Studies on the production, extraction, purification and use of hydantoinase

Ph. D. THESIS

IN

CHEMISTRY

BY

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CHEMICAL ENGINEERING DIVISION
NATIONAL CHEMICAL LABORATORY
PUNE-411008. INDIA

MAY 2003

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A

THESIS SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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CONTENTS

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"STUDIES ON THE PRODUCTION, EXTRACTION, PURIFICATION AND USE OF HYDANTOINASE"** submitted by Mr. Bhaskar G. Gaikwad was carried out by the candidate under my guidance at National Chemical Laboratory, Pune. Such material as has been obtained from other sources, has been duly acknowledged in this thesis.

Date: 16th May 2003

(Dr. B.D. Kulkarni)
Research Guide

Acknowledgement

I am sincerely and heartily obliged to Dr. B. D. Kulkarni (FNA) Deputy Director, Head, Chemical Engineering Division, for his guidance, constant support and encouragement. It would not been possible without his support.

I am deeply thankful to Mr. Sanjay Nene Head, Biochemical Engineering Group for his support and valuable suggestions throughout this research.

I am thankful to Mr. V.V. Jogdand and Miss Rita Varma for their cooperation, help and valuable suggestion during this research work.

I am thankful to Dr. R. V. Gadre and Dr. H. V. Adikane for their cooperation during this work.

I thank to other staff of Biochemical Engineering Group for their support during this research work.

I thank to Head, NCIM, Pune for providing me cultures for this study.

I thank to my family members for cooperating me and adjusting me while this work was going on.

I am thankful to Director, National Chemical Laboratory for permitting me to do this research work. I am thankful to CSIR for this opportunity.

I thank to those who have helped me directly or indirectly but by mistake not mentioned in this acknowledgement.

Bhaskar Gaikwad

Chapter 1

General Introduction

Introduction

Man's need for chemicals is fulfilled in various ways. While some chemicals are present in nature in a free state or in a derivatized form the remaining are synthesized either by a chemical or biochemical route. Chemical synthesis accounts for a large number of commercially important products such as plastics, drugs and drug intermediates, pesticides and bulk organic chemicals. Plants, animals and microorganisms synthesize many chemicals using enzymes, which are nature's catalysts.

Synthesis of chemicals using cells/enzymes is termed biosynthesis. Depending upon the method of biosynthesis such reactions are referred to as biotransformations, bioconversions or fermentations. Many chemicals can be prepared by either chemical or biochemical routes. Economy of manufacture dictates the choice of route that is preferred. Bioconversions or biotransformations generally occur under mild conditions and are safe to handle. Enzymes carry out specific reactions therefore minimizing the risk of side product formation; hence purity of product is generally assured. The biosynthetic route is used to obtain many chemicals viz.. organic and amino acids, alcohols, ketones, antibiotics, proteins and lipids etc [Gutcho (1973), Atkinson and Mavituna (1991)]. Some are listed in table 1. Besides this many types of compounds are formed by biosynthesis.

Stereoselective hydroxylation, ketoreduction reduction, isomerization, biotransformation of steroids, alkaloids and terpenoids are some examples of reactions carried out by enzymes. Synthesis of chemicals, where one or more of the steps employed use enzymes are referred to as chemo-enzymatic processes. The enzymatic step is performed either by using cells or enzyme directly. Often immobilized cells or enzyme are used for this purpose. Use of a biochemical route for preparation of chemicals is increasingly used to minimize the hazards and risks involved in chemical synthesis.

Table 1: Fermentation products

Product Category	Individual examples
Acids	acetic acid, propionic, lactic, itaconic, tartaric, fumaric, malic, maleic, gluconic, succinic, gallic acid etc.
Alcohols	ethanol, propanol, butanol etc.
Amino acids	glycine, alanine, arginine, aspartic acid, cysteine, glutamic acid, histidine, leucine, lysine, methionine etc.
Carbohydrates	ribose, fructose etc.
Enzymes	glucoamylase, alpha-amylase, protease, lipase, fumarase, glucose isomerase, cellulase, hydantoinase etc.
Hydroxy compounds	glycerol, arabitol, mannitol, xylitol, 2-3 butanediol etc.
Ketones	acetone, dihydroxyacetone, alkanone etc.
Polysaccharides	xanthan, gellan, alginate
Others	lipids, carotenoids, vitamins, antibiotics etc.

Alpha-amino acids are an important building block of biological tissue. Most α-amino acids have an asymmetrical carbon atom because of which they show optical rotation. In nature they occur in the free form mostly as the L-isomer, however natural D-forms of amino acids are also known to occur in the combined state.

