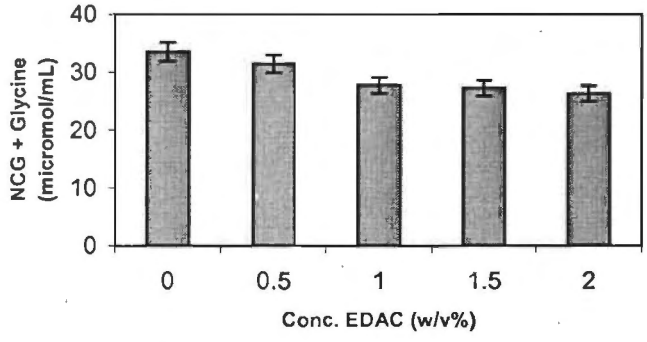


Fig. 5.6 Effect of EDAC concentration on hydantoinase activity of RUKM3s crude extract

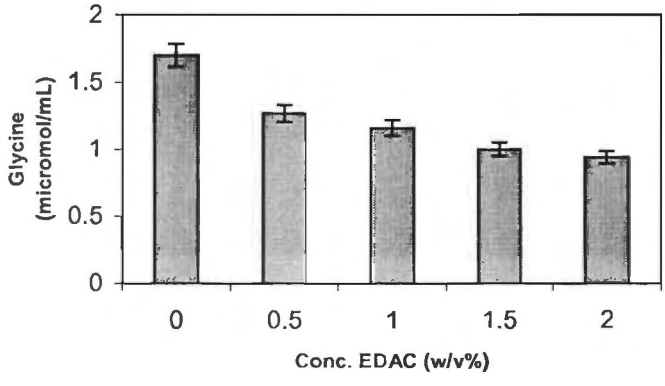


Fig. 5.7 Effect of EDAC concentration on NCAAH activity of RUKM3s crude extract

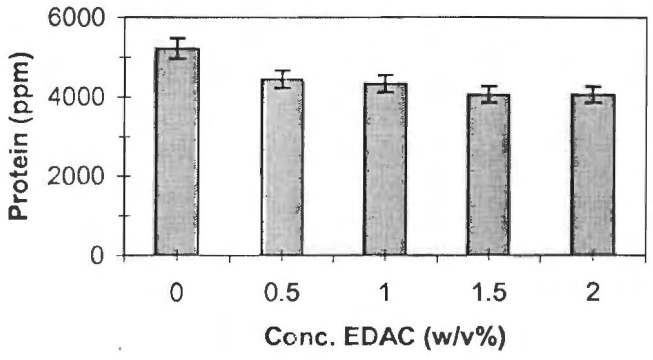


Fig. 5.8 Effect of EDAC concentration on protein in the supernatant of RUKM3s crude extract

5.3.2 Immobilisation by calcium alginate bead-entrapment and micro-encapsulation

5.3.2.1 Effect of immobilisation on the enzyme activity of resting cells and crude extract

While the immediate effect of immobilisation can be an increase or a decrease in the activity for different enzymes, the common effect of immobilisation is increased storage stability and re-usability of the immobilised enzymes, which makes them better than their non-immobilised forms (Chang *et al.*, 1998). Experiments to determine the effect of immobilisation on the activity of resting cells of RUKM3s demonstrated that both hydantoinase (Fig 5.9) and NCAAH (Fig. 5.10) activity in calcium alginate beads and capsules were lower than in the non-immobilised resting cells and crude extract. This may be a result of reduced interaction between substrate and enzymes due to diffusion limitations after the surfaces of the microcapsules and beads were hardened with calcium chloride (Fan and Lee, 2001). The efficiency of the biocatalysts could be improved by reducing the size of the beads and capsules to increase the surface area to volume ratio. The diameter of the beads and capsules obtained in this work was 2-3 mm. From literature, the optimal diameter of the biocatalysts is approximately 1 mm (Fan and Lee, 2001).

The activity of both enzymes was higher in the beads than in the capsules. This may be due to the fact that interaction of substrate with the enzymes and the diffusion of products out of the matrix occur more readily in beads than capsules. Diffusion through beads is more extensive than in capsules (Lee *et al.*, 1995). The total surface area available for substrate-enzyme interaction is

larger in beads due to their porous nature, than in capsules where interaction is limited to the surface of the outer sphere. A comparison between whole cells and crude extract revealed no significant differences in their respective hydantoinase activities, whether immobilised in beads or in capsules.

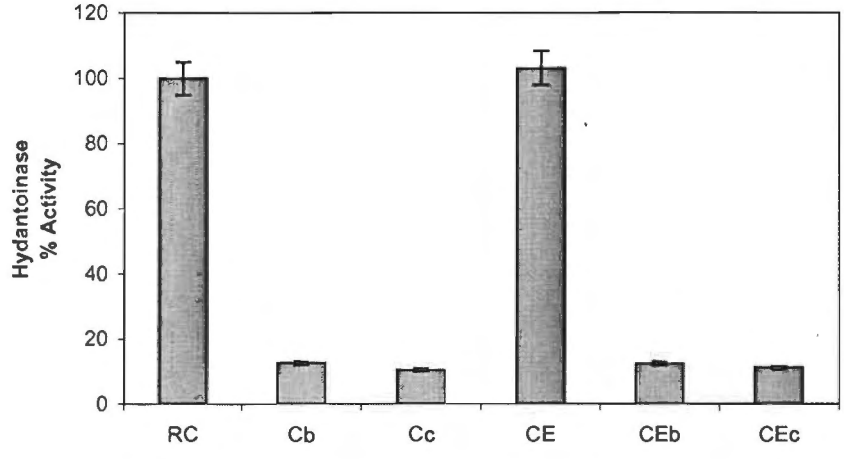


Fig. 5.9 Hydantoinase activity of immobilised biocatalysts as % activity of RUKM3s resting cells (RC) shown for equivalent amounts of resting cells in beads (Cb) and in capsules (Cc), crude extract (CE), crude extract in beads (CEb) and in capsules (CEc)

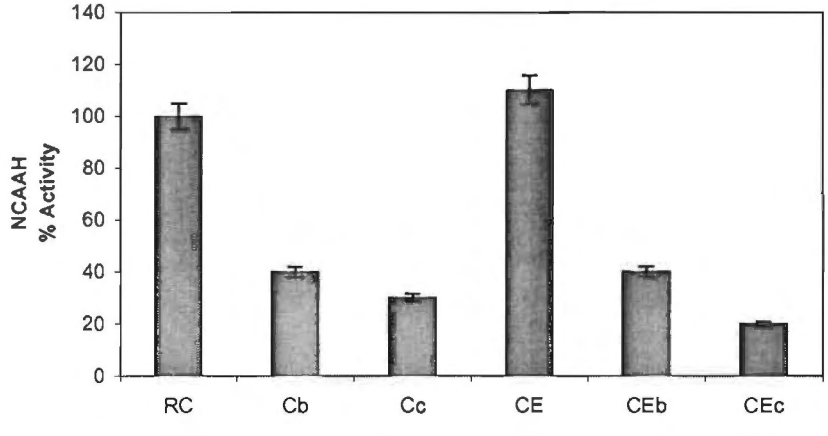


Fig 5.10 NCAAH activity of immobilised biocatalysts as % activity of RUKM3s resting cells (RC) shown for equivalent amounts of resting cells in beads (Cb) and in capsules (Cc), crude extract (CE), crude extract in beads (CEb) and in capsules (CEc)

5.3.2.2 Storage stability of calcium alginate beads and capsules

The enzyme activities of the immobilised biocatalyst increased during storage for 3 h at room temperature in Tris-HCl buffer. This result may suggest that that upon immobilisation, activity is not lost; instead it is reduced, possibly by diffusion limitations. The result could also indicate that the structure of the calcium alginate matrices weakened during the 3 h of storage. This situation might be remedied by adjusting the concentrations of sodium alginate and calcium chloride to optimise the diffusibility of the biocatalyst's surface. Foster *et al.*, (2003) succeeded in increasing the hydantoinase and *N*-carbamoylase activities in *Agrobacterium tumefaciens* crude extract immobilised in calcium alginate by varying the concentrations of calcium chloride and sodium alginate. When the enzyme activity of RUKM3s cells and crude extract immobilised in calcium alginate beads and capsules were measured after 14 days of storage at 4 °C in Tris-HCl buffer, it was found that the hydantoinase and NCAAH activity in both cells and crude extract, immobilised in beads and capsules, were higher after a period of storage than immediately after preparation. This may indicate an improvement in the diffusibility of the immobilisation matrix, resulting in better transport of substrate and product molecules into and out of the biocatalyst respectively. The apparent increase in enzyme activity during storage may be used as a method of conditioning the calcium alginate biocatalyst prior to use. The advantage of the immobilised biocatalyst is the longer period of stability of the enzyme activity compared to the non-immobilised state. This is important for process development.

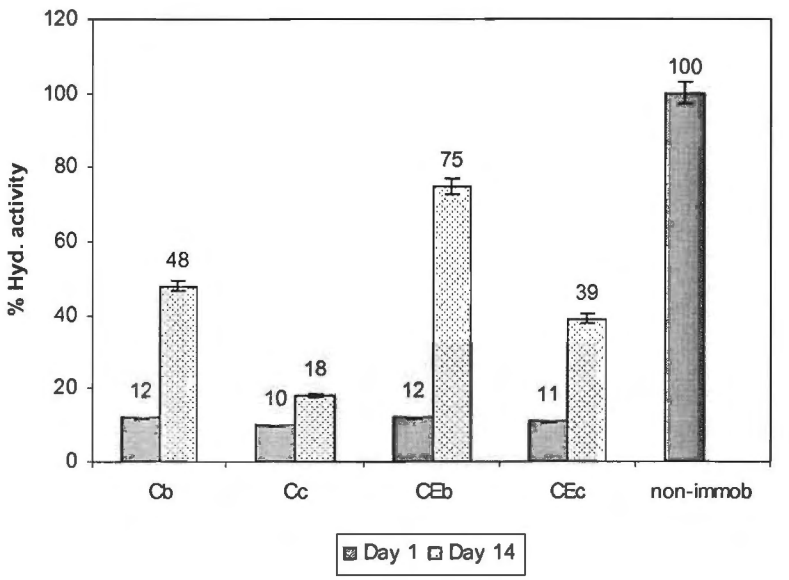


Fig 5.11 Effect of storage on hydantoinase activity of RUKM3s cells in beads (Cb) and in capsules (Cc), and RUKM3s crude extract in beads (CEb and in capsules (CEc) compared with non-immobilised crude extract (100%)

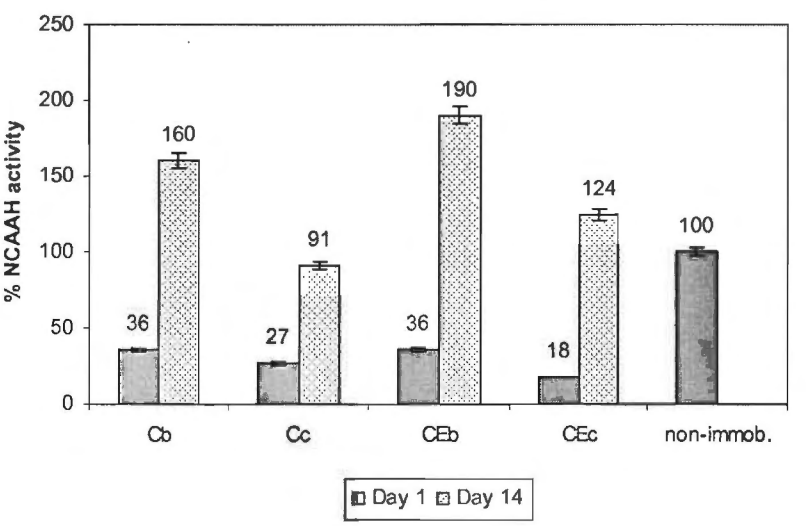


Fig 5.12 Effect of storage on NCAAH activity of RUKM3s cells in beads (Cb) and in capsules (Cc), and RUKM3s crude extract in beads (CEb) and in capsules (CEc) compared with non-immobilised crude extract (100%)

5.3.2.3 Reusability of calcium alginate immobilised biocatalyst

Experiments were devised to determine the reusability of the immobilised biocatalysts. In these experiments, the beads or capsules were reacted with substrates under standard assay conditions. At the end of the reaction period, the substrate was decanted, the biocatalyst washed with buffer and fresh substrate added. The reaction cycle was the repeated. Results of these experiments with immobilised calcium alginate biocatalyst show that for both beads and capsules, hydantoinase activity levels (Fig. 5.13) decreased to approximately half the initial levels in the second cycle, and then to approximately a third in the third cycle. The NCAAH activity (Fig. 5.14) decreased by approximately 50% in the second cycle, and down to zero by the third cycle for both capsules and beads. A major problem with the re-use of the alginate capsules and beads is the progressive physical disintegration of the matrices during incubation. This causes enzyme activity loss through leakage, and impairs the recovery of the biocatalyst for re-use. This makes the calcium alginate impractical for scale-up. In related work reported in literature, Fan and Lee (2001) used hydantoinase in calcium alginate beads to convert phenyl hydantoin and found the operational stability of the hydantoinase biocatalyst to be good up to five cycles of reuse. These authors also found that the operational stability of the biocatalyst was improved by cross-linking the enzyme with polyglutaraldehyde prior to immobilisation.

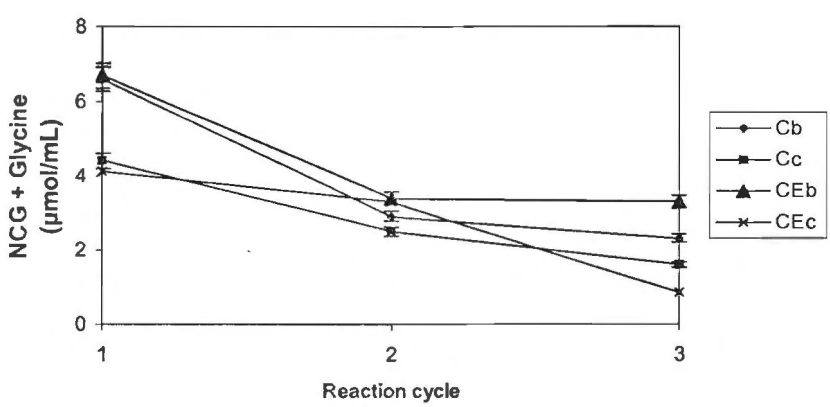


Fig 5.13 Effect of reuse of alginate-based RUKM3s biocatalyst on hydantoinase activity over three cycles showing crude extract in beads (CEb), crude extract in capsules (CEc), resting cells in beads (Cb) and resting cells in capsules (Cc).

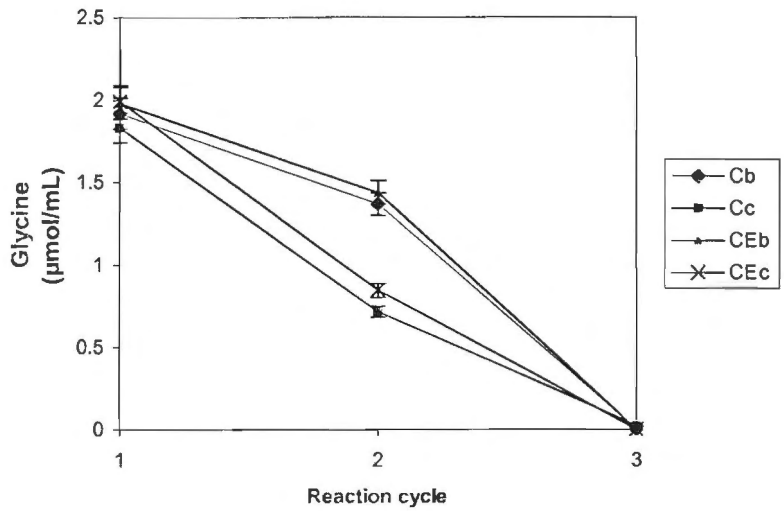


Fig 5.14 Effect of reuse of alginate-based biocatalyst on NCAAH activity over three cycles showing crude extract in beads (CEb), crude extract in capsules (CEc), resting cells in beads (Cb) and resting cells in capsules (Cc).

5.3.3 Immobilisation of RUKM3s in Eupergit® C (oxirane Acrylic beads)

Eupergit® C is a carrier that consists of macro-porous beads. It has been identified as a suitable matrix for covalent immobilisation of enzymes for industrial application. Crude extracts of RUKM3s were immobilised in Eupergit® C, and the immobilisation and operational parameters of the resulting biocatalysts were evaluated. Experiments were designed to determine the optimal operating conditions of the biocatalyst, protein binding capacity and activity yield of the carrier, the dependence of binding capacity on the weight of the carrier, the dependence of the coupling yield on protein concentration, the effect of immobilisation on hydantoinase and NCAAH activities, the effects of cross-linking agents on the binding yield, the operational stability and the storage stability of the biocatalyst.

5.3.3.1 Dependence of binding capacity on weight of carrier

An important factor in the optimisation of the use of a carrier for covalent immobilisation is the binding capacity of the matrix, which is the amount of protein that can be immobilised per unit mass of the carrier. The amount of bound protein was determined by evaluating the concentration

of protein in the supernatant before and after immobilisation. Experiments were conducted (section 5.2.5.1) to determine how the amount RUKM3s protein bound to Eupergit® C changed with the weight of the carrier. The highest amount of protein that can bind per unit weight of carrier represents the optimal binding capacity of the matrix. It was found that above 1000 mg of carrier per 5 mL of the crude protein suspension, with a protein concentration of 5000 μg/mL, no increases in the amount of bound protein were observed (Fig. 5.15). Therefore, use of the carrier in excess of 1000 mg/5mL (or 200 mg/mL) would be uneconomical.