L-Amino acids

Though proteins contain as many as 25 different amino acids [Fruton and Simmond (1961)], only 20 naturally occurring amino acids are known to exist (Rozzell and Wagner 1992). Except for glycine all naturally occurring amino acids are optically active with L-configuration. The L-amino acids are listed in table 2 [after Aida et al. (1986) and Atkinson and Mavituna (1991)] are produced either by a bioconversion or biotransformation route. Monosodium glutamate, a flavour/taste enhancer was prepared first on a large scale. L-cysteine was synthesized as a cattle feed ingredient. L-

methionine, L-phenylalanine, L-tryptophan, L-tyrosine, L-glutamate and L-lysine were synthesized by both chemical and biochemical methods.

Table 2

Amino acids prepared by Fermentation or Biotransformation

alpha amino acids	**uses**	**references**
L-alanine	Human food supplement	Chibata et al.(1986a)
L-arginine	animal feed supplement	Yoshida (1986)
L-aspartic acid	in preparation of synthetic sweetener, aspartame	Chibata et al.(1986b)
L-glutamic acid	Taste enhancer	Kikuchi & Nakao (1986)
L-histidine	Human food supplement	Akai (1986)
L-leucine	animal feed supplement	Komatsubara & Kisumi (1986)
L-lysine	animal feed supplement	Tosaka & Takinami (1986)
L-methionine	in preparation of plant hormone	Tanaka & Soda (1986)
L-phenyl-alanine	in preparation of peptide sweetener	Shio (1986)
L-tryptophan	Human food supplement	Shio (1986)
L-valine	animal feed supplement	Komatsubara & Kisumi (1986)

Some natural L-amino acids are also formed by a semi-synthetic route e.g. Hydantoin based substrates are synthesized chemically and then subjected to hydrolysis, using a specific hydantoinase enzyme to obtain N-carbamoyl-derivatives. The N-carbamoyl derivative produced can either be chemically transformed to L-amino acids, or in some using the enzyme carbamoylase to form L-amino acids. Ishii and Takinami

(1978) prepared L-tryptophan from D,L-tryptophanhydantoin. Synthesis of L-phenylalanine from D,L-5-benzylhydantoin was reported by Ishii et al.(1978). Miyoshi et al. (1985) synthesized L-phenylalanine, L-tryptophan and L-tyrosine from D,L-5-benzylhydantoin, D,L-5-indolylmethlhydantoin and D,L-5-p-hydroxybenzylhydantoin respectively. Some derivatives of amino acids are also formed from hydantoin-based substrates. Formation of L-3,4-hydroxyphenylalanine and L-piperonylglycine from 5-(3',4')-dihydroxy-benzylhydantoin and 5-(3',4')-methylene-dioxybenzylhydantoin respectively was studied by Sano et al.(1977). Syldatk et al. (1992b) reported the synthesis of L-amino acids from D,L-benzylhydantoin and related compounds. L-alpha-naphthylalanine and L-beta-naphthylalanine were synthesized from D,L-5-alpha-naphthylmethylhydantoin and D,L-5-beta-naphthylmethylhydantoin respectively by Syldatk et al.(1992c).

L-amino acids can be prepared from alpha-ketocarboxylic acid e.g. L-leucine was synthesized from keto-isocaproate [Groeger et al.(1986)]. L-Alanine and its derivatives were synthesized by Harada and Tamura (1979). L-Aspartic acid was synthesized by Tamura and Harada (1980). Some amino acids are prepared from N-acetylamino acid by aminoacylase enzyme. In some cases D,L-amino acids mixtures were used as a starting material to obtain the L-amino acid. L-methionine was obtained from D,L-methionine where D-methionine was converted to a keto acid [Ishiikawa and Kumara(1988)]. In some cases only L-form is obtained, from the D,L-mixture, eg. aminotransferase enzyme selectively converts the D-form to L-form. L-leucine, L-phenylalanine and L-homophenylalanine were synthesized by this way [Fotheringham et al. (1991)]. L-homophenylalanine is used in the preparation of ACE (angiotensin-converting enzyme) inhibitor. L-amino acids are also prepared from 2-amino nitriles and 2-amino ylidene acetonitrile derivatives. L-leucine was prepared by this way [Wakamoto et al.(1989)]. L-amino acids are also obtained from amides of D,L-amino acid. Dotani et al. (1989) prepared L-valine from D,L-valine amide. Asano et al.(1994) converted mesaconic acid, ethylfumaric acid and chlorofumaric acid to threo-3-methyl-L-aspartic acid, threo-3-ethyl-L-aspartic acid and threo-3-chloro-L-aspartic acid respectively. Sutherland and

Willis (1998) synthesized optically pure 4-amino-2-hydroxy acids. These are some of the references for preparation of L-amino acids from different types of substrates.