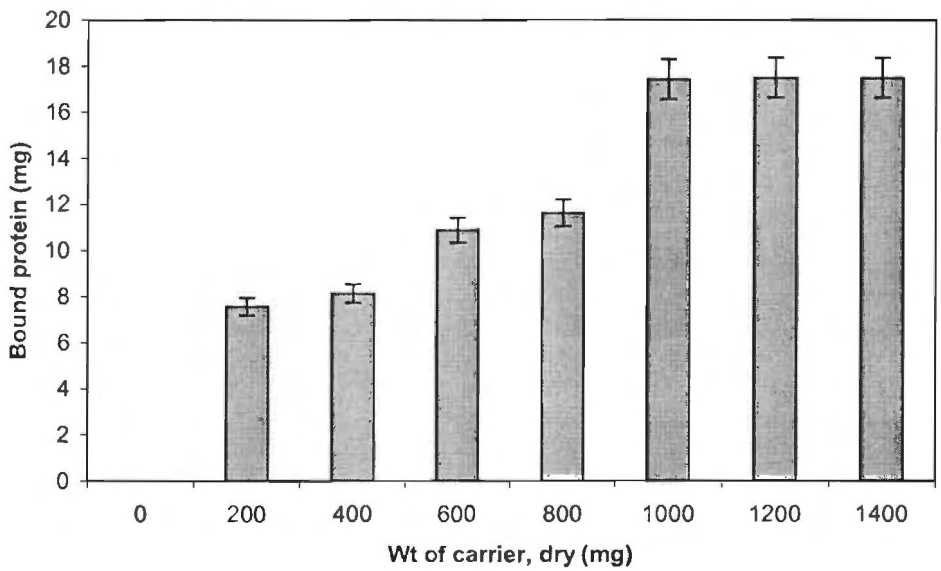


Fig. 5.15 Amount (mg) of bound protein for different weights of Eupergit® C

5.3.3.2 Dependence of coupling yield on protein concentration

The amount of protein that binds to a matrix is dependent on the nature of the covalent binding groups on the surfaces both the matrix and the protein. In addition to the amount of carrier used, economical and optimal binding of protein to carrier is dependent on the concentration of the protein in solution. This dependence varies for different proteins and therefore needs to be optimised for different biocatalysts. Various concentrations of protein were used for immobilisation on a unit of Eupergit® C carrier and the amount of protein successfully

immobilised was determined. The results (Fig. 5.16) show that the coupling yield (the amount of protein immobilised), declined significantly for solutions of protein concentrations above 4g/L.

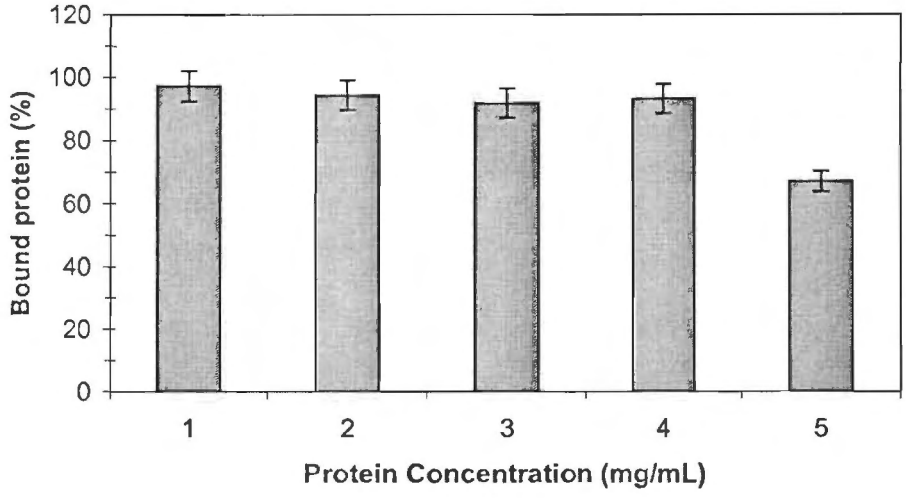


Fig. 5.16 Fraction (%) of protein bound to Eupergit® C for different concentrations of protein in solution

5.3.3.4 Effects of covalent immobilisation of RUKM3s in Eupergit® C on enzyme activity

Successful immobilisation is judged by the amount of enzyme activity retained in the post-immobilisation period, and the stability of the retained activity during both storage and biocatalytic operation. The amounts of enzyme activity exhibited by the immobilised RUKM3s-Eupergit C biocatalyst and the concentration of protein bound to the matrix were determined (Table 5.3). Compared with activities in the non-immobilised state, it was found that covalent immobilisation in Eupergit® C caused a 14% decrease in the hydantoinase activity (Fig. 5.17) and a four-fold increase in the NCAAH activity (Fig. 5.18) of RUKM3s crude extracts. This increase in the activity of the NCAAH upon immobilisation was also observed in alginate beads and capsules. Therefore, immobilisation is a good way to enhance NCAAH stability and activity for application in industrial processes. Approximately 63 % of the protein suspended prior to immobilisation protein was bound to the beads (Fig. 5.19).

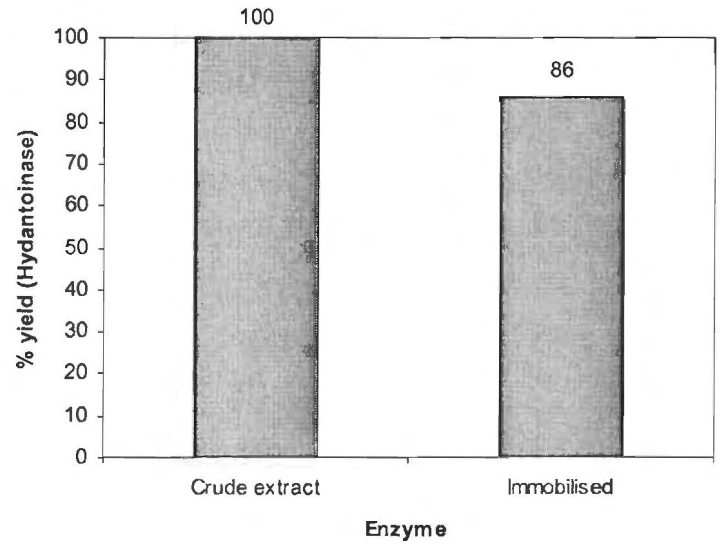


Fig. 5.17 Effect of covalent immobilisation in Eupergit® C on the hydantoinase activity of RUKM3s crude extract, showing product yield (NCG plus Glycine) from immobilised and non-immobilised extract. Product of crude extract = 100%.

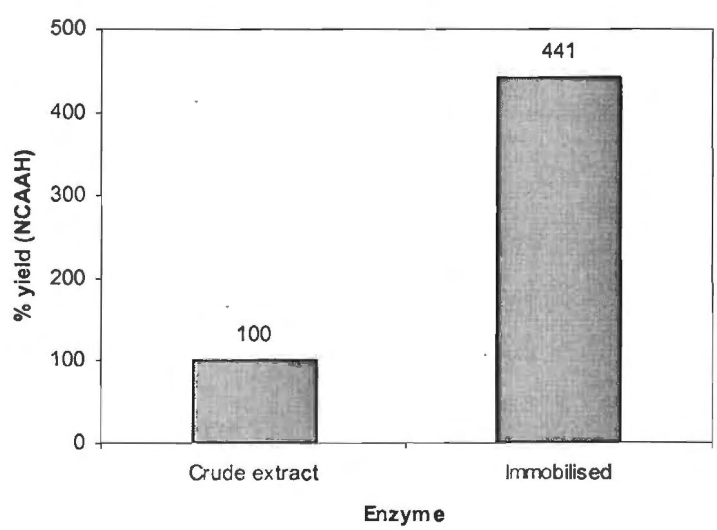


Fig. 5.18 Effect of covalent immobilisation in Eupergit® C on the NCAAH activity of RUKM3s crude extract, showing yield (Glycine) from immobilised and non-immobilised extract. Product of crude extract = 100%.

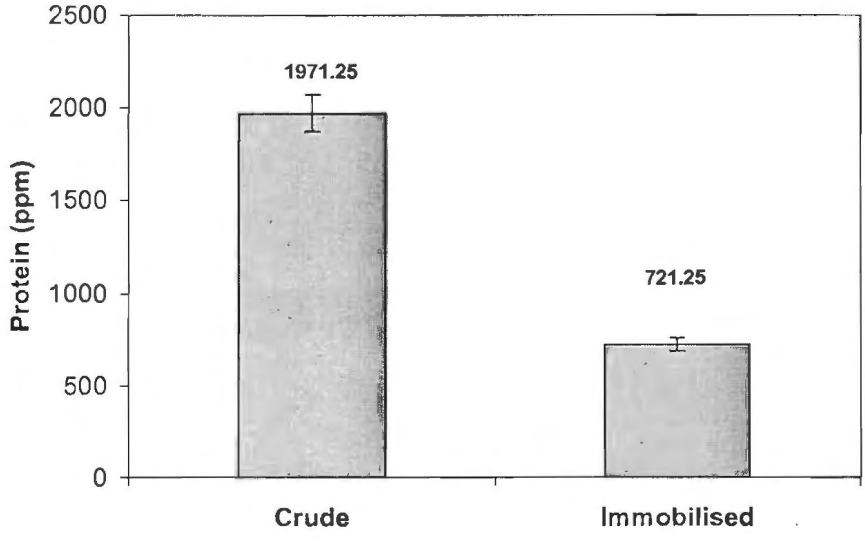


Fig. 5.19 Effect of covalent immobilisation in Eupergit® C on the residual protein concentration of RUKM3s crude extract; reduction in protein in solution after immobilisation

One of the common effects of immobilisation of enzymes, cells and crude extracts is an apparent shift in the conditions of optimal operation of the biocatalyst. In earlier studies (Chapter 3) it was determined that the optimal pH for the NCAAH of RUKM3s was 9 and that of the hydantoinase was 9-10. The optimal temperature for activity of both enzymes was determined to be 40 °C. The optimal operating pH and temperature for the RUKM3s-Eupergit C biocatalyst were determined in order to evaluate any apparent shifts in these optima due to immobilisation. The optimal pH for NCAAH activity and hydantoinase activity in the biocatalyst were found to be to be 9 and 10 respectively (Fig. 5.20), and the optimal temperature for both enzymes in the biocatalyst was found to be 40 °C (Fig. 5.21). Thus, immobilisation caused no shift in the temperature and pH optima for the hydantoinase and NCAAH of RUKM3s.

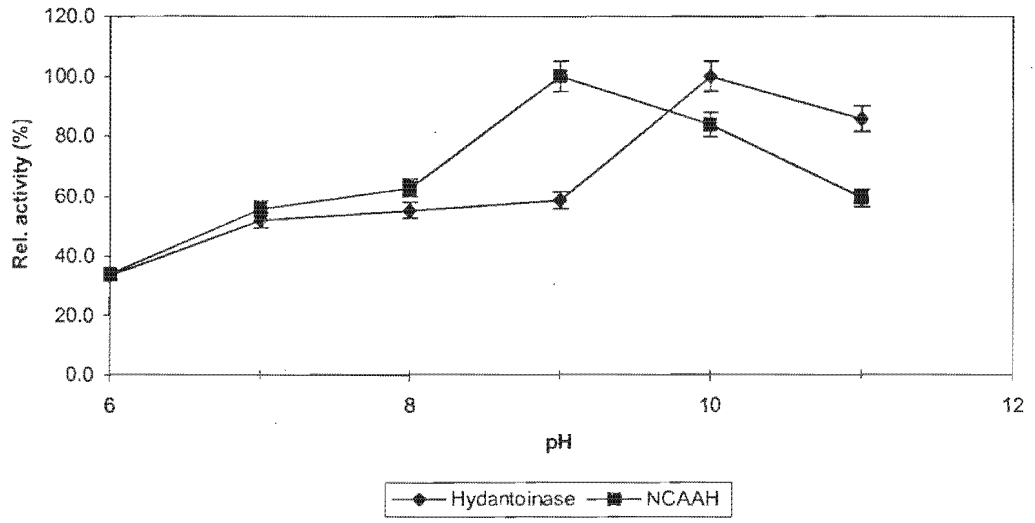


Fig. 5.20 Effect of pH on hydantoinase and NCAAH activity of the RUKM3s-Eupergit® C biocatalyst

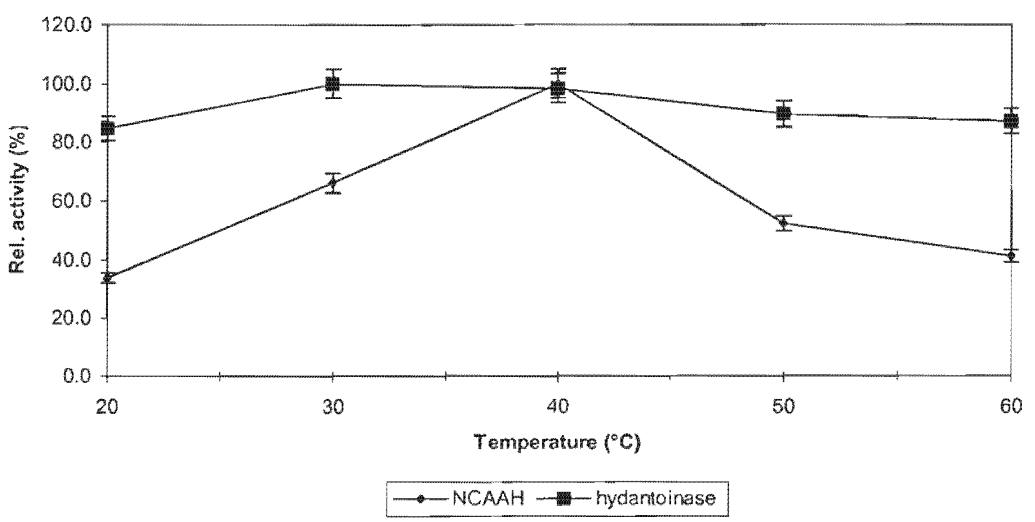


Fig. 5.21 Effect of temperature on hydantoinase and NCAAH activity of the RUKM3s- Eupergit® C biocatalyst

5.3.3.5 Storage and Operational Stability of the RUKM3s-Eupergit® C biocatalyst

In order to determine the degree to which bound activity is stably retained during storage and use in the post-immobilisation period, it is important to characterise biocatalysts in terms of their storage and operational stability. The storage stability is an indicator of the shelf life of the biocatalyst under optimal conditions. The operational stability is a measure of the repeatability of use of the biocatalyst under its normal or optimal operating conditions. The storage stability of the RUKM3s-Eupergit® C biocatalyst was evaluated by regular assays of the hydantoinase (Fig. 5.22) and NCAAH (Fig. 5.23) activities of samples of biocatalyst stored at 4 °C over 4 weeks..

It was found that the half-life of the RUKM3s-Eupergit® C biocatalyst, stored in the wet state at 4 °C was approximately two weeks, for both hydantoinase and NCAAH. Approximately 25% of the enzyme activity of both enzymes was retained beyond the 4-week experimental period.

The determination of the effects of lyophilisation on the enzyme activities of the biocatalyst revealed that the RUKM3s-Eupergit® C biocatalyst retained 98% of hydantoinase (Fig. 5.24) but only 17% of the NCAAH (Fig. 5.25) activity. As a reflection on the stability of the two enzymes, this is consistent with the earlier finding where resting cells retained 81% of hydantoinase activity and 39.7 % of NCAAH activity after lyophilisation (Section 2.3.3.3).

The RUKM3s-Eupergit® C biocatalyst was re-used in repeated biocatalytic reactions to determine the number of use-cycles, and therefore, its operational stability. The hydantoinase retained half of its maximum activity up to 28 cycles of reuse (Fig. 5.26) and the NCAAH up to 18 cycles (Fig. 5.27). It was also noted that the enzyme activities were low in the period immediately after immobilisation, but increased steadily during re-use to reach maximum levels after approximately 7 cycles. The low activities of immobilised biocatalyst in the period immediately after immobilisation, and subsequent increase during reuse could imply that the biocatalyst requires a period of conditioning in the substrate environment before it can attain optimal activity levels. This has been observed by other workers. The low activities at the start of use of the biocatalyst can be overcome by pre-incubation of the biocatalyst in a solution containing the target substrate under the intended operating temperature and pH.

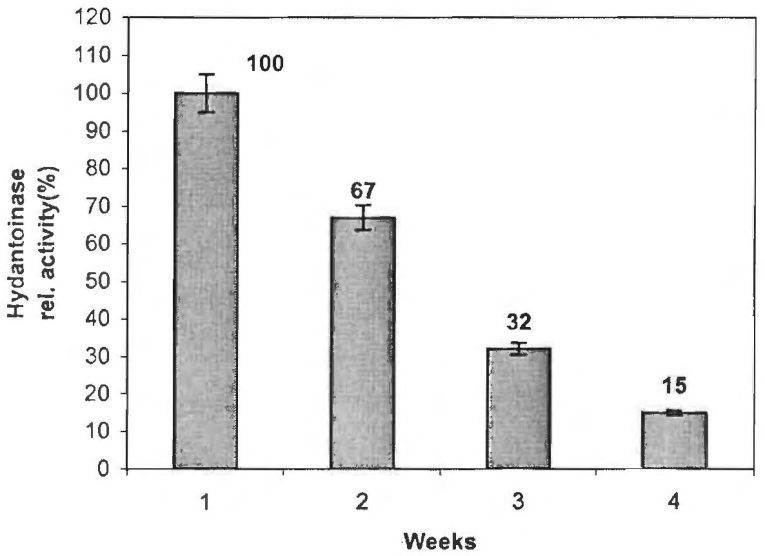


Fig. 5.22 Storage stability of the hydantoinase in RUKM3s-Eupergit® C biocatalyst at 4 °C over 4 weeks shown as activity relative (%) to start of storage period

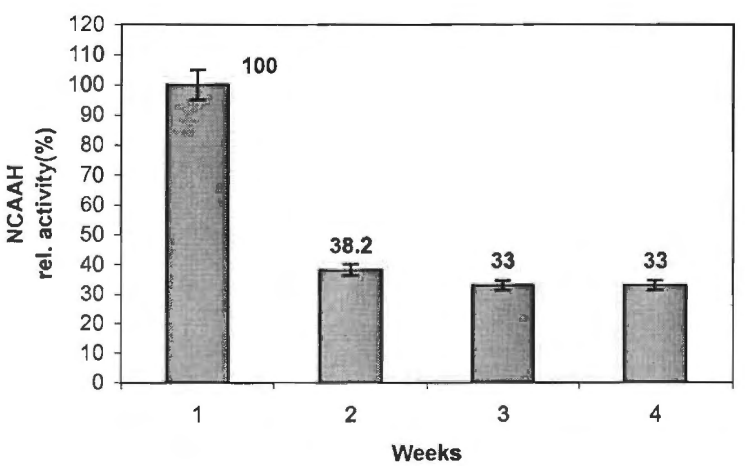


Fig. 5.23 Storage stability of the NCAAH in RUKM3s-Eupergit® C biocatalyst at 4 °C over 4 weeks shown as activity relative (%) to start of storage period

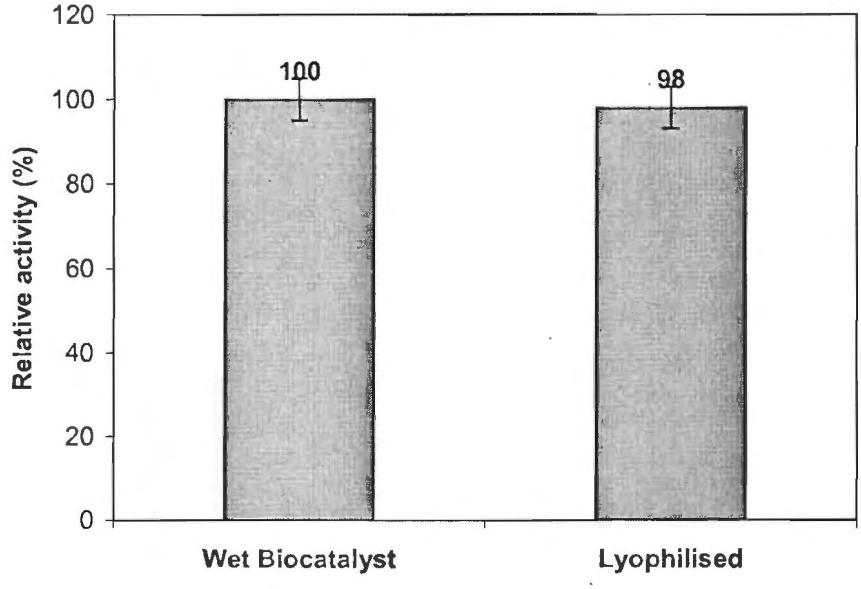


Fig. 5.24 Effect of lyophilisation on the hydantoinase in RUKM3s-Eupergit® C biocatalyst

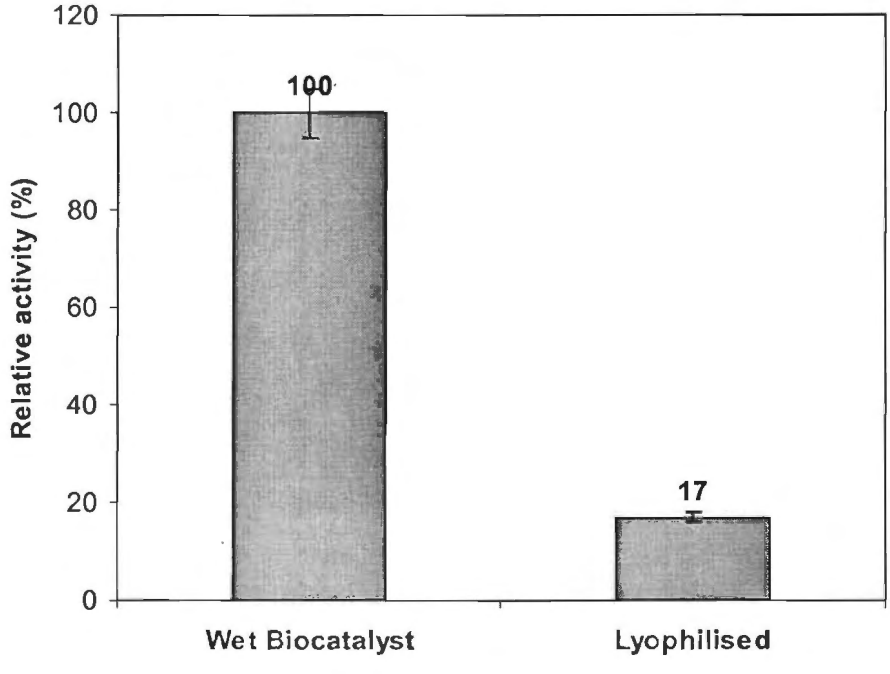


Fig. 5.25 Effect of lyophilisation on the NCAAH in RUKM3s-Eupergit® C biocatalyst

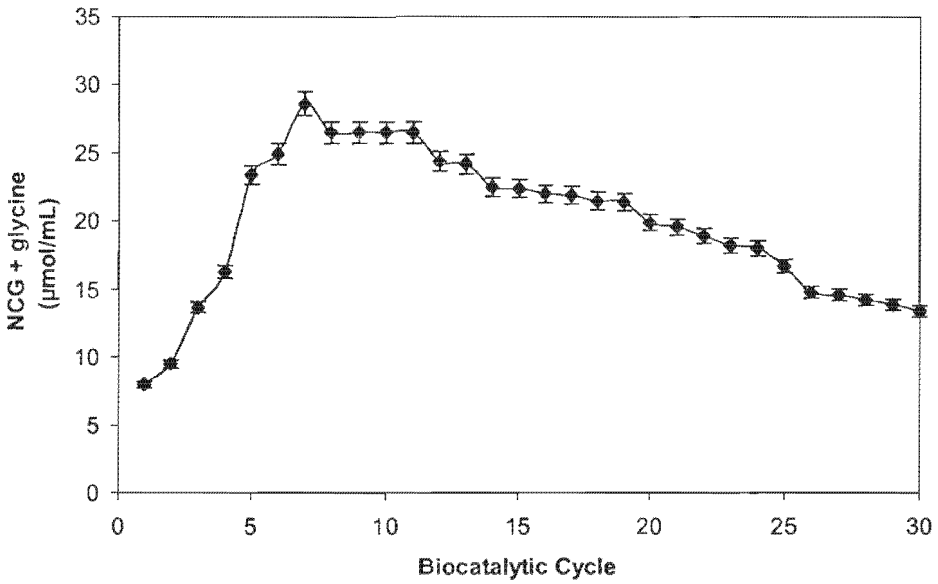


Fig. 5.26 Operational stability of hydantoinase in RUKM3s-Eupergit® C: activity of biocatalyst during cycles of reuse

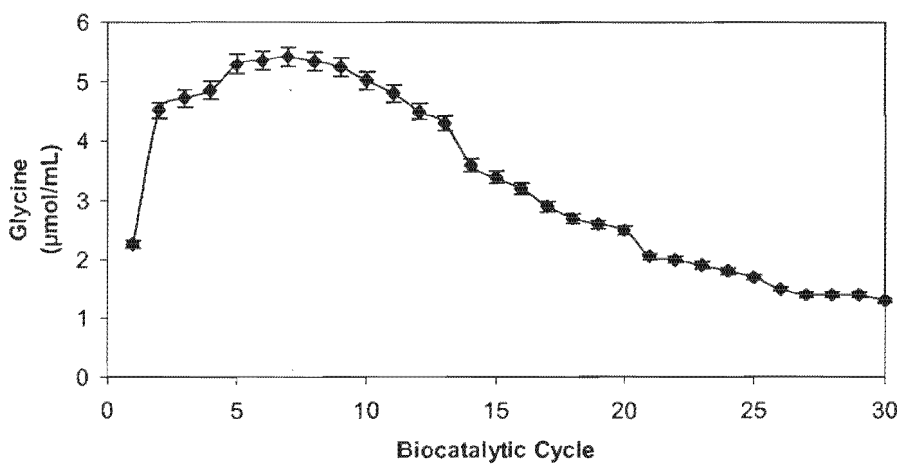


Fig. 5.27 Operational stability of NCAAH in RUKM3s-Eupergit® C: activity of biocatalyst during cycles of reuse

5.3.3.6 Effects of carrier modification on the binding yield of Eupergit® C

Covalent coupling of proteins to Eupergit C occurs via oxirane groups, which are known to react with amino, thiol and hydroxy groups forming covalent bonds. The reactive groups in oxirane beads, like those in other covalent carriers can be modified by preactivation of the carrier with suitable cross-linking agents to stabilise linkages with enzymes or create linkages at sites other than the active sites of enzymes. Cross-linking agents such as glutaraldehyde and EDAC (Ragnitz *et al.*, 2001) can be used to initiate linkages between carriers and functional groups in biomolecules. EDAC has been used to mediate covalent coupling of hydantoinase to Sepharose 4B and an L-carbamoylase to Eupergit® C, where the use of supports preactivated with glutaraldehyde resulted in complete loss of activity of the L-carbamoylase activity (Ragnitz *et al.*, 2001).

To investigate the possibility of improved binding and activity yield of RUKM3s enzymes due to carrier modification, Eupergit® C was preactivated with EDAC and glutaraldehyde respectively, prior to immobilisation. The amount of protein (Fig. 5.28) bound to the preactivated carrier and the enzyme activities retained in the matrix were determined at various concentrations of the cross-linking agents. It was found that no binding of protein to the matrix occurred in the presence of EDAC, whereas 60-80% of the protein was bound in the presence of glutaraldehyde. However, biocatalyst from both treatments contained neither hydantoinase nor NCAAH activity. This could mean that enzymes were inactivated by the treatments. Further investigations on the use of modified carrier could test the use of lower concentrations (1-25 mM) of the cross-linking agents. The present work used 0.5- 2 % (w/v) (or 25-100mM) concentrations of glutaraaldehyde and EDAC. In work reported by other researchers, increases in *N*-carbmoylase activity were noted by Ragnitz *et al.* (2001) using 1-90 mM concentrations of EDAC.

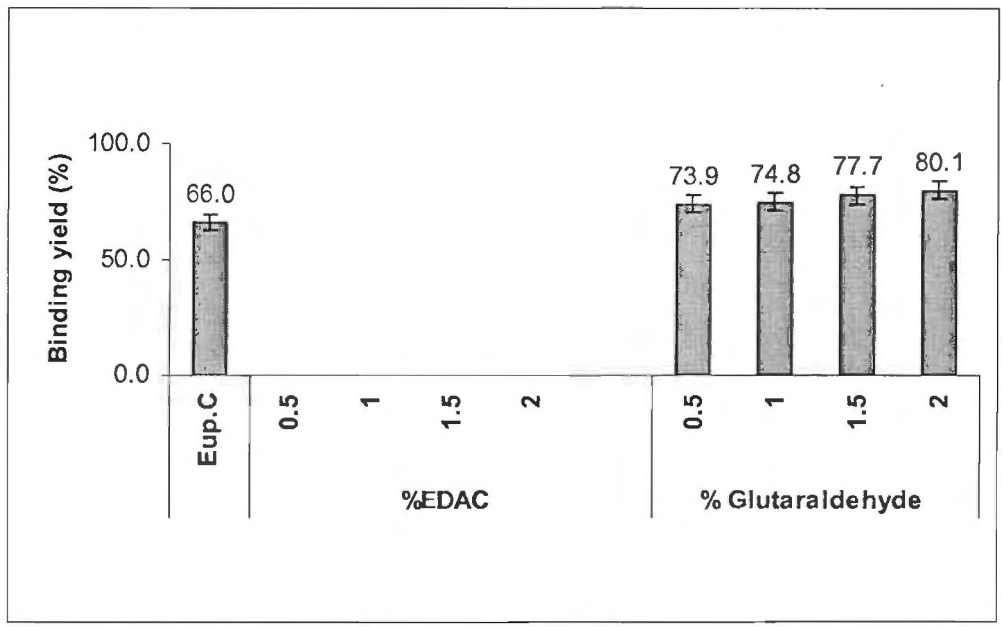


Fig. 5.28 Effect of pre-treatment of carrier with cross-linking agent (%) on the amount (%) of protein in RUKM3s extract bound to Eupergit® C

5.3.4 Immobilisation of RUOR-PN1 in Eupergit® C

5.3.4.1 Effects of covalent immobilisation of RUOR-PN1 in Eupergit® C on enzyme activity

The strain RUKM3s has been shown to have high hydantoinase activity. However, the efficient production of amino acids using RUKM3s can be limited by the relative instability and low activity of its NCAAH. These characteristics of the NCAAH have been noted in previous reports (Skepu, 2000; Buchanan *et al.*, 2001). One of the ways by which the overall productivity of a RUKM3s biocatalyst could be enhanced is the use a dual bioreactor system combining the high hydantoinase activity of RUKM3s in the first stage to produce NCG, followed by a high NCAAH activity in the second stage, from a different strain.

In other work in our research group, the strain RUOR-PN1 was identified as a high NCAAH-producing strain (Foster, 2002). RUOR-PN1 is a mutant strain of RUOR, an *A. tumefaciens* strain that has a higher *N*-carbamoylase activity than many other strains. It has been found to convert hydantoins to amino acids using an enzyme system that involves a racemase, a hydantoinase and a

D-selective *N*-carbamoylamino acid amidohydrolase. Significantlly, its *N*-carbamoylase was not inhibited by *N*-carbamyl amino acids (Hartley *et al.*, 1998). The high NCAAH activity of RUOR-PN1 makes it an ideal candidate for use in a combined bioreactor system with RUKM3s, to improve the overall yield of amino acids. Since the RUKM3s biocatalyst has a higher hydantoinase activity than the RUOR-PN1 biocatalyst, the former could be used in the first stage of the combined bioreactor system to achieve close to 100% conversion of hydantoin to NCG plus glycine by manipulating the amount of biocatalyst. Thereafter, the high NCAAH activity of the RUOR-PN1 biocatalyst could be used in the second stage to achieve 100% conversion of the residual NCG from the first stage to glycine. The two processes would occur in the two reactors as shown below (Fig. 5.29). This scheme was developed and tested experimentally in chapter 6. Where chiral substrates are used, the conversions of substrate to products illustrated in Fig. 5.29 also has potential for use in stereoselective synthesis of D- or L- amino acids.

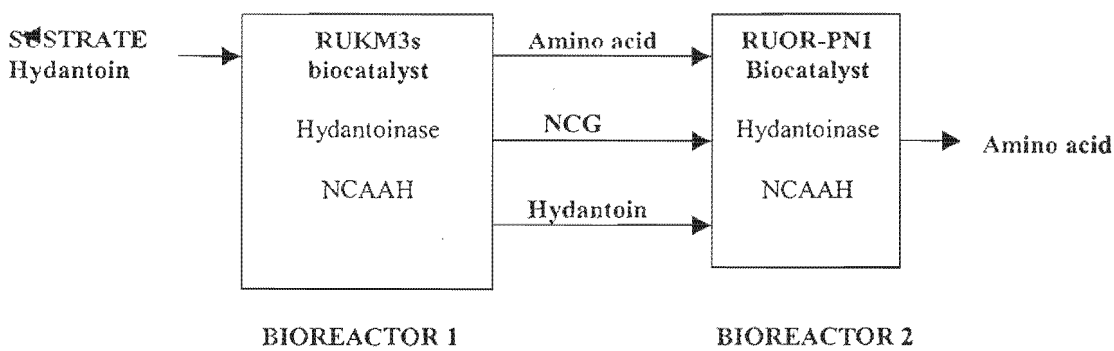


Fig. 5.29 Proposed scheme of process to achieve 100% conversion of substrate to product

In order to prepare RUOR-PN1 for use in the continuously-operated packed-bed bioreactor system, the immobilisation parameters of crude extract of the strain in Eupergit® beads were investigated. The binding and activity yields of RUOR-PN1 were determined and compared with those of RUKM3s (Table 5.3). It was found that whereas the hydantoinase activity of immobilised RUKM3s was higher than that of immobilised RUOR-PN1, the NCAAH of the latter was almost twice that of RUKM3s. During immobilisation, the hydantoinase of RUOR-PN1 was not

significantly affected (Fig. 5.30), while the NCAAH activity increased by 20% (Fig. 5.31) and 75% of protein in the crude extract was bound to the carrier (Fig. 5.32).