D-Amino acids

Earlier D-amino acids were considered as unnatural and of limited use. As research into pharmaceutical intermediates progressed, there was a change in thinking . It was observed that microbial cells were capable of synthesis of D-amino acids. D-alanine, D-glutamic acid and D-2-aminoadipic acid were found in cell wall of some microorganisms [Rozzel and Wagner (1992)]. Some plants and microorganisms were found to synthesize metabolites which yielded D-amino acids on enzymatic hydrolysis. *Bacillus brevis* synthesizes gramicidins which on hydrolysis gives D-leucine. *Bacillus polymyxa synthesizes* polymyxins which yields D-leucine. D-amino acid was also obtained by resolution of D,L-amino acid [Fruton and Simmond (1961)]. Ogawa et al (1977) reported presence of D-aspartic acid and D-glutamic acid in pea seed.

Nagata (1999) has cited in his review that free D-amino acids are present in bacteria and some animals. Free D-serine, D-aspartate, D-proline, D-alanine and D-glutamate were found in *P. islandicum* and *H. salinarium* [Nagata et al.(1999)]. Innocente and Palla (1999) have reported presence of D-alanine, D-lysine, D-ornithine and D-tyrosine in cheese. There are many references to the formation of D-amino acid by biosynthetic pathways. D-cysteine and D-methionine are prepared by semisynthetic methods. D-cysteine and D-valine are used in the preparation of cephalosporin and fluvalinate respectively. Hydantoin based substrates are used in many cases for synthesis of D-amino acids. D-alanine is prepared from 5-methylhydantoin [Nakamori et al.(1978)]. Makryleas and Drauz (1990) have obtained D-valine from D,L-isopropylhydantoin. Gillonier and Guivarch (1980) have synthesized D-phenylalanine from corresponding hydantoin. Yamada et al. (1978b) have prepared N-carbmoyl-D-methionine from hydantoin derivative.

Tagawa et al.(1994) prepared D-phenylalanine from N-benzoylphenylalanine. Alpha-keto acids, phenylpyruvate and hydroxyphenylpyruvate were used to prepare aromatic D-amino acids, D-phenylalanine and D-tyrosine respectively [Bae et al. (1999)]. Isobu and Hirose (1999) have prepared D-leucine from D,L-leucine. N-acetylamino acids were used for the preparation of D-amino acids by using the enzyme D-aminoacylase. D-tryptophan, D-phenylalanine, D-leucine, D-valine and D-methionine were synthesized by this method [Mitsuhashi et al.(2000)]. D-amino acids can be synthesized from corresponding amides. Dotani et al. (1985) prepared D-valine from D-1-isopropylaminoacetamide.

There are compounds in which an amino acid features as a chiral center e.g. glutathione; D-phenylglycine; p-hydroxyphenylglycine; 3,4-hydroxyphenylalanine (L-dopa); beta-lactam antibitoics; lipo-amino acids etc. Wang and Potter (1976) have separated D-α-phenylglycine from its racemic mixture. D-α-phenylglycine is used in the synthesis of penicillin. Ichikawa and Shibukawa (1986) have reviewed the formation of intermediates of β-lactam antibiotic. Glutathione used in the treatment of hepatic ailments is prepared by both synthetic and chemo-enzymatic methods [Kumagai 1986].

D-phenylglycine and D-p-hydroxyphenylglycine are prepared by semisynthetic method from hydantoin derivatives [Olivieri et al. (1979b); Tawaki et al.(1986); Kanegafuchi (1980)]. They are used as a precursor in the synthesis of semi-synthetic penicillin, ampicillin and amoxicillin respectively [Kamphuis et al.(1990)]. Sato et al.(1988) have cited the uses of hydantoin derivatives as precursors in various applications. D-m-methoxyphenylglycine is prepared from D,L-m-methoxy-phenylhydantoin [Ajinimoto Co. Japan(1980)]. 3,4-dihydroxyphenylalanine (L-dopa) is synthesized by both synthetic and chemo-enzymatic methods and is used in the treatment of Parkinson's disease [Enei and Yamada (1986)]. Amino acid antimetabolites, synthesized by biosynthetic methods, can be used as anticancer drugs or plant growth regulators [Sakai (1986)].

Tao and Li (1984); Li (1985) have reveiwed uses of amino acids in agrochemicals. Xu (1984) has reviewed the use of amino acids in various fields. Hudson (2000) has reviewed the use of aminophosphonic acid, aminophosphinic acid and its derivatives as agrochemicals. Poduska et al. (1976) have synthesized derivatives of amino acids, which have insecticidal properties. Nath et al.(1997) have prepared dibutyltin complexes which show antimicrobial and antitumour properties. They have used amino acids in the preparation of these complexes, penicillin preparation, novel peptides preparation etc.

Examples of amino acids used as precursors (table 3) and final products (table 4) are cited below.