Table 5.3 Comparison of the binding and activity yields of RUOR-PN1 and RUKM3s crude extracts immobilised in Eupergit® C

	Bound Protein		Product yield (% of theoretical yield)	
	% of initial concentration	mg/g dry support	Hydantoinase	NCAAH
RUOR-PN1	75 ± 1	6.5 ± 0.5	45 ± 1	40 ± 1
RUKM3s	63 ± 1	9.5 ± 0.5	57 ± 1	22 ± 1

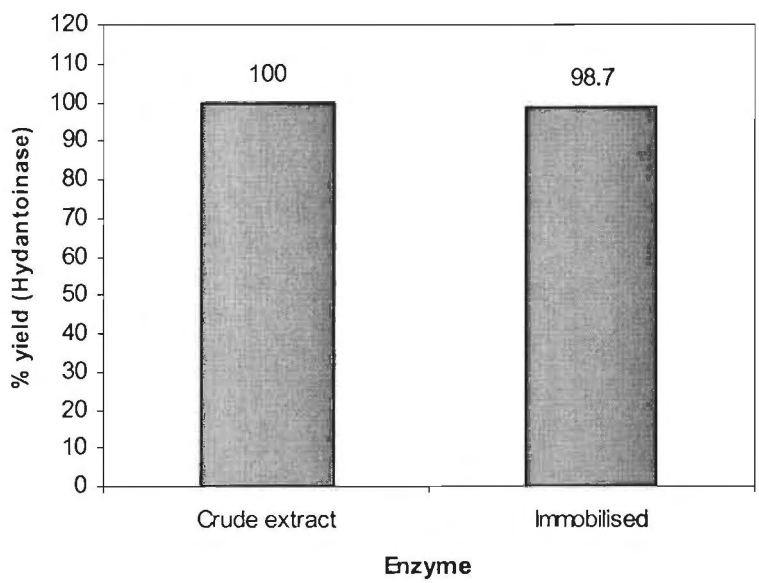


Fig. 5.30 Effect of immobilisation on hydantoinase activity of RUOR-PN1 crude extract in Eupergit® C

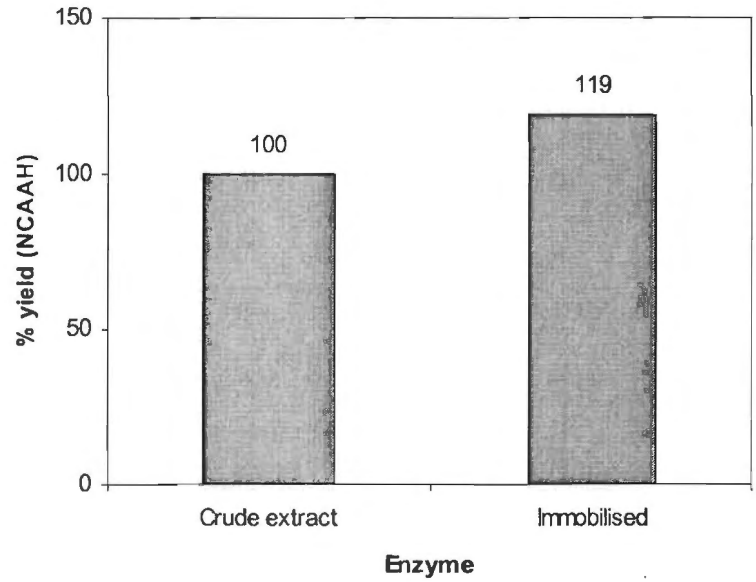


Fig. 5.31 Effect of immobilisation on NCAAH activity of RUOR-PN1 crude extract in Eupergit® C

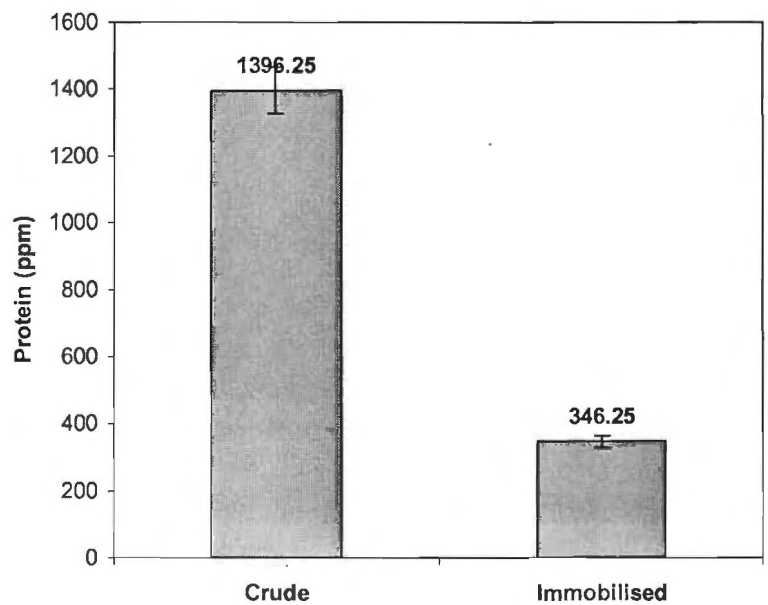


Fig. 5.32 Effect of immobilisation in Eupergit® C on protein concentration of supernatant of RUOR-PN1 crude extract

5.3.4.2 Storage and Operational Stability of the RUOR-PN1 -Eupergit® C biocatalyst

The storage and operational stability of RUOR-PN1 were also determined. It was found that significant levels of hydantoinase (Fig. 5.33) and NCAAH (Fig. 5.34) activity were retained beyond 4 weeks of storage at 4 °C. The hydantoinase activity from RUOR-PN1 had decreased by only 33 % after 30 cycles of reuse (Fig 5.35), and the NCAAH activity reached half of the maximum activity after 19 cycles (Fig. 5.36).

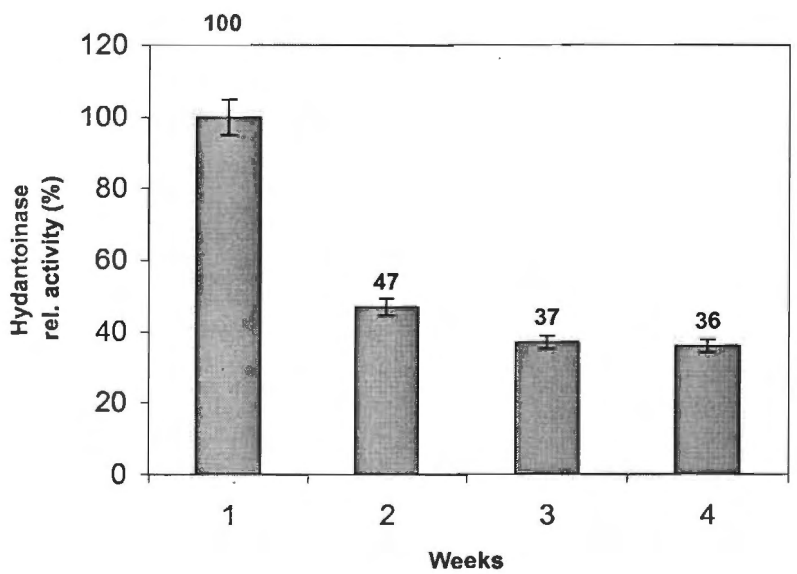


Fig. 5.33 Storage stability of RUOR-PN1 hydantoinase in Eupergit® C at 4 °C

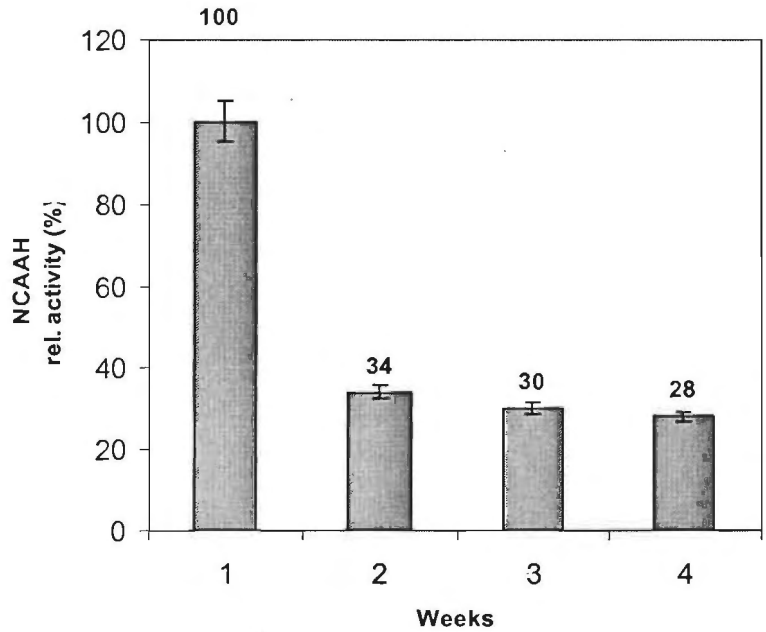


Fig. 5.34 Storage stability of RUOR-PN1 NCAAH in Eupergit® C at 4 °C

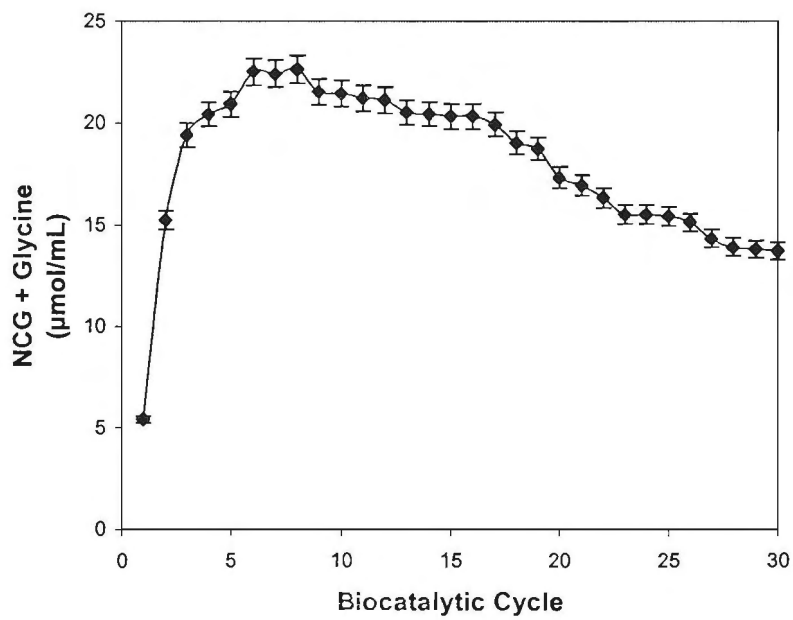


Fig. 5.35 Operational stability of RUOR-PN1 hydantoinase in Eupergit® C: activity of biocatalyst during cycles of reuse

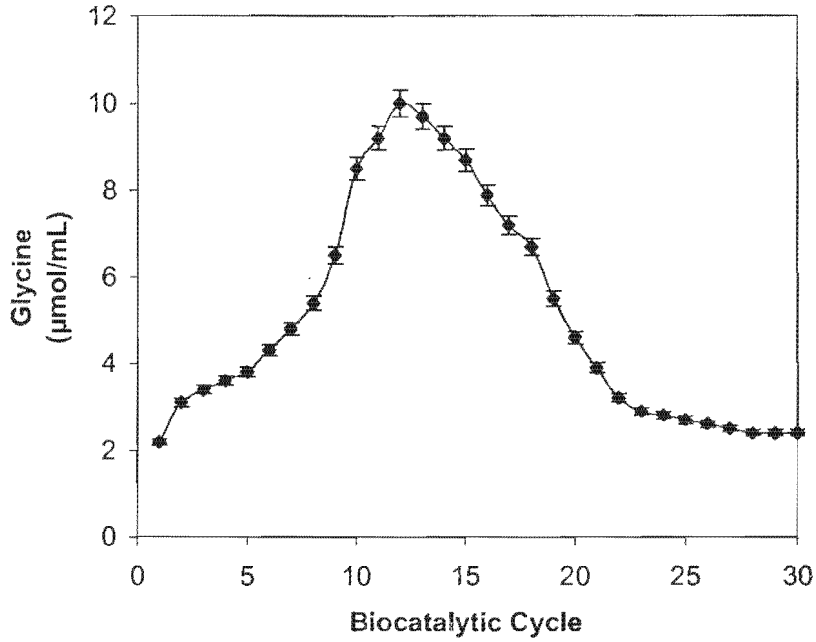


Fig. 5.36 Operational stability of RUOR-PN1 NCAAH in Eupergit® C: activity of biocatalyst during cycles of reuse

5.3.4.3 Comparison of immobilisation and operational parameters of RUKM3s biocatalysts

The various methods of immobilisation were evaluated to determine their suitability for use in the production of biocatalyst for industrial application. A comparison of some of the operational parameters of RUKM3s biocatalyst produced by the methods in this chapter is provided in Table 5.4. In terms of operational stability of the biocatalyst, which could have serious economic implications in the overall process costs, the Eupergit® C-RUKM3s biocatalyst was the best. Calcium alginate beads and capsules yielded a biocatalyst with a good storage stability, but poor operational stability due to disintegration of the matrix during use. Immobilisation by adsorption to membranes is not suitable for industrial application because the weak electrostatic forces that bind the enzymes to the support seem to be broken during the first cycle of use. The addition of cross-linking agents caused lower activities in the crude extract, which may suggest inactivation of the enzymes. The method is therefore not suitable for stabilisation, except when used to

improve adsorption to other matrices or to provide enzyme crystals that can be further immobilised by bead entrapment or microencapsulation (Fan and Lee, 2001; Ragnitz *et al.*, 2001).

Table 5.4 Comparison of immobilisation and operational parameters of RUKM3s biocatalysts prepared by various methods

Method	Matrix	% protein bound	Hydantoinase Yield (%)	NCAAH Yield (%)	Storage stability (weeks to ½ life)	Operational stability (cycles to ½ life)
Adsorption	Nylon	13.4	22.6	26.4	-	1
	Nylon + GL	11.9	19.8	28.4	-	1
Cross-linking	EDAC	22	-	-	-	-
	Glutaraldehyde	45	-	-	-	-
Encapsulation	Ca-alginate -cells	100	11.2	12.0	4+	2
	-crude extract	100	25.0	16.4	4+	2
Entrapment	Ca-alginate -cells	100	30.6	21.2	4+	2
	-crude extract	100	47.8	25.6	4+	2
Covalent	Eupergit C	63	57.2	21.6	2-3	18-28

5.4 CONCLUSIONS

A variety of immobilization methods have been developed for the purpose of increasing productivity in enzymatic reactions. In particular, enzymatic reactions employing immobilized crude extracts of enzymes have the following advantages: the catalytic power of the enzyme is noticeably stabilized; the enzymes are easily recycled, which naturally cuts down the expense incurred in the process; and, products can be isolated in a simple manner (Chang *et al.*, 1998). In this chapter, the suitability of five different techniques of enzyme immobilisation for the stabilisation of RUKM3s hydantoin hydrolysing enzymes was investigated. Adsorptive membranes, cross-linking agents, encapsulation and entrapment within matrices, and covalent bonding were all used to produce RUKM3s biocatalysts whose immobilisation and operational parameters were all evaluated. It was found that among the methods tested, covalent

immobilisation to Eupergit® C produced the most suitable biocatalyst in terms of stability, ease of separation of products and recovery of the biocatalyst.

The investigation of immobilisation by surface adsorption to membranes revealed that among the five membrane filters used, nitrocellulose adsorbed the most protein, followed by nylon, polysulphone and cellulose acetate, and polypropylene adsorbed no protein at all. The presence of glutaraldehyde reduced the amount of protein adsorbed to the membranes. Nylon adsorbed the most hydantoinase, followed by nitrocellulose, while polysulphone and cellulose acetate adsorb the same amounts. Nylon also adsorbed the most NCAAH activity, followed by cellulose acetate, nitrocellulose and polysulphone with the least. Nylon, polysulphone and nitrocellulose adsorb less hydantoinase in the presence of glutaraldehyde. Nylon adsorbs more NCAAH in the presence glutaraldehyde than without. Nylon was therefore the best membrane for immobilisation by adsorption. However, compared to other methods of immobilisation such as entrapment and microencapsulation, adsorption is not convenient. Adsorbed biocatalyst is only available on the plane of the immobilisation surface, whereas matrices are three-dimensional. Increasing the amount of biocatalyst when a nylon membrane is used would involve stacking sheets of membrane one on top of another, which could reduce the flow rate of the substrate, or require big reactors.

One of the most common methods of immobilization methods developed so far is bead entrapment, where cells, enzymes or their crude extract are entrapped in gel-cored beads made of calcium alginate, polyacrylamide, carrageenan, agarose, gellan gum, or polytetrafluoroethylene (PTFE) (U.S. Pat. Nos. 5,175,093; 5,288,632; 5,093,253; 4,722,898; 4,828,997; 5,070,019). However, a major disadvantage of the bead entrapment method is that the immobilised enzymes are known to gradually leak out of the carriers (Chang *et al.*, 1998).

Another common method of immobilisation is microencapsulation, which has been applied in various ways for immobilization of animal, plant, bacterial, algal, or fungal cells, crude extracts and enzymes (U.S. Pat. Nos. 5,286,495; 4,806,355; 4,689,293). In this method, the enzyme is trapped within a liquid-cored spherical capsule of calcium alginate. A major weakness of this method is that the slightest structural defect in the capsule can lead to instant leakage of the enzyme from the capsule.

When used in this study, immobilisation by bead entrapment and microencapsulation in calcium alginate produced a biocatalyst in which hydantoinase and NCAAH activity were initially lower than in the non-immobilised resting cells and crude extract. The reduced activity may have been due to the fact that the gel matrices were hardened in calcium chloride solution after immobilisation, which could have resulted in lower diffusion rates for substrate and product. Diffusion through alginate beads and capsules is a function of the concentrations of sodium alginate and calcium chloride. Higher concentrations lead to less leakage of the enzyme, but also reduce the permeability of the matrix to substrate and product (Blandino *et al.*, 2000).

The activity of both enzymes was higher in beads than in capsules. The beads had a higher operational stability than capsules, which tended to disintegrate more easily. Reuse of the immobilised calcium alginate biocatalysts showed that for both beads and capsules, the NCAAH decreased in the second cycle, and declined to almost zero in the third cycle in both capsules and beads. Hydantoinase activity levels decreased to about half the initial levels in the second cycle, and then to about 30% in the third cycle. A major problem with the reuse of the alginate capsules and beads was the gradual disintegration of the matrices during incubation. This may have been due to phosphate or other ions which cause disintegration of calcium alginate. The same effect has been reported in literature, and it is avoided by using reagents, media and substrate that do not contain phosphates (Ivanova *et al.,* 2002). The efficiency of the immobilisation by microencapsulation and bead entrapment can be improved by reducing the bead or capsule size, which increases the surface area available for diffusion of substrate and products. This is best achieved by use of an Electrostatic droplet generator operating between 3000 and 6000 V, which can produce beads or capsule of 2.4 mm to 0.8 mm diameter (Ivanova *et al.,* 2002).

An investigation of the suitability of cross-linking as a method of stabilisation showed that varying the concentrations of glutaraldehyde and EDAC affected the hydantoinase and NCAAH activity. Even at the lowest concentration used (0.5 %w/v or 25mM) glutaraldehyde reduced the hydantoinase activity of the suspension to about 30%, and the NCAAH activity to 0% of the enzyme activity levels before cross-linking; the corresponding effect on protein concentration of the supernatant was 50% reduction. EDAC at the highest (100mM) concentration used reduced hydantoinase activity by about 25 %, the NCAAH activity by 40% and the protein by 20%. As a method of producing a biocatalyst for industrial application, cross-linking is not suitable because

at the concentrations of cross-linking agent used in this work, there was a loss of activity. In addition, separation of the biocatalyst from the products and recovery of the biocatalyst for reuse could be a difficult and costly undertaking.

Covalent immobilisation in Eupergit® C was found to be the most suitable method for the stabilisation of the hydantoin hydrolysing enzymes of RUKM3s. The immobilisation and operational parameters of the biocatalyst were considered in detail. When experiments were conducted to determine the dependence of binding capacity of RUKM3s-Eupergit® C on the weight of the carrier, it was found that the optimal weight of Eupergit® C was 200 mg/mL of protein suspension, and the study of the dependence of protein binding on protein concentration revealed that the optimal coupling yield of RUKM3s protein on Eupergit® C was 4g/L. Compared with activities in the non-immobilised state, it was found that covalent immobilisation in Eupergit® C caused a small decrease in the hydantoinase activity but a three-fold increase in the NCAAH activity of RUKM3s crude extracts. Approximately 63 % (1250 µg/mL) of the protein in suspension prior to immobilisation was bound to the beads, and the biocatalyst could give a yield of 28.6 µmol/mL from 50 µmol/mL hydatoin, and 5.4 µmol/mL from 25 µmol/mL NCG under standard operating conditions (that is, weighed biocatalyst equivalent to 100 mg crude extract, used in 2.5 mL substrate, for 3 h, 40 °C).

The optimal operating pH and temperature for the RUKM3s-Eupergit® C biocatalyst were determined for NCAAH activity and hydantoinase activity in the biocatalyst to be 9 and 10 respectively, and the optimal temperature for both enzymes in the biocatalyst was found to be 40 °C. These results showed that immobilisation caused no shift in temperature and pH optima for the two enzymes. Investigations of the storage and operational stabilities of the RUKM3s-Eupergit C biocatalyst revealed that the half-life of the Eupergit® C biocatalyst stored at 4 °C is about two weeks; that the biocatalyst retains 98% of hydantoinase and 17% of the NCAAH activity after freeze-drying; and that the hydantoinase lost half of its maximum activity after 28 cycles of reuse and the NCAAH after 18 cycles. Similarly favourable reports of the high operational stability of enzymes immobilised in Eupergit® C have been cited in literature for lipases (Katchalski-Katzir and Kraemer, 2000) and cyclodextrin glucosyltransferases (Martin *et al.*, 2001).

The immobilisation parameters of the high NCAAH-yielding mutant strain RUOR-PN1 in Eupergit® C beads were also investigated. When the binding and activity yields of RUOR-PN1 were determined and compared with those of RUKM3s, it was found that whereas the hydantoinase activity of immobilised RUKM3s is higher than that of immobilised RUOR-PN1, the NCAAH of the latter is almost twice that of RUKM3s. During immobilisation, the hydantoinase of RUOR-PN1 was not significantly affected, while the NCAAH activity increased by 20%, and 75% of protein in the crude extract was bound to the carrier. The storage and operational stability of RUOR-PN1 biocatalyst were also determined. It was found that significant levels of hydantoinase and NCAAH activity were retained beyond 4 weeks of storage at 4 °C. The hydantoinase activity from RUOR-PN1 had decreased by only 33 % after 30 cycles of reuse, and the NCAAH activity reached half of the maximum activity after 19 cycles.

The two biocatalysts of RUKM3s and RUOR-PN1 covalently immobilised in Eupergit® C used in a process optimised for production of amino acids in a bioreactor system. Details of the process are reported in chapter 6.

CHAPTER 6

MODELING THE HYDANTOINASE REACTION AND OPTIMISING PRODUCTION OF AMINO ACIDS IN CONTINUOUSLY OPERATED PACKED-BED BIOREACTORS

6.1 INTRODUCTION

6.1.1 Enzyme Kinetics

In the work reported in the previous chapter, a suitable immobilisation matrix was chosen for the stabilisation of the hydantoin-hydrolysing enzymes of RUKM3s and the operating conditions of the biocatalyst were established. Immobilisation conditions were also optimised for a biocatalyst based on the hydantoin hydrolysing activity of RUOR-PN1, a mutant strain of *Agrobacterium tumefaciens* that has a relatively high NCAAH activity. The possibility of designing and using two reactors in series to produce amino acids from the two biocatalysts represents an attractive proposition. In order to optimise such a system for maximum productivity with minimum experimental effort, a mathematical model of the hydantoinase reaction was developed. However, it is impossible to optimise bioprocesses on the basis of theoretical assumptions alone (Berkholz *et al.,* 2000). Therefore, in this study, empirical experimental data on the catalytic activities of the biocatalysts was used in addition to model-based experimental design.

An important aspect of the development of biotechnological processes is the construction of mathematical models of the behaviour of real systems. This is because optimisation of all the possible variables by experimentation can take a long time and involve a lot of costs. The validity of the model (and its assumptions) for the system can be evaluated by comparison with empirical data. The advantage of the model is in that, if valid for the system, it can be used in a predictive sense to cut down on experimental work. In general one starts with formulation of a verbal model of a process, which is then translated into a mathematical expression. However, in bioprocess engineering, reality is often too complex to be modelled in all its intricacies, with the result that the model obtained may become too complex and scarcely easier to handle than reality itself. Therefore a reduction in the complexity of reality is necessary (Roels, 1983). In this chapter, the model developed was simplified by making assumptions about the bioreactor

environment. In the model it is assumed that the reactor volume and the volumetric flow rate, temperature, pH and yields are constant. It is also assumed that the biocatalyst does not disintegrate during the processing period.

Modelling enzyme kinetics starts with formulating unique mathematical expressions which relate expected changes in the reality of a system with time or with the concentrations of components such as substrate, product and enzyme. This is followed by measurements of actual or estimated experimental data, manipulation of the data to derive values of parameters to compare with the model expectations, evaluation of the extent of deviations from the expected and confirmation of the validity or otherwise of the model.

Although enzyme kinetics investigations are mainly based on non-linear models, most common enzyme kinetic rate laws can be transformed to linear models. Estimations of initial-rate data are used to obtain kinetic constants for enzyme characterization. The experimental procedure consists of measuring the concentration of the substrate or product over discrete time intervals in a stirred-tank reactor under controlled reaction conditions. These discrete concentration/time data have to be transformed into an initial rate at the beginning of the experiment. If the concentration/time or conversion/time data show a strict linear dependence, an initial rate may be obtained by a simple linear regression of a straight line. The initial rate should be obtained by taking into account the enzyme concentration and the initial substrate concentration. If there are uncertainties with respect to the beginning of the reaction due to mixing or other time-consuming operations, the model should account for the time delay (Shuler and Kargi, 1992).

Where straight lines are not observed, and the observed data shows curved lines with decreasing slopes, initial rates must be obtained by mathematical means which allow for extrapolation to time zero. Departure from linearity can arise from physical causes such as lag phases due to toxic or deactivating impurities, subunit association or dissociation and limitations in the response of analytical devises. Non-linearity in the time dependence of product formation is most often due to the change in the concentration of reactants. Severe substrate inhibition may yield concave time dependence, whereas product inhibition may give a convex profile. Mathematical models for reproducing non-linear time dependence of product formation must have a realistic finite derivative at time zero. This can be achieved by linear regression (Shuler and Kargi, 1992).

Earlier experiments (Chapter 3) evaluated the dependence of activities of RUKM3s hydantoin hydrolysing enzymes on substrate concentration. It was found that the hydantoinase and NCAAH activities increased with initial substrate concentrations for both enzymes, and that at higher concentrations the rate of product formation was less affected by increases in substrate concentration. The ideal concentrations of substrate for the assay of amino acids produced by these enzymes were therefore chosen to be 25 mM for NCG and 50 mM for hydantoin as substrates.

In this study data was generated on the initial reaction rates and production rates of the hydantoinase and NCAAH enzymes of RUKM3s and RUOR-PN1 immobilised biocatalysts using stirred-tank bioreactors. The data was used to predict the individual and combined productivity of the two biocatalysts in continuously operated fixed-bed reactors and to test the predictions by evaluation of empirical findings. The hydantoinase and NCAAH activities of the RUKM3s-Eupergit® C biocatalyst were compared with those of the second biocatalyst formulated from the hydantoin hydrolysing enzymes of *Agrobacterium tumefaciens* strain RUOR-PN1, which has higher NCAAH and lower hydantoinase activity than RUKM3s. The two biocatalysts were used separately and then combined in batch and continuously operated bioreactor systems, and data from their performance was used to develop a model and then test it as a model of the biocatalytic production of amino acids.

Experiments were conducted to obtain the rates at which the substrates, hydantoin and NCG, are converted to NCG and glycine respectively. For two organisms with differing activities of the hydantoinase and NCAAH, and therefore differing rates of production of both NCG and glycine, it was suggested in Section 5.3.4.1 that a combined reactor system could be set-up for optimal production of the intermediate, NCG and the final product, glycine. The optimisation was based on the assumption that productivity is a function of substrate concentration [S] and the concentration of enzyme, [E] in an enzyme-substrate system. A second assumption made in the optimisation was that the bioconversion to amino acids follows first order rate kinetics, and it was expected that the amount of biocatalyst can be increased linearly to match desired yields by altering proportions of the different biocatalysts.

6.1.2 Modelling the hydantoinase reaction

On the basis of existing knowledge of the reactions that produce amino acids from hydantoin and *N*-carbamyl amino acid, the following theoretical model of the hydantoinase or carboxylic acid amide hydrolysis reaction was developed.

The **hydantoin hydrolysis** reaction is represented by the general equation:

$$\text{Hydantoin} \longrightarrow \text{NCG} \longrightarrow \text{Glycine} \qquad (1)$$

For a reactor based on bioconversions by the RUKM3s-Eupergit® C biocatalyst:

If the rate of conversion of Hydantoin to NCG and Glycine is represented by the equation:

$$-\frac{dH}{dt} = \frac{dNCG}{dt} + \frac{dG}{dt} \qquad (2)$$

then, the rate of production of NCG from hydantoin, $\mathbf{r_1}$ is represented by:

$$\mathbf{r_1} = \frac{dNCG}{dt} = -\frac{dH}{dt} - \frac{dG}{dt} \qquad (3)$$

and, if the rate of conversion of NCG to glycine is represented by:

$$\frac{-dNCG}{dt} = \frac{dG}{dt} \qquad (4)$$

then, the rate of formation of glycine $\mathbf{r_2}$ is represented by:

$$\mathbf{r_2} = \frac{dG}{dt} = \frac{-dNCG}{dt} \qquad (5)$$

For a second reactor based on bioconversions by the RUOR-PN1-Eupergit® C biocatalyst, the reactions that produce NCG and glycine would be represented by equations 2, 3, 4 and 5, but the corresponding rates of production for NCG and glycine would be $\mathbf{r_3}$ and $\mathbf{r_4}$ respectively.

In both reactors:

$$-\frac{dH}{dt} = \frac{dNCG}{dt} + \frac{dG}{dt}$$

and $$\frac{-dNCG}{dt} = \frac{dG}{dt}$$

However, not all the NCG formed in each reactor is necessarily consumed in the production of glycine. Therefore, the rates of production and consumption of NCG from the system are not equal:

$$\left[\frac{dNCG}{dt}\right]_1 \neq -\left[\frac{dNCG}{dt}\right]_2 \qquad (6)$$

For a combined continuously operated packed-bed bioreactor system using RUKM3s-Eupergit® C and RUOR-PN1-Eupergit® C biocatalysts, the overall rate of production of glycine, $\mathbf{r_g}$ expected becomes:

NCG ⟶ Glycine

$$\mathbf{r_g} = \mathbf{r_2} + \mathbf{r_4} \qquad (7)$$

The overall rate of production of NCG, $\mathbf{r_{NCG}}$ is total of all the NCG produced including that subsequently converted to glycine. Hence the expected total amount of NCG produced is essentially the total amount of hydantoin converted, and is a sum of all the rates represented by:

Hydantoin ⟶ NCG

$$\mathbf{r_{NCG}} = \mathbf{r_1} + \mathbf{r_2} + \mathbf{r_3} + \mathbf{r_4} \qquad (8)$$

For each reactor, the experimental value of the total rate of production of NCG is the sum of the NCG and glycine produced from using hydantoin as a substrate. The experimental value of the rate of production of glycine is the rate of utilisation of NCG as a substrate. From the

experimental data, the expected rates of overall production of NCG and glycine can be calculated. On the basis of the productivity of the two biocatalysts when used separately in single bioreactors, the two can be combined in a dual bioreactor system, where the optimal amounts of each biocatalyst required to maximise the yield of glycine have been determined.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Hydantoin, *N*-carbamylglycine, glycine and all other chemicals used in the biocatalytic reactions were obtained from the same sources as reported in previous chapters.

6.2.2 Determination of initial rates for non-immobilised RUKM3s enzymes

Experiments were conducted in batch cultures of 5 mL to determine the initial reaction rates of the hydantoinase and NCAAH in RUKM3s crude extract. 2.5 mL substrate was reacted with 2.5 mL of crude extract prepared as in the last chapter. Triplicate sets of reactions were set to end at 5 min intervals from 0 to 30 min. The rates of formation of the products NCG and glycine by the RUKM3s crude extract were determined from the product yields after 0, 5, 10, 15, 20, 25, and 30 min.

6.2.3 Rates of substrate conversion for RUKM3s- and RUOR-PN1- Eupergit® C biocatalysts in batch reactions

Experiments to determine the productivity of RUKM3s and RUORPN1- Eupergit® C biocatalysts, prepared as described in the last chapter, were conducted in batch reactions of 2.5 mL substrate, 2 g biocatalyst and 0.5 mL potassium phosphate buffer (0.1 M, pH 8). Reactions based on sets of triplicate hydantoin and NCG substrates were set to end at 5-min (and later 30 min) intervals from 0 to 90 min. The rates of formation of the products NCG and glycine by the RUKM3s and RUOR-PN1-Eupergit® C biocatalysts were determined from the product yields at 0, 5, 10, 15, 20, 25, 30, 60 and 90 min.

6.2.4 Yields of separate RUKM3s- and RUOR-PN1- Eupergit® C biocatalysts in continuously operated packed-bed bioreactors

The second set of experiments to determine the productivity of the biocatalysts was carried out in 50 mL bioreactors with continuous substrate feed. Hydantoin (50 mM or μmol/mL) was pumped at a rate of 1.5 mL/min from a 500 mL reservoir through a reactor containing 20 g RUKM3s-Eupergit® C biocatalyst in a 50 mL volume. Samples of 90 mL were collected from the outlet stream on an hourly basis for 5h and stored at 4 °C until assayed for concentrations of NCG and glycine. Trichloroacetic acid (TCA) was added to the sample collection bottles (to a final concentration of 4%) to stop the reactions and prevent further biocatalytic activity by leaked enzymes. The results were used to determine the estimated rate of hydantoin utilisation for the RUKM3s-Eupergit ® C biocatalyst, $\mathbf{r_1}$ (mM/h or μmol/mL/h).