Uses of amino acids

Table 3: Precursors:

Precursors	**fields**	**references**
imino acid-peptides	agrochemicals, drugs	Ofuna et al.(1987)
D-N-carbamylvaline	agrochemicals, drugs	Tawaki et al.(1988)
D-b-hydroxyamino acids	agrochemicals, drugs	Kato et al.(1989)
[(Tert-Butyldimethylsilyl) oxy] carbonyl amino acids	agrochemicals, antibacterial and central nervous system agents	Ohfune and Sakaitani (1987)
(2S)-CH_2:$CHCH_2CH(NHCO_2R6)CO$-(L)-$NHCH(CHMeOR7)CO_2Me$ where R^6=R^7= $SiMe_2Cme_3$		
dihaloisonicotinoyl derivatives of amino acids.	herbicide	Kenney and Stein (1980)
N-(2,6-dialkylphenyl)-N-formylglycine	herbicide, fungicide	Schmierer et al.(1986)
L-leucine	insecticide ((4S)-4-isobutyl-2-methoxy-1,3,2-oxazphospholidine 2-sulfide)	Eto et al.(1978)

5-Arylidenehydantoin (Benzalhydantoin; 5-benzylidenehydantoin)	pesticide, medicine	Imaki and Takuma (1986a,b)
Azotidinones	cephems, penicillin and nocardicin	Wasserman & Lipshutz (1978)
N-Acyl-a-aromaticglycine derivatives.	semisynthetic penicillin	Christidis & Schouteeten (1979)
Hydroxyphenylglycine	penicillin, cephalosporins	Senuma et al.(1980)
Amino acid derivative of 2,3 dioxopiperazine	penicillins, cephalosporins	Saikawa et al.(1981)

Table 4

Compounds	**uses**	**referencas**
[(1,2,3,4-tetrahydro-4-oxo-2-thioxoquinazolin-3-yl)-alkanoates]	agrochemicals	Suesse et al.(1987)
Amino acid conjugate Amino acids= leucine, isoleucine, valine etc.	herbicide	Feung et al.(1977)
N-carbamoyl-N-phenylamino acid	herbicide	Hashimoto et al.(1976)

derivative		
Thiocarbonyl amino acid derivative	herbicide	Okii et al.(1980) Nakamizo et al.(1977)
Benzothiazolyl amino acid derivative	herbicide, fungicide, anticholesteremics and antiarrhythmics	Kyowa Hakko Kogyo Co., Ltd., Japan(1982b)
Halogenated a-amino acids RCF2CFR1CHR2CH(NH2)CO2H; where R=Cl, Br; R1=H,F,Cl; R2=H, Me	herbicide, insecticide	Muramatsu and Ueda(1982)
a-amino fatty acid anilide derivative RR1C6H3NHCR2MeCONHC6H4R3-4 where R=H, alkyl, alkoxy, CF3, halo; R1=H, Me, Et; R2=H, Me; R3=alkyl	herbicide	Mitsui Toatsu Chemicals, Inc., Japan (1983)
Phosphonomethylated amino acids (HO)2P(O)CH2NHCH2CO2H and derivatives	herbicide	Nagubandi(1985a,b)
N-(carboxyphosphinomethyl)-amino acids	herbicide	Issleib et al.(1985)
N-(2-hydrxyalkyl)amino acids and derivatives	herbicide, fungicide	Lautenschlaeger et al.(1986)

Table 4

L-2-amino-4-hydroxy(methyl)phosphinyl-butyric acid	herbicide	Kuwahara et al.(1990)
Amino acid esters of milbemycins	insecticide, acarides and nematocides	Terada et al.(1985)
Difluoromethylhomocysteine	insecticide	Tsushima et al.(1990)
Acetylpolyamino acids	insecticide	Astruc et al.(1992)
N-(O,S-Dimethylthiophosphoryl) a-L-amino acid esters	insecticide	Tang et al.(1999)
Pyridyl esters of a-substituted amino acid	pesticide	Henrick(1980)
(E)-isomers of N-a-[2-cyano-2-(alkoxyimino)acetyl]amino acid derivatives	pesticide, fungicide	Lunkenheimer et al.(1986)
Fluorobutenyl esters of amino acids	pesticide	Kraatz et al.(1997)
3-Phenyl-2-acetylaminoprop-2-enoic acid 3,4,4-triflurobut-3-enyl ester		

Routes for enzymatic synthesis of amino acids

Aminoacylase

Various researchers have used aminoacylase enzyme for the preparation of amino acids. Acetyl-D,L-isomers are converted to their L-amino acids by aminoacylase (EC 3.5.1.14) from *Aspergillus oryzae* [Chibata et al.(1976)]. Meiller and Mirabel (1979) and Rhone-Poulenc Industries (1977) obtained L-methionine from N-acetyl-D,L-methionine using immobilized L-aminoacylase from porcine kidney. Tripathi et al.(2000) hydrolysed N-acetyl-D-amino acids to D-amino acids (D-methionine, D-valine, D-phenylalanine) using cells. Sugie and Suzuki (1980) studied the conversion of N-acetyl-D,L-phenylglycine to D-phenylglycine using D-aminoacylase. Smalla et al. (1985) obtained L-phenylalanine from chloacetyl-D,L-phenylalanine using immobilized aminoacylase. Kung et al.(1986) have obtained L-phenylalanine from N-acetyl-D,L-phenylalanine methyl ester by using aminoacylase and esterase.

Sakai et al. (1991) used D-aminoacylase for the hydrolysis of N-acetyl-D-phenylalanine to D-phenylalanine. Moriguchi et al.(1993) obtained D-leucine by hydrolysis of its N-acetyl, N-formyl, N-butyryl and N-propionyl derivatives. They synthesized D-phenylalanine, D-norleucine and D-methionine from their N-acetyl derivatives using D-aminoacylase from *A. xylosoxydans sub sp. xylosoxydans* A-6. Mitsuhashi et al. (2000, 2001) studied the hydrolysis of N-acetyl derivative of D-amino acids to D-amino acids (D-tryptophan, D-phenylalanine, D-valine, D-leucine and D-methionine) using D-aminoacylase. Biselli (1992) obtained L-amino acids from their racemic mixture. Olama (1999) obtained L-alanine from N-acetyl-L-alanine. Shintani et al.(1984) studied the hydrolysis of N-long chain acyl amino acids using N-long chain acyl aminoacylase. Sambale et al.(1987) obtained L-valine from N-(methoxycarbonyl)-D,L-valine using strain.

Racemase

The enzyme Racemase is used to obtain enantiopure D or L-amino acid from a racemic mixture and/or interconversion of amino acids from D to L -form or vice versa

Klages and Weber (1989) prepared L-phenylalanine from D,L-a-amino-b-phenyl-propionitrile using cells (*A. calcoaceticus and Arthrobacter* ATCC 31652) containing amino acid racemase and L-amino acid amide amidase. Hermes et al.(1990) studied the racemization and hydrolysis of D-valine amide using cells containing amino acid amide-racemase. Godtfredsen et al.(1989) converted D,L-phenylglycine amide to L-phenylglycine using microbial cells containing amino acid racemase and amidase enzyme. Pietzsch et al. (1990) converted D-3-methyleneindolyl-5-hydantoin to L-tryptophan using hydantoin racemase. Terasawa et al.(1990) studied the conversion of D-serine to L-serine using an amino acid racemase.

Aminotransferase

In some cases amino acids are prepared by using an aminotransferase enzyme. Lewis and Farrand (1985) have prepared L-phenylalanine from Na-phenylpyruvate and Na aspartate, using an aminotransferase.

Hydantoin

Allantoin is synthesized by most higher plants. Baeyer (1861) carried out the hydrogenation of allantoin. He named the formed compound as hydantoin (fig.1). It is also known as imidazolidine-2,4-dione or 2,4-diketotetrahydroimidazole. Hydantoin is crystalline and sweet in taste [Syldatk et al.(1992a)]. Syldatk et al. (1992a) have discussed the uses of hydantoin and its derivatives. Hydantoin and its derivatives are widely used as substrates for amino acid preparation. Both natural and unnatural amino acids are synthesized from these substrates Hydantoin have found applications in the medical field [Triadan (1968); Spurlock (1945)] as anticonvulsant, antimicrobial, virucidal, tuberculostatic, herbicidal and fungicidal agents [Mehta et al.(1981); Tahara et al. (1979); Thibault and St. Clair (1982); Tielemann (1971); Ohta et al. (1980); Mappes et al. (1980); Syldatk et al. (1992a)]. Ware (1950) reported use of 5,5-disubstituted hydantoins as hypnotic and narcotic agents.

Fig.1: Formation of hydantoin

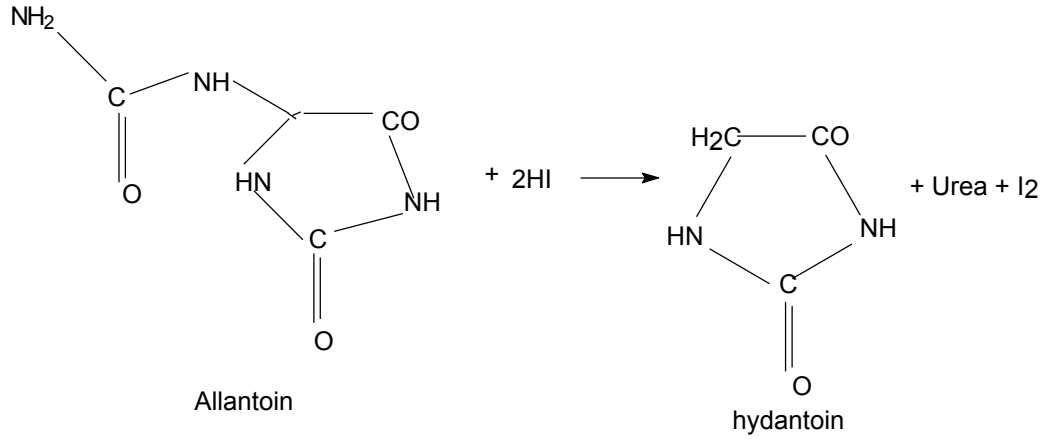

Fig. 1: *Formation of hydantoin from allantoin [Syldatk et al. (1992a); Baeyer (1861)].*