NCG (25 mM or μmol/mL) was pumped at a rate of 1.5 mL/min from a 500 mL reservoir through a reactor containing 20 g RUKM3s-Eupergit® C biocatalyst in a 50 mL volume. Samples of 90 mL were collected from the outlet stream on an hourly basis for 5 h and stored at 4 °C until assayed for concentration of glycine. The results were used to determine the rate of glycine production for the RUKM3s, $\mathbf{r_2}$ (μmol/mL/h or mM/h).

Hydantoin (50 mM) was pumped at a rate of 1.5 mL/min from a 500 mL reservoir through a reactor containing 20 g RUOR-PN1-Eupergit ® C biocatalyst in a 50 mL volume. Samples of 90 mL were collected from the outlet stream on an hourly basis for 5 h and stored at 4 °C until assayed for concentrations of NCG and glycine. The results were used to determine the rate of hydantoin utilisation for the RUOR-PN1-Eupergit ® C biocatalyst, $\mathbf{r_3}$ (mM/h).

NCG (25mM) was pumped at a rate of 1.5 mL/min from a 500 mL reservoir through a reactor containing 20 g RUORPN1-Eupergit ® C biocatalyst in a 50 mL volume. Samples of 90 mL were collected from the outlet stream on an hourly basis for 5 h and stored at 4 °C until assayed for concentration of glycine produced. The results were used to determine the rate of glycine production for the RUOR-PN1-Eupergit® C biocatalyst, $\mathbf{r_4}$ in (mM/h).

6.2.5 Yields of combined RUKM3s- and RUOR-PN1- Eupergit® C biocatalysts in continuously-operated packed-bed bioreactors

A combination of two reactors (Fig. 6.1) separately containing the two biocatalysts was assembled and used to convert hydantoin and NCG to glycine. Hydantoin (50 µmol/mL or mM) was pumped at a rate of 1.5 mL/min from a 500 mL reservoir through two reactors containing 20g RUKM3s-Eupergit® C in the first bioreactor and 20 g RUOR-PN1-Eupergit ® C in the second bioreactor in 50 mL volumes. Samples of 90 mL were collected from the outlet stream on an hourly basis for 5 h and stored at 4 °C until assayed for concentrations of NCG and glycine produced. The reaction was stopped by addition of TCA to a final concentration of 4%. The results were used to determine the rate of total NCG production, r_{NCG} for the combined bioreactor system in µmol/mL/h.

NCG (25mM or µmol/mL) was pumped at a rate of 1.5 mL/min from a 500 ml reservoir through a reactor containing 20g RUKM3s-Eupergit ® C in the first bioreactor and 20 g RUOR-PN1-Eupergit® C in the second bioreactor in 50 mL volumes. Samples of 90 mL were collected from the outlet stream on an hourly basis for 5 h and stored at 4 °C until assayed for concentration of glycine produced. The results were used to determine the total rate of glycine production, r_G for the combined bioreactor system.

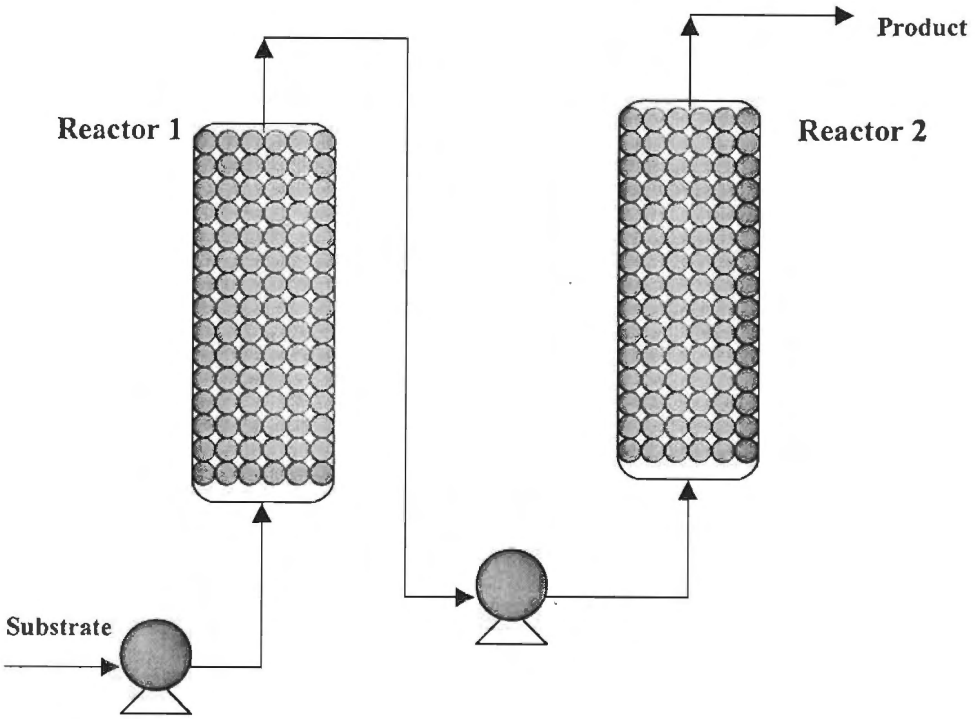


Fig. 6.1 Dual-column bioreactor system used to produce amino acids from hydantoin substrates hydrolysed by RUKM3s and RUOR-PN1 enzymes covalently immobilised in oxirane acrylic beads (Eupergit ® C)

6.3 RESULTS AND DISCUSSION

6.3.1 Initial rates of reaction by RUKM3s crude extract in batch reaction

The starting point in the investigation of the biocatalytic activity was determination of product yields of the enzymes in their free state. The initial rates of substrate conversion by RUKM3s hydantoin hydrolysing enzymes were determined using crude extract (Fig. 6.2 and 6.3). To stabilise the enzymes for storage and reuse, immobilisation is used. To that end, the yields of the enzymes of RUKM3s (Fig. 6.4 and 6.5) and RUOR-PN1 (Fig. 6.6 and 6.7) covalently immobilised in Eupergit® C were evaluated. The aim of these experiments was to compare the productivity of the enzymes in their free and immobilised forms, as well as to compare the

productivity of RUKM3s- and RUOR-PN1- Eupergit® C biocatalysts. The experimental data for the two immobilised biocatalysts was used to determine an ideal combination for the construction of a bioreactor system for the highest yield of amino acids.

In Figs. 6.2 and 6.3, it was found that the rate of NCG production was 0.291 µmol/mL/min and the rate of production of glycine was 0.037µmol/mL/min for the free crude extract of RUKM3s. The productivity of the immobilised RUKM3s hydantoinase was less than that of the free crude extract by 25 %. In the immobilised form, the NCAAH produced glycine at a rate 35% higher than in the free crude extract.

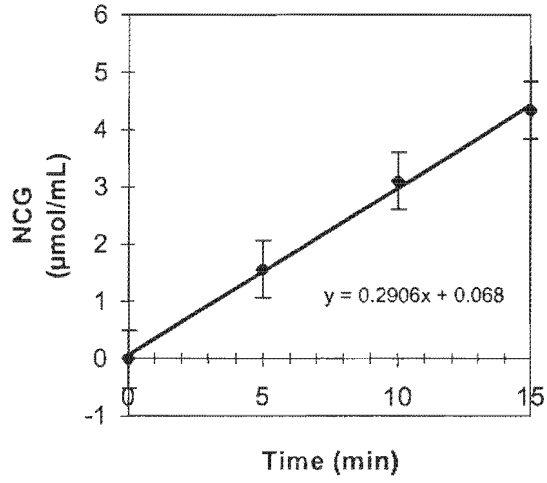


Fig. 6.2 Rate of NCG production by non-immobilised RUKM3s crude extract

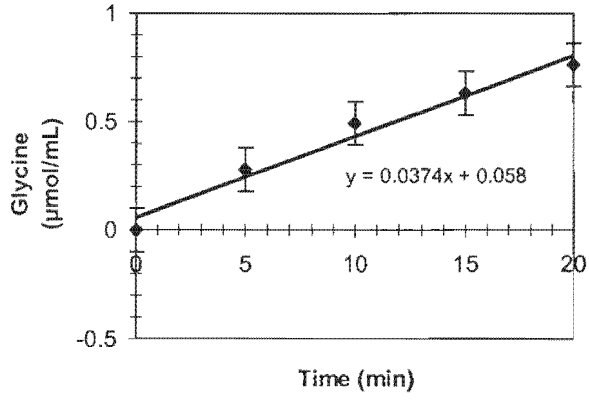


Fig. 6.3 Rate of glycine production by non-immobilised RUKM3s crude extract

6.3.2 Rates of conversion by RUKM3s and RUOR-PN1 Eupergit ® C biocatalysts in batch reactions

A comparison of the separate productivities of the covalently immobilised biocatalysts of RUKM3s and RUOR-PN1 (Figs. 6.4-6.7) was made. The results, summarised in Table 6.1, revealed that while the NCG production of immobilised RUKM3s (0.182 μmol/mL/min) is almost twice that of immobilised RUOR-PN1 (0.102 μmol/mL/min), the glycine production of immobilised RUKM3s (0.042 μmol/mL/min) is less than that of immobilised RUOR-PN1 (0.064 μmol/mL/min).

Table 6.1 Initial rates of product formation by free and immobilised RUKM3s and immobilised RUOR-PN1 in batch reactions

Biocatalyst	Enzyme	Productivity (μmol/mL/min)	
RUKM3s- Free	Hydantoinase	0.291	
	NCAAH	0.037	
RUKM3s-Eupergit ® C	Hydantoinase	0.182	**(r_1)**
	NCAAH	0.042	**(r_2)**
RUOR-PN1-Eupergit ® C	Hydantoinase	0.102	**(r_3)**
	NCAAH	0.064	**(r_4)**

These experimental data in Table 6.1 were extrapolated to determine hourly production (Table 6.2), and on its basis, the expected (model) values of initial rates of production of NCG and glycine for the two reactors in μmol/mL/h were calculated. The results in Table 6.2 are comparable to those obtained experimentally over 3 h in earlier studies (chapter 3). This was done because the larger volumes used in industrial scale bioreactors require sustained productivity over hours rather than minutes.

Table 6.2: Initial productivity rates of RUKM3s-Eupergit C and RUOR-PN1-Euergit® C biocatalysts in separate batch experiments over 30 min

Reaction		Rate (μmol/mL/hr)
KM3s: Hyd ⟶ NCG	r_1	**10.9**
KM3s: NCG ⟶ Gly	r_2	**2.5**
PN1: Hyd ⟶ NCG	r_3	**6.1**
PN1: NCG ⟶ Gly	r_4	**3.9**

The expected combined rate of glycine production, r_G represented by equation (11) is

$$r_G = r_2 + r_4 = 6.4 \text{ μmol/mL/h}$$

The expected combined rate of production of NCG, r_{NCG} as represented by equation (12) is:

$$r_{NCG} = r_1 + r_2 + r_3 + r_4 = 23.4 \text{ μmol/mL/h}$$

In Fig 6.10, the rate of NCG production of the combined system bioreactor system during the first hour was recorded as 22.5 μmol/mL. This experimental value is only slightly (by 0.9 μmol/mL) below the expected (model) value of 23.4 μmol/mL calculated above. The experimental value of glycine produced in the first hour in the combined bioreactor (Fig 6.11) was 8.3 μmol/mL. This is higher than the expected (model) value of 6.4 μmol/mL glycine calculated in Table 6.2 by 1.9 μmol/mL (29.7%). However, taking into account the margin of error for the experimental results of *each* bioreactor (10%) and that of the two-reactor system (20%), the experimental value was not much higher than the expected value. The model of the hydantoin hydrolysis proposed in this work is therefore validated empirically.

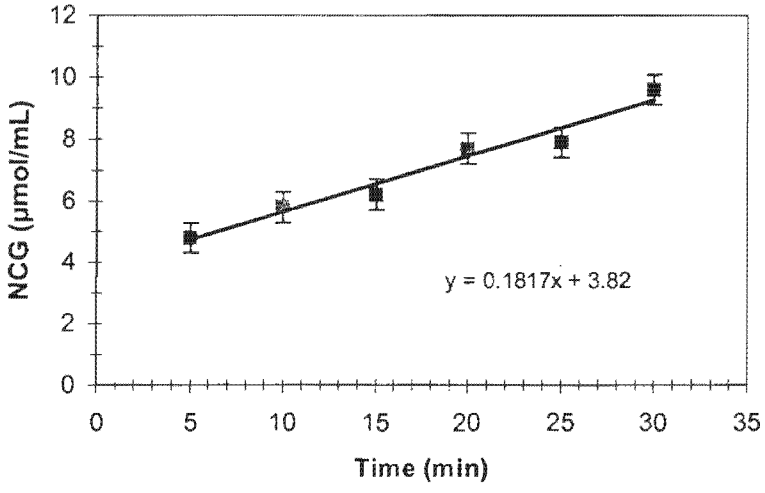


Fig. 6.4 Rate of production of NCG by RUKM3s-Eupergit ® C biocatalyst

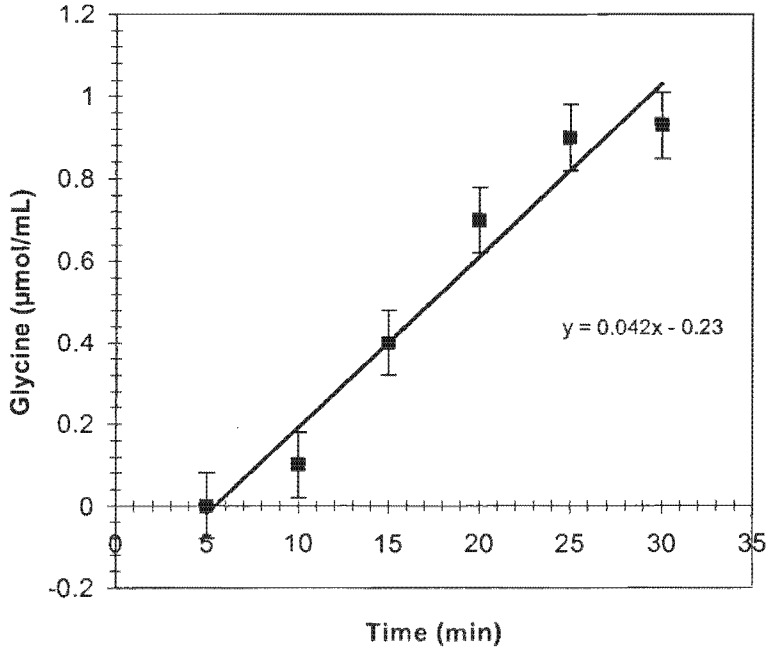


Fig. 6.5 Rate of production of glycine by RUKM3s-Eupergit ® C biocatalyst

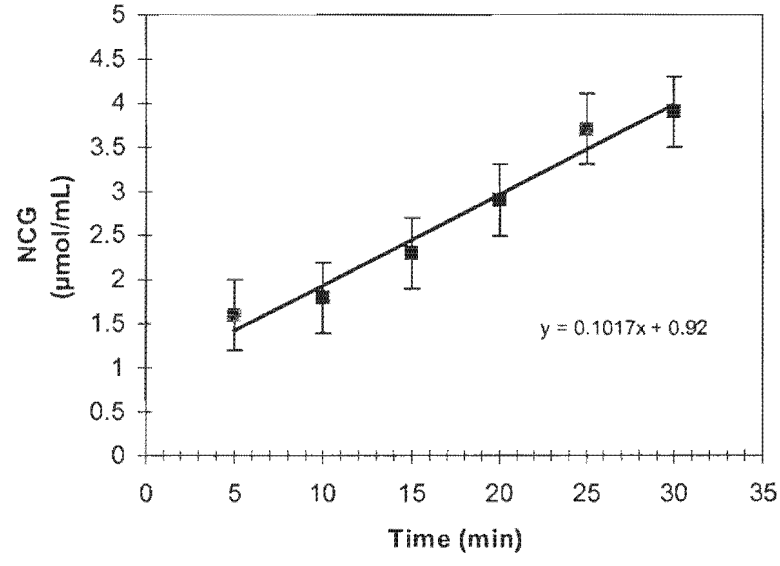


Fig. 6.6 Rate of production of NCG by RUOR-PN1-Eupergit ® C biocatalyst (reaction conducted in batch mode)

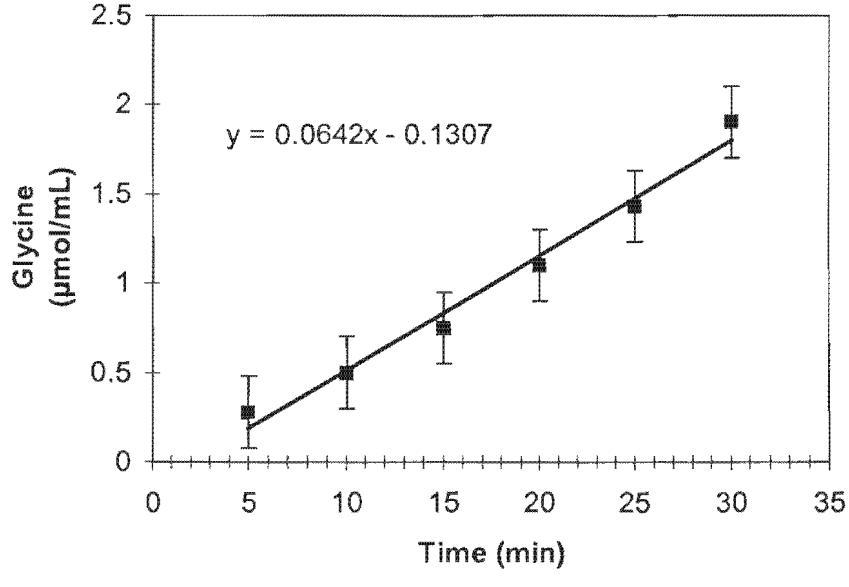


Fig. 6.7 Rate of Production of glycine by RUOR-PN1-Eupergit ® C biocatalyst (reaction conducted in batch mode)

6.3.3 Yields achieved in separately operated RUKM3s and RUOR-PN1-Eupergit® C biocatalysts in continuously operated packed-bed reactors

The yields of NCG (Fig. 6.8) and glycine (Fig. 6.9) for hourly samples collected from separate reactors containing RUKM3s and RUOR-PN1 biocatalysts were monitored over five hours and recorded for later comparison with the productivity of the combined bioreactor system. The yields from the combined bioreactor system are shown for NCG (Fig. 6.10) and glycine (Fig. 6.11).