Hydantoin synthesis: 5-substituted hydantoins are synthesized using different methods. Three ways are given below :

i)Bucherer-Bergs synthesis:

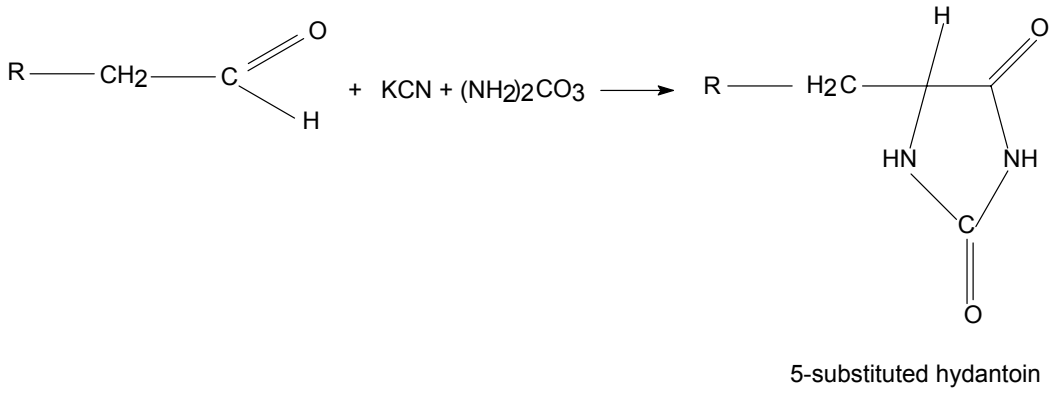

Fig. 2 : *Synthesis of 5-substituted hydantoin [Syldatk et al. (1990)].*

ii) From an aldehyde and hydantoin:

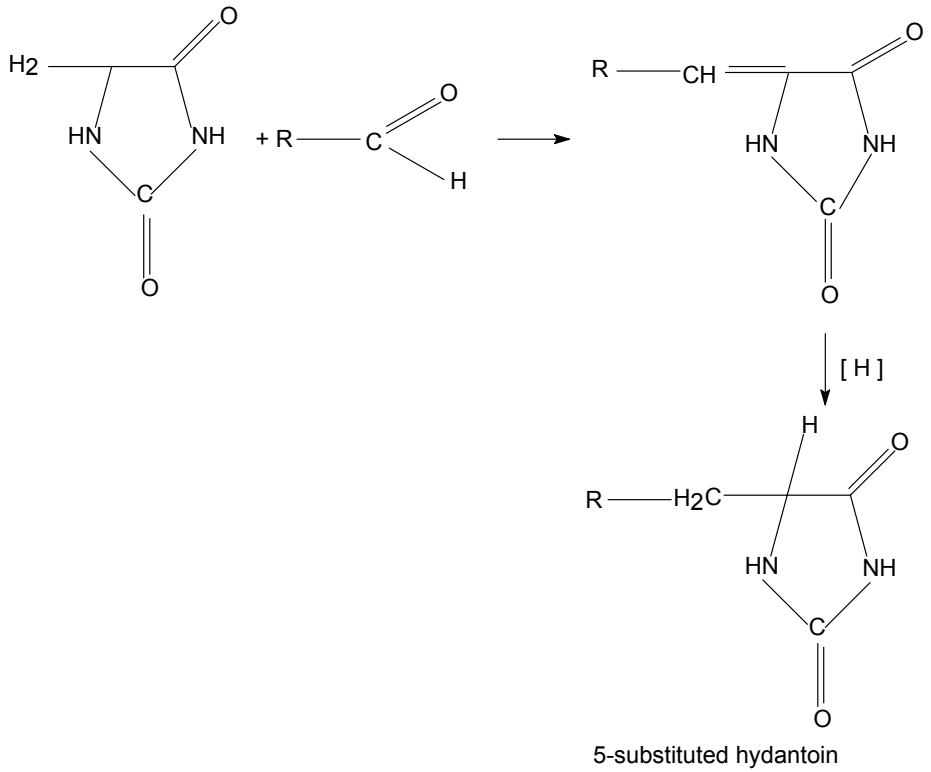

Fig. 3: *Synthesis of 5-substituted hydantoin [Syldatk et al. (1990)*

iii) Glyoxylic acid, urea and phenol in presence of acid yield 5-(p-hydroxyphenyl) hydantoin [Takahashi (1983)]