A summary of the results comparing the productivity of the biocatalysts in batch and in continuous reactors, as well as for single and combined reactors is presented in Table 6.3. The productivity of the RUKM3s-Eupergit® C biocatalyst in batch reactions over 5 h was 28.6 µmol/mL NCG and 5.42 µmol/mL glycine. In a continuously operated packed-bed bioreactor, the RUKM3s-Eupergit® C biocatalyst produced more NCG (38.8 µmol/mL) and glycine (10.9 µmol/mL) over 5 h. The RUOR-PN1-Eupergit® C biocatalyst produces significantly less NCG in the continuously operated reactor than in batch reaction, and almost the same amount of glycine in both reactors.

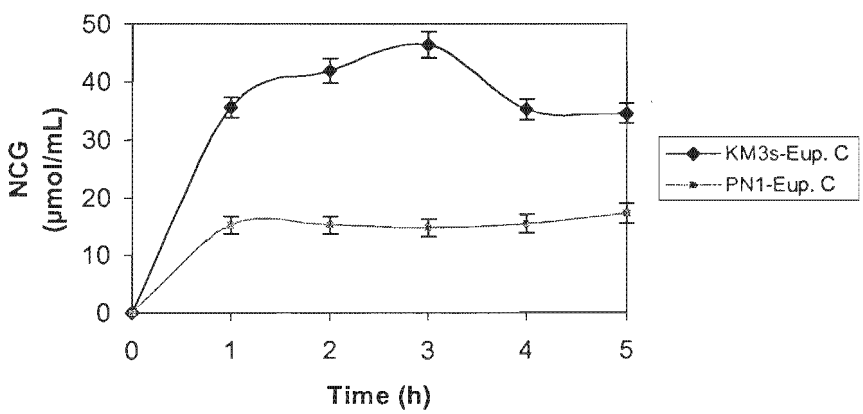


Fig. 6.8 Comparison of NCG production by RUKM3s and RUOR-PN1- Eupergit ®C biocatalysts in two separate continuously operated packed-bed bioreactors

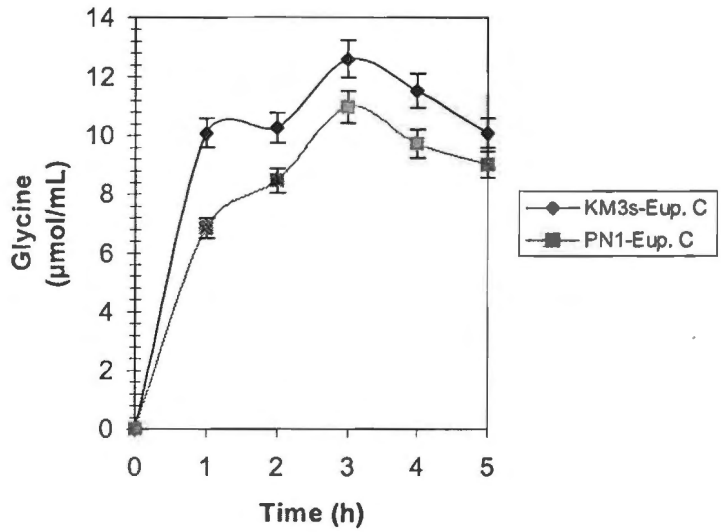


Fig. 6.9 Comparison of glycine production by RUKM3s and RUOR-PN1- Eupergit ® C bioctalysts in two separate continuously operated packed-bed bioreactors

6.3.4 Yield by a continuously operated packed-bed bioreactor system combining RUKM3s- and RUOR-PN1-Eupergit ® C biocatalysts.

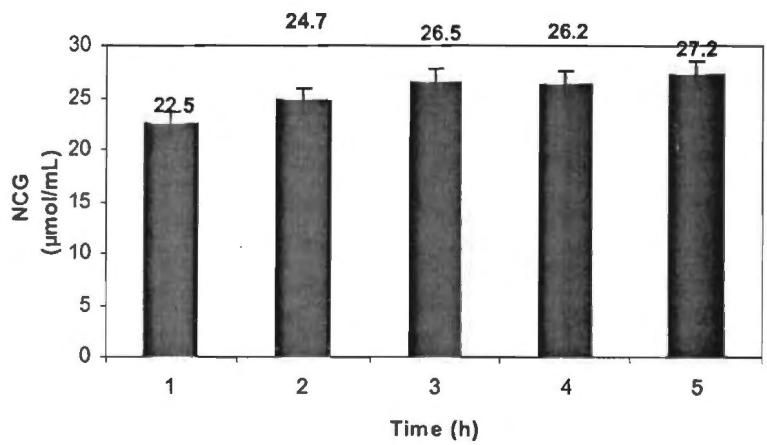


Fig. 6.10 Production of NCG by an RUKM3s- and RUOR-PN1-Eupergit ® C combined bioreactor system

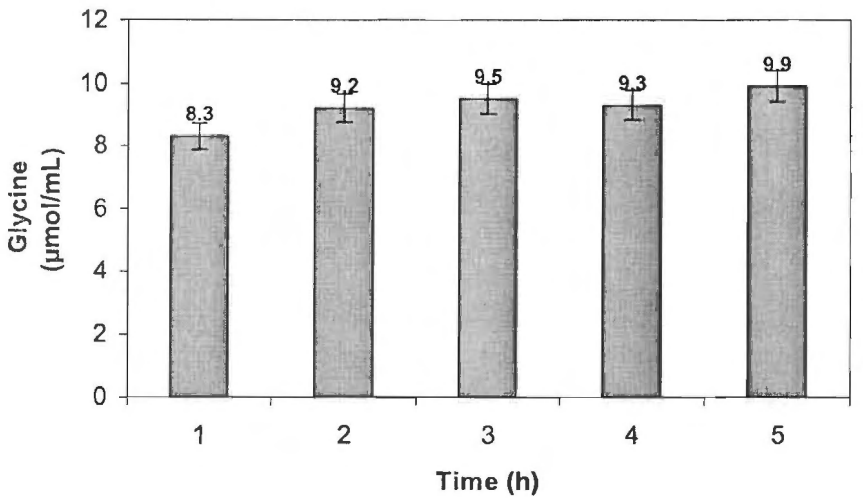


Fig. 6.11 Production of Glycine by an RUKM3s- and RUOR-PN1-Eupergit®C combined bioreactor system

A comparison of the productivity data for the biocatalysts from RUKM3s and RUOR-PN1 (Table 6.3) further showed that when the two biocatalysts were used in a combined bioreactor system, the total output 45.8 µmol/mL NCG (from 50 mM hydantoin) and 16.6 µmol/mL glycine (from 25 mM NCG) after five hours. The total conversion of hydantoin to NCG for the combined bioreactor system was 91 %, and the total conversion of NCG to glycine was 66.5 % of theoretical yield calculated on the basis of total feed. This could be increased to 100% yield, by reducing the substrate concentration, increasing the amount of biocatalyst or increasing the residence time.

The observed yield of NCG from the first bioreactor was 77.5 %. Assuming that the amount of yield is proportional to the amount of biocatalyst used, if the amount of RUKM3s-Eupergit®C was increased by a factor of 1.3 (to become 26g for the 500 mL substrate), the yield from the first bioreactor could be increased to 50 mM NCG (or 100%). Of that amount, if 43.6 % is converted to glycine in the first bioreactor, the remaining 56.4 % or 28.2 mM NCG could be converted to glycine by the RUOR-PN1-Eupergit®C biocatalyst in the second bioreactor. The observed yield of glycine from the second bioreactor was 36.0%. Assuming the amount of yield is proportional to the amount of biocatalyst used, if the amount of RUOR-PN1-Eupergit®C

biocatalyst was increased by a factor of approximately 3 (to become 60 g for 500 mL substrate), the yield of glycine after conversion in the second bioreactor would be 100% for the system. Additional experiments to confirm these assumptions about the amount of biocatalyst, and model the effects of residence time and substrate concentration on total output are still ongoing.

Table 6.3: Yields of products by RUKM3s and RUOR-PN1-Eupergit ® C biocatalysts after 5 h operation

Biocatalyst	Substrate	Theoretical Yield (μmol/mL)	Total Product (μmol/mL)		% Conversion	
			Batch	Continuous	Batch	Continuous
RUKM3s-Eupergit®C	Hydantoin	50	28.6	38.8	57.2	77.5
	NCG	25	5.42	10.9	21.7	43.6
RUOR-PN1-Eupergit® C	Hydantoin	50	22.6	15.6	45.2	31.2
	NCG	25	10.0	9.0	40	36.0
Combined RUOR-PN1 & RUKM3s	Hydantoin	50	-	45.8	-	91.5
	NCG	25	-	16.6	-	66.5

6.4 CONCLUSIONS

Hydantoinase levels in most microbial systems are lower than NCAAH levels. This means that, the rate of the first step of the hydantoinase reaction is generally faster than the rate of the second step. In some of the reports cited in literature, it was been found that the ratio of hydantoinase to *N*-carbamoylase can be as much as 1: 3 on a weight basis (Kim and Kim, 1995). In their study, Kim and Kim (1995) went on to optimise the ratio of the two enzymes to minimise accumulation of the intermediate product (*N*-carbamyl-D-*p*-hydroxyphenylglycine) in the enzymatic

production of D-hydroxyphenylglycine. In this study, a model of the hydantoinase reaction to improve substrate conversion based on the rates of substrate conversion for two different enzyme systems (RUKM3s and RUOR-PN1) was proposed, and used to calculate the expected rates of production of NCG and glycine in a continuous flow dual column bioreactor system. The modelled values closely approximated the experimental data obtained for the combined bioreactor system. Therefore the model of the hydantoinase reaction is validated by empirical data. The model can be used to increase the yields of enantiomeric products if chiral substrates and complementary, stereospecific enzyme systems are used in the bioreactor system.

The hydantoinase activity of RUKM3s-Eupergit ® C biocatalyst was lower than that of the free crude extract while the NCAAH activity of the immobilised form was 35% higher than that of the free crude extract. This leads to the conclusion that covalent immobilisation stabilises the NCAAH in RUKM3s and slightly reduces the activity of the hydantoinase.

The productivity of the RUKM3s-Eupergit® C biocatalyst in batch reactions over 5 h is 28.6 mM NCG and 5.42 mM glycine. The lower glycine yield from RUKM3s may be due to product inhibition of the NCAAH of RUKM3s. In the continuously operated packed-bed bioreactor over the same time (5 h), the RUKM3s-Eupergit® C biocatalyst produced more NCG (38.8 mM) and glycine (10.9 mM) than in the batch mode. The RUOR-PN1-Eupergit® C biocatalyst produced significantly more glycine (10.0 mM) than the RUKM3s-Eupergit C biocatalyst in batch reactions over 5 h. The yields of the RUOR-PN1-Eupergit® C were not significantly affected by operation in the continuous mode.

The RUKM3s biocatalyst showed higher hydantoinase activity than the RUOR-PN1 biocatalyst, and the RUOR-PN1 biocatalyst had a higher NCAAH activity than the RUKM3s biocatalyst. The total conversion of hydantoin to NCG for the combined bioreactor system was 91 %, and the total conversion of NCG to glycine was 66.5 % of theoretical yield calculated on the basis of total feed. As expected, this was higher than the yields from each bioreactor operating alone. The system could be optimised to achieve 100% conversion by use of approximately 26 g RUKM3s-Eupergit® C biocatalyst, and 60 g RUOR-PN1-Eupergit C biocatalyst per 500 mL substrate flowing through the bioreactors over 5 hours.

Other researchers have managed to achieve 100% conversion by using substrate concentrations as low as 1% (w/v) (Fan and Lee, 2001). However, this is not useful for industrial application where high substrate and hence product yields are required. In the present work we have demonstrated that by manipulating the relative amounts of the two biocatalysts one could achieve a 100% conversion system without lowering the substrate concentration. Using 26g of the RUKM3s-Eupergit®C biocatalyst and 60 g of the RUOR-PN1-Eupergit®C biocatalyst for 500 mL substrate (hydantoin), the yield of glycine after conversion in the second bioreactor would be 100% for the system. To the best of our knowledge, this approach has not been reported previously. In future work, the theoretical model of the hydantoinase reaction proposed in this chapter could be improved by extending the model from the 'reactions' to a simulation of scaled-up conditions. This would ascertain the industrial applicability of the system.

CHAPTER 7
GENERAL DISCUSSION

The major aim of the work reported in this thesis was to develop and evaluate a stabilised biocatalyst based on the hydantoin-hydrolysing enzymes of *Pseudomonas putida* RUKM3s, which could be used industrially to produce amino acids from 5-monosubstituted hydantoin substrates. Experiments were devised to optimise conditions for the production of RUKM3s biomass with high levels of hydantoin-hydrolysing activity, and to evaluate techniques of protein extraction, enzyme isolation and purification. The purified enzymes were characterised to establish a basis for comparison with hydantoin-hydrolysing enzymes isolated by other research groups from other microbial, plant and animal sources.

The application of a microbial system in the development of a biocatalyst is dependent on the successful screening and isolation of strains, and the cultivation of large quantities of biomass with high levels of enzyme activity. The screening and isolation of RUKM3s and other hydantoin-hydrolysing strains has been subject of earlier studies (Phehane, 1998; Hartley *et al.*, 1998; Buchanan *et al.*, 2001). In other work, the amide bond hydrolysis reaction by enzymes in the RU-series of hydantoin-hydrolysing strains was studied for various hydantoin substrates (Skepu, 2000). In the present work physical and chemical conditions were optimised for the cultivation of RUKM3s biomass and the synthesis of the enzymes.

It was found that at a laboratory scale, significant quantities of RUKM3s biomass could be obtained within 24 h from nutrient broth (NB) and from a specially-formulated medium for *Pseudomonas putida* (PP2 medium). Comparison of growth in NB and in PP2 media showed that PP2 provided higher concentration (11.6 g /L) than NB (3.3 g/L) on the basis of dry cell weight. The enzymes of RUKM3s are known to be inducible and hydantoin minimal medium (HMM) has been found to provide the necessary induction of enzyme expression (Hartley *et al.*, 1998). However, growth in HMM is slow (3-4 days), compared to growth in nutrient broth and in PP2 which takes 18-24 h. Nutrient broth supplemented with 0.1 % hydantoin (HNB) and PP2 media have been shown to induce enzyme synthesis. It was found that the addition of such inducer-level concentrations of hydantoin did not affect the growth characteristics or the final biomass concentration.

The optimal fermentation conditions for yield of RUKM3s biomass were found to be 28 °C, pH 7 and 25-40 % relative dissolved oxygen (DO_2) saturation. Analysis of the oxygen utilisation rate (OUR) and the carbon dioxide emission rate (CER), combined with the growth curves obtained for RUKM3s in NB and PP2 media indicated that the exponential phase of growth was complete and the deceleration phase started after about 8 h of cultivation.

When the influence of growth conditions on ultimate enzyme activity were evaluated, it was found that biomass grown at 28 °C had the highest hydantoinase activity. NCAAH activity did not demonstrate dependence on the growth temperature of the biomass. Biomass grown at pH 7.5 had the highest hydantoinase activity, but the NCAAH activity was not dependent on the pH during growth. Enzyme activities did not show dependence on the DO levels during growth. The same amount of enzyme activity was recorded per unit biomass above 10 % DO.

Grown under optimal conditions, RUKM3s cells gave the highest hydantoinase activity after approximately 16 h of growth and the highest NCAAH activity after 10 h of growth. The enzyme activity of RUKM3s cells was not affected negatively by storage of the wet cells at -20 °C. Lyophilisation of the cells caused 20 and 60 % reductions in hydantoinase and NCAAH activities respectively. Freeze-dried whole cells stored at -20 °C retained sufficient levels of viability to be revived by rehydration and incubation in nutrient broth during the three-week testing period.

The evaluation of techniques of protein extraction revealed that sonication was the most efficient among the methods tested in terms of the amount of protein extracted and the amount of hydantoinase activity apparent in the cell-free supernatant after treatment. However, French pressing was the better method for the isolation of high NCAAH from extracted protein.

The induction of the synthesis or expression of hydantoinase and NCAAH were studied further. It was found that NCG in growth media decreased NCAAH production, and had a very small inductive effect on hydantoinase. Hydantoin hydrolysing enzymes are known to be dependent on metals. The effects of additions of various divalent ions to growth media were studied. It was found that the addition of manganese to growth medium induced higher enzyme activity, while magnesium and zinc inhibited the activities of the two enzymes.

The optimal temperature for the activity of both hydantoinase and NCAAH during biocatalytic reaction was found to be 40 °C. The optimal pH for NCAAH activity during biocatalytic reaction was found to be 9, and the optimal pH for hydantoinase was 9-10. The amount of product from NCAAH was highest after 5 h of reaction under standard conditions. The amount of product from hydantoinase reaches its peak after 3 h of reaction.

In investigations of optimal substrate concentration, the enzyme activity increased with initial substrate concentrations for both NCAAH and hydantoinase. At higher concentrations the rate of product formation was less affected by increases in substrate concentration. In experiments to produce amino acids from various 5-monosubstituted hydantoin substrates, it was found that the yield decreased with increases in the steric size of the side groups on the hydantoin substrates. It was also shown that the NCG intermediates were more readily produced than the amino acids.

After the conditions of biomass production, enzyme induction and synthesis, and protein extraction had been optimised, various methods were used to isolate and purify the hydantoin-hydrolysing enzymes of RUKMs. It was found that ammonium sulphate at 40% saturation co-precipitated the hydantoinase and NCAAH. Some NCAAH activity was detectable in the precipitate from 60% ammonium sulphate. Fractional precipitation using acetone resulted in better discrimination between hydantoinase and NCAAH. Most of the NCAAH was precipitated by 20% acetone and most of the hydantoinase was precipitated by 60 % acetone. Cumulative precipitation using acetone co-precipitated both enzymes at 60% solvent concentration. Isoelectric precipitation of the proteins indicated that the isoelectric point of hydantoinase was at pH 6, while that of NCAAH was at pH 8.

Enzymes co-precipitated by salt or solvent have been separated and purified by other researchers combining the precipitation step with gel filtration and preparative gel electrophoresis (Moriyoshi, *et al.*, 1999). In our study, it was found that perfusive chromatography using a Sephacryl S-100 HR column eluted the two enzymes in two distinct fractions. Fractions showing enzyme activity and relatively high concentration of protein were pooled, concentrated by dialysis and freeze-dried before further characterisation. It was found that the hydantoinase was still active in the pooled fractions, while NCAAH was inactive. The NCAAH became slightly

active after the dialysis step, indicating that the enzyme's subunits possibly reassociated into the native enzyme molecule during removal of salt.

The purified enzymes were characterised in terms of molecular weight, number and size of subunits and stereoselectivity. SDS-PAGE analysis and size exclusion chromatography indicated that the NCAAH of RUKM3s is a dimer of molecular weight approximately 60 kDa, with two subunits of approximately 30 kDa each. Hydantoinase was found to be approximately 210 kDa. The characterisation of the chiral selectivity of the enzymes of RUKM3s indicated that the system had a non-selective or D-selective hydantoinase activity and an L-selective *N*-carbamoylase activity. This has important implications for the potential usefulness of the hydantoin-hydrolysing enzymes of RUKM3s in the stereoselective synthesis of L-amino acids and the required optical purity of the starting substrate. This result supports earlier work on the L-selective nature of the NCAAH of RUKM3s (Skepu, 2000). If the hydantoinase was exclusively D-selective or non-selective, the presence of L-alanine in the final product suggests that there was possibly a racemase in the system. The presence of racemases in microbial systems bearing hydantoin-hydrolysing activity has been reported on by many research groups (Rozzell and Wagner, 1992).

Stabilisation of biocatalysts by immobilisation is technically important for ease of separation of products from the biocatalyst and recovery of the biocatalyst for re-use. Among five methods of immobilisation used, it was found that covalent immobilisation in Eupergit® C provided the most suitable biocatalyst from RUKM3s enzymes. The matrix coupled with 63% of the solubilised protein and achieved yields of 28.6 μmol/mL NCG and 5.4 μmol/mL glycine when operated in batch reactions without continuous substrate feeding. The biocatalyst was re-used in 18 cycles before reaching 50% of the initial NCAAH activity, and in 28 cycles before 50% of the initial hydantoinase activity. During storage, the biocatalyst was found to lose approximately 50% of its initial NCAAH and hydantoinase activities after 2-3 weeks.

Covalent immobilisation in Eupergit® C was also used to produce a biocatalyst based on the enzymes of RUOR-PN1. The protein binding yield of RUOR-PN1 was found to be 75 %. For the RUOR-PN1-Eupergit® C biocatalyst, the yield from hydantoinase was 22.6 μmol/mL NCG and that from NCAAH was 10 μmol/mL glycine in batch reactions under standard conditions. Thus

the RUKM3s-Eupergit® C biocatalyst has higher hydantoinase activity and lower NCAAH activity than the RUOR-PN1-Eupergit® C biocatalyst. The operational and storage stabilities of the two biocatalysts are almost similar.

These two biocatalysts compare favourably with other immobilised biocatalysts reported in literature. Kim *et al.*, (1994) immobilised a strain of *Pseudomonas* sp. (isolated from soil) within polyacrylamide gel, and used it for the synthesis of D-*p*-hydroxyphenylglycine from D, L-5-substituted hydantoin. The half-life of immobilised whole cell D-hydantoinase was found to be about 50 h, which is much less than the three weeks achieved for the RUKM3s biocatalyst. The storage stability of the RUKM3s–Eupergit® C biocatalyst is therefore unique and novel. As far as we know, the immobilisation of hydantoin-hydolysing enzymes in Eupergit® C and the use of Eupergit® C –based biocatalysts in amino acid synthesis has not been reported previously.

A model of the hydantoinase reaction, based on the activity of the stabilised biocatalyst, was developed and tested by empirical studies in a bioreactor system. In the system, the benefits of high hydantoinase activity from RUKM3s were combined with the high NCAAH activity of an *Agrobacterium tumefaciens* strain, RUOR-PN1 (Hartley *et al.*, 1998; Foster *et al.*, 2002) to achieve high conversion efficiencies and enhance the overall product yield. The reaction rates of the biocatalysts from RUKM3s and RUOR-PN1 were used to model the hydantoin hydrolysis reaction, and empirical data from their performances in batch and continuously operated reactors were used to design a two-stage bioreactor system. The model of the hydantoinase reaction was used successfully to calculate expected rates of production of NCG and glycine. The modelled values closely approximated the experimental values obtained for the combined bioreactor system. Therefore the model of the hydantoinase reaction was successfully validated by empirical data.

When the two biocatalysts were used in a combined bioreactor system, it was found that the total conversion of hydantoin to NCG was 91 % and the total conversion of NCG to glycine was 66.5 %. These conversion levels could feasibly be achieved in larger scale bioreactors in an industrial process. The system could readily be optimised to achieve 100% conversion by reducing the initial concentration of substrate and hence reducing the probability of product inhibition. Further, by manipulating the amounts of the two biocatalysts in a dual column system, 100%

conversion can be achieved at higher concentrations. The system could be optimised to achieve 100% conversion by use of approximately 26 g RUKM3s-Eupergit® C biocatalyst, and 60 g RUOR-PN1-Eupergit® C biocatalyst for 500 mL substrate flowing through the bioreactors over 5 hours. The development of the model upon which this finding is based has not been reported previously, and is therefore a novel finding for the hydantoinase process.

The strain RUKM3s is a novel *Pseudomonas* sp. with high enzyme activity, and an ability to produce L-amino acids. The biocatalyst from RUKM3s therefore has potential industrial application in the stereoselective synthesis of amino acids. The productivity and stability of the immobilised RUKM3s biocatalyst is higher than that of other reported biocatalysts. The biocatalyst performed efficiently, for relatively longer periods of time in both batch and continuous reaction systems. The combination of an RUKM3s biocatalyst with an RUOR-PN1 biocatalyst in a dual column packed-bed bioreactor system achieved yields close to 100% in a novel process which could be used to improve the industrial productivity of the hydantoinase process. To the best of our knowledge, this innovation has not been reported previously.

In conclusion, this study achieved its five objectives of developing and optimising the biocatalytic reaction conditions of hydantoin-hydrolysing enzymes of RUKM3s; investigations of amino acid synthesis using a range of 5-monosubstituted hydantoins; extraction, isolation, partial-purification and characterisation of the enzymes; stabillisation of the enzymes by immobilisation and modelling of the hydantoinase reaction. The study also went beyond the above initial objectives to combine the hydantoinase and NCAAH activities of RUKM3s with those of RUOR-PN1 to produce a very efficient bioreactor system for amino acid synthesis in a way that has not been investigated previously. The evidence gathered in the above investigations supports the hypothesis of this study; that the hydantoin-hydrolysing enzymes of RUKM3s can be optimally extracted, isolated, purified and stabilised for industrial application as a biocatalyst for stereospecific production of amino acids from 5-monosubstituted hydantoin substrates.

APPENDICES

APPENDIX A
COMPOSITION OF CULTURE MEDIA

A.1 HNB medium

Nutrient broth (0.1% hydantoin) 16 g nutrient broth plus 1 g hydantoin in 1000 ml distilled deionised water. Autoclave.

A.2 *Pseudomonas putida* medium (PP2)

Citric acid	1.12 g
$MgSO_4.7H_2O$	1.96 g
KH_2PO_4	5.60 g
Yeast Extract	14.00 g (or nutrient broth at 16g/litre)
Sunflower oil	1.40 g
Hydantoin	1.40 g
Trace elements	14.00 ml
Antifoam	1.40 ml
Tap water to:	1400 ml

A.3 Hydantoin minimal medium agar

10 X M9 salts	100 mL
40% Glucose	25 mL
4% Hydantoin	250 mL
$MgCl_2$ (1M)	200 µL
$CaCl_2$ (1M)	200 µL
Trace Elements	10 mL
ddH_2O	615 mL
Agar-agar	20 g

Add agar to distilled deionised water, autoclave and cool. Add the rest of the ingredients using sterile technique. Pour into sterile plates.

A.4 Hydantoin minimal media broth

Same as HMM agar, but without the agar-agar.

A.5 Nutrient broth

16 g nutrient broth in 1000 mL distilled deionised water. Autoclave.

A.6 12% Trichloroacetic acid (TCA)

12 g TCA/ 100 mL dd water (filter)

APPENDIX B

REAGENTS

B.1 Ehrlich's reagent

10% p-aminobenzaldehyde in 6 N HCl.

B.2 Ninhydrin reagent

0.8g Ninhydrin + 0.12g hydridantin in 30 mL 2-methoxyethanol. Add 10 mL of acetic acid (4 M, pH 5.5). Prepare fresh in a brown bottle.

B.3 Bradford's reagent

Use protein dye reagent from Sigma; Catalogue No. B6919; or prepare own reagent by dissolving 100mg of coomassie blue G250 in 50 ml of 95% ethanol. The solution is then mixed with 100 mL of 85% phosphoric phosphoric acid and made up to 1 L with distilled water (Walker, 1996).

APPENDIX C

STANDARD SOLUTIONS

C.1 50 mM N-carbamyl glycine

0.5905g N-carbamyl glycine in 100 ml phosphate buffer

C.2 100 mM Hydantoin

1.0008g Hydantoin in 100 mL phosphate buffer

C.3 1mM Glycine

First prepare 10 mM Glycine by dissolving 0.07507g Glycine in 100 mL phosphate buffer. Dilute 10 ml of 10 mM Glycine to 100 mL to obtain 1 mM Glycine.

C.4 BSA standard (0.5 mg/ml)

0.05g (or 50 mg) BSA in 100 mL dd Water

C.5 Protein molecular weight markers for ND-PAGE

Product No. MW-ND-500 from Sigma, USA (14, 200 to 545, 000 Da)

C.6 Protein molecular weight markers for SDS-PAGE

Product No. SDS-6H from Sigma, USA (6500 to 205, 000 Da)

APPENDIX D

STOCK SOLUTIONS AND BUFFERS

D.1 10 X M9 salts:

Na_2HPO_4	60 g	
KH_2PO_4	30 g	
NaCl	5 g	
ddH_2O	make up to 1 litre	(Autoclave)

D.2 40% Glucose

Glucose (AR) 400 g/1000 mL ddH_2O (Autoclave)

D.3 4% Hydantoin

Hydantoin 40g/1000 mL ddH_2O (Autoclave)

D.4 Trace Elements

Boric acid	50 mg
$MnSO_4.2H_2O$	40 mg
$ZnSO_4$	40 mg
$(NH_4)Mo_2O_{24}.4H_2O$	20 mg
KI	10 mg
$CUSO_4$	4 mg

Make up to 990 mL with ddH_2O. Autoclave. Add 20mg $FeCl_3$ dissolved in 10 mL distilled deionised water, autoclaved separately.

D.5 Magnesium chloride (1M)

$MgCl_2$ 20.33 g/ 100 mL distilled deionised water , Autoclave

D.6 Calcium chloride (1 M)

$CaCl_2$	14.70 g/ 100 mL distilled deionised water. Autoclave

D.7 Potassium Phosphate buffer, (0.1M, pH 8.0)

0.2 M K_2HPO_4	34.84 g/Litre ddH_2O
0.2 M KH_2PO_4	27.2 g/Litre ddH_2O

Place + 400 mL 0.2 M KH_2PO_4 (aq) in a beaker, add 0.2 M K_2HPO_4 (aq) until pH 8.0. Autoclave.

D.8 Sodium Acetate buffer (pH 5.5; 4M)

Mass= 4 x 82.04 x (50/1000) = 16.41g for 50 mL. Dissolve 16.41g in 20 mL dd Water. Add 4 M acetic acid until pH 5.5 is reached. Make up to 50 mL with water. 4 M acetic acid: mass per 100 ml = 4 x 60.05 x (100/1000) = 24.02g. 1 litre acetic acid = 1.05 kg; therefore, 22.89 mL acetic acid gives 24.02g

APPENDIX E

PROTOCOL FOR SDS & ND POLYACRYLAMIDE GEL ELECTROPHORESIS

E.1 Reagents

1. Resolving gel buffer (1 M, pH 8.8)

   For 500 ml:

Tris	60.6g
HCl (conc.)	7.3 ml

   Dissolve in water, check pH and adjust if necessary

2. Stacking gel buffer (1M, pH 6.8)

   For 500 ml:

Tris	60.6 g
HCl (conc.)	41.0 ml

   Dissolve in water, check pH and adjust if necessary

3. 30% Acrylamide Stock

   For 250 ml:

Acrylamide	75g
bis-Acrylamide	2 g

   dissolve in 250 ml water

4. Running/bath buffer (stock solution)

   for 1000 ml:

tris	30.3 g
glycine	144.1g
SDS	10.0 g

   Dissolve in 1000 ml water

5. Running buffer (working solution)

   Dilute stock 1 in 10

6. Dissociation buffer

7. for 50 ml

SDS	5 g
Mercaptoethanol	5.0 ml

Glycerol	7.5 ml
0.2% Bromophenol blue	2.5 ml
Tris-HCl (1M, pH 6.8)	6.3 ml
distilled water	28.7 ml

8. Sample buffer (for Non-denaturing)

   For 50 ml:

   Same as for dissociation buffer, less SDS and Mercaptoethanol

9. Coomassie Staining solution

10. for 1000 ml

methanol	450 ml
glacial acetic acid	100 ml
Coomassie brilliant blue (R250)	2.0 g
Water	450 ml

11. Destain solution

Methanol	450 ml
Glacial acetic acid	70 ml
Water	480 ml
Glycerol	100 ml

E.2 Preparation of Gel

a. Plug

1. Mix in the following order:

   3 ml of 30% acrylamide stock solution

   80 µl of 10 % ammonium persulphate (APS) (Prepare fresh)

   40 µl of TEMED

2. Immediately place plug at the bottom of the gel plate with Pasteur pipette and allow to set.

b. 10% Resolving Gel

13.35 ml of acrylamide stock solution

15.0 ml of Tris-HCl buffer (pH 8.8)

9.25 ml distilled water

400 µl 10% SDS (omit and replace with water for Non-denaturing)

300 µl of 10% APS (prepare fresh)

20 µl of TEMED

-Pour resolving gel into gel plate with Pasteur pipette

-Pour a thin layer of water across the top of the gel and allow to set for approximately 30 minutes

c. 4% Stacking gel

2 ml of the acrylamide stock solution

1.9 ml Tris-HCl buffer (pH 6.8)

9.25 ml distilled water

1.0 ml of 80% glycerol (replace with water for non-denaturing)

100 µl of 10% APS (prepare fresh)

150 µl 10% SDS (replace with water for non-denaturing)

20 µl TEMED

-remove the water from the surface of the resolving gel and pour stacking gel with Pasteur pipette.

-add a 10-tooth comb and leave to set for about 20 minutes

-once set, place the gel in the base of the electrophoresis apparatus

-remove comb and fill apparatus with running buffer

E.3 Sample loading

1. ND-PAGE: In a microcentrifuge tube, mix 50 µl of enzyme solution with 50 µl solution of sample buffer dye. Load onto the PAGE gel. (about 30-50 µg protein/well).
2. SDS-PAGE. In a microcentrifuge, mix 50 µl of enzyme solution with 50 µl of dissociation buffer. Heat the solution for 5 minutes in a boiling water bath. Allow to cool to room temperature and load.
3. Boil 20 µl of molecular weight markers in dissociation buffer for 2 minutes.

E.4 Electrophoresis

Electrophorese at 100-120 V until the dye is within 1 cm of the bottom (3 hours)

E.5 Staining and Destaining

Cover the gel with coomassie staining solution for 0.5-1 hour.

Destain for 2 x 20 minutes with destain solution I.

Destain overnight with destain solution II.

Take photograph

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