Hydantoin hydrolysis

Hydantoin is a stable compound whose hydrolysis occurs under only extreme conditions i.e. 60% H_2SO_4 at 120-130^0 C. Degussa prepared D,L-phenylalanine by treating 5-(phenylmethyl)hydantoin with HCl [Hoppe and Martens (1984)]. Elks et al. (1948) and Deulofen (1936) had used $Ba(OH)_2$ for hydrolysis. Allantoin, carboxymethylenehydantoin, carboxyethylene-hydantoin are naturally occuring hydantoin derivatives. Allantoin is hydrolyzed by allantoinase or 5-ureidohydantoinase [EC 3.5.2.5] to allantoic acid. This enzyme is present in plants, animals and microorganisms. Syldatk et al. (1999) have reviewed hydantoinase. EC 3.5.2 which is a class of enzymes cleaving hydantoin and derivatives of hydantoin. It is further sub-classified into:

dihydropyrimidinase (hydantoinase) EC 3.5.2.2; carboxymethylhydantoinase EC 3.5.2.4; allantoinase EC 3.5.2.5; N-methylhydantoinase EC 3.5.2.14; (imidase, carboxyethylhydantoinase etc) 5,6-dihydrouracil and 5,6-dihydrothymine have a pyrimidine ring. Hydantoinase cleaves this ring. Therefore it is called as dihydropyrimidinase EC 3.5.2.2. It also cleaves 5-monosubstituted hydantoins (fig. 4).

Fig. 4: Cleavage of dihydrouracil and 5-monosubstituted hydantoin

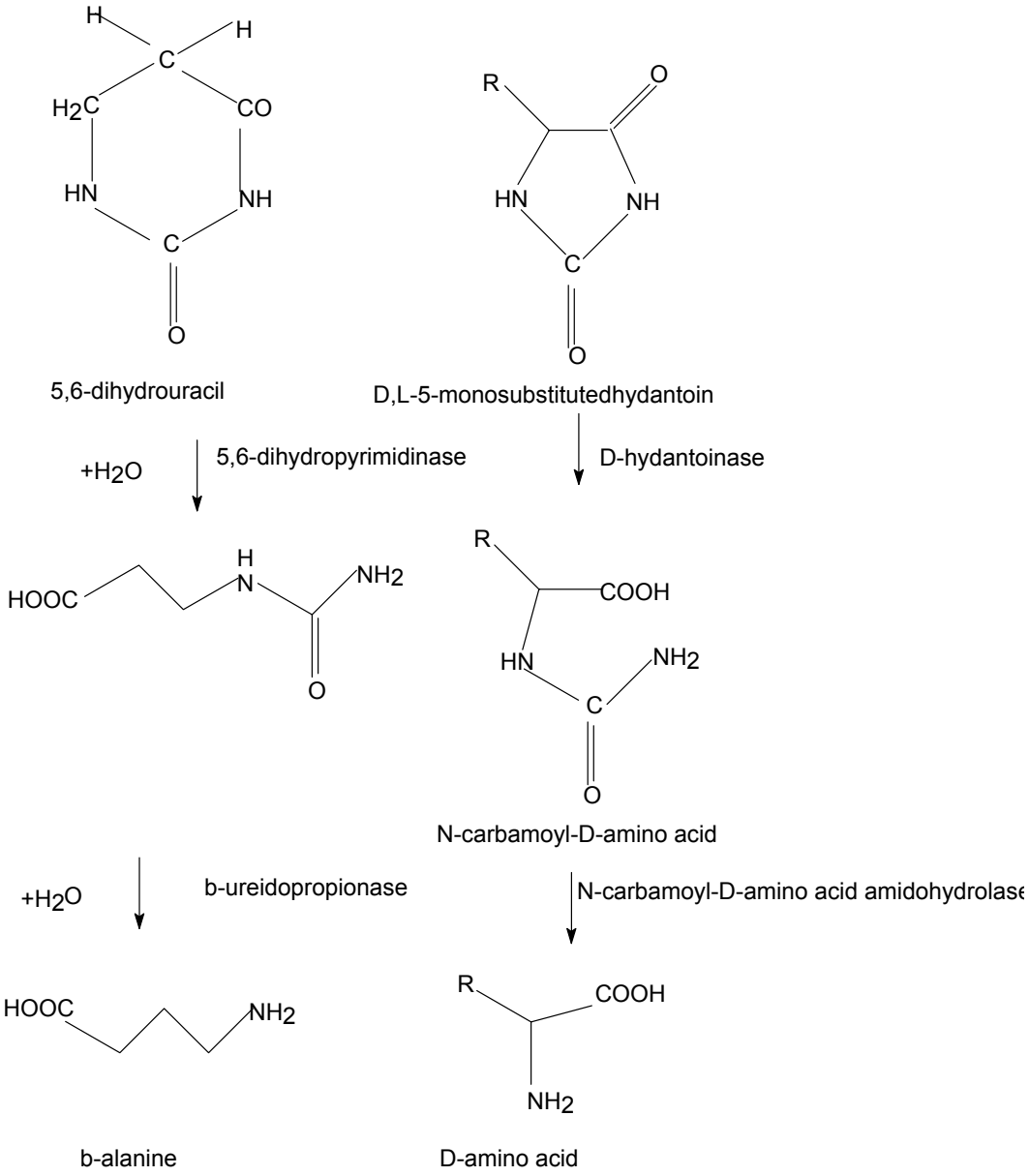

Fig. 4: *D-selective cleavage of 5,6-dihydrouracil & D,L-5-monosubstitutedhydantoin [Syldatk et al. (1992a)]*

Hydantoinase is widely distributed in nature either as D or L selective and accordingly it is called-hydantoinase or L-Hydantoinase. It is present in plants (*Pisum sativum, lentil seed, Vigna angularis, Phaseolus vulgaris*, jack beans, peas, melon seeds, cucumber seeds etc.); animals (liver of veal, calf, rat, pig etc.); microorganisms (*Peudomonas, Aerobacter, Agrobacterium, Arthrobacter, Actinoplanes, Bacillus, Nocardia, Streptomyces* etc.). The N-carbamoyl-derivative is formed as a result of action of hydantoinase on hydantoin or its derivatives. It is further converted to amino acid by either a chemical method or by using the enzyme called as N-carbamoylase. In some microorganisms e.g. *Agrobacterium* both enzymes are present. These enzymes are stereospecific. The manner in which research on hydantoinase has progressed is summarized in Table 6 [Syldatk et al. (1992a)].

Table 6

Year	**Researchers**	**Invention**
1926	Gaebler & Keltch	Hydantoic acid (N-carbamoylglycine) formation from hydantoin in dog body.
1932	Sobotka	L-5-phenyl-5-ethylhydantoin hydrolysis by *Aspergillus sp.*
1934	Wada	Hydantoin + milk/tissue suspension→ urea.
1946	Bernheim & Bernheim	Hydantoin is cleaved by liver extract of ominivorous animals.
1947	Eadie et al. (1949)	Hydantoin is cleaved by plant seed extract.
1950	Grisolia et al.	These enzymes are involved in primidine metabolism [Wallach & Grisolia (1957); Caravaca & Grisolia (1958)].
1970 & 1973	Dudley et al.	N-sub.-5-phenylhydantoin→ D-selective hydrolysis
1975	Cecere et al.	Conversion of 5-monosub. hydantoin to its N-carbamoyl
1978	Dinelli et al.	N-carbamoyl-phenylglycine preparation by immobilized enzyme.

D-amino acids are synthesized using microbial hydantoinase. It has been extensively reviewed by Yamada et al. (1980a); Tani et al. (1978) etc. Yamada et al. (1978a) have used various microorganisms [*Pseudomonas, Aerobacter, Agrobacterium, Actinoplanes, Nocardia* and *Streptomyces*] for dihydropyrimidinase preparation. Takahashi et al. (1978) and Takahashi (1983) obtained N-carbamoyl-D-amino acids by hydrolysis of D,L-5-substituted hydantoin using hydantoinase from *Pseudomonas straita*. Maguire and Dudley (1978) hydrolyzed hydantoin, 5-phenylhydantoin, dihydrouracil and a-phenylsuccinimide to its N-carbamoyl derivative using dihydropyrimidinase from calf and rat liver. Yamada et al.(1980b) synthesized D-phenylglycine-based amino acids from hydantoin derivatives using dihydropyrimidinase.

Production of D-(-)-4-hydroxyphenylglycine was studied by Tramper and Luyben (1984). Preparation of D and L-amino acid, by using hydantoinase, discussed by Syldatk et al.(1986). Syldatk et al.(1987) studied the hydrolysis of 5-substituted hydantoin by D-hydantoinase from *Arthrobacter sp.* Morin et al (1987, 1990) studied D-hydantoinase production from various species e.g. *Pseudomonas, Serratia, Corynebacterium* etc. Young-Jin Pharmaceutical Industries, Ltd., S. Korea, (1991) have used D- hydantoinase from *Streptomyces* Y-183 for hydrolysis of D,L-5-phenylhydantoin and D,L-5-(hydroxyphenyl)-hydantoin. Chevalier et al. (1989a,b) studied the conversion of dihydrouracil to N-carbamoylamoyl-alanine by using immobilized cells of *Pseudomonas putida.* Yamada et al.(1994) have prepared N-carbamoylamoyl-b-aminobutyric acid, from D,L-6-methyldihydrouracil using dihydropyrimidinase of *P. putida* IFO12296, which is further converted into (S)-b-aminobutyric acid chemically.

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Burtscher et al. (1995) have produced D-N-carbamoyl-α-amino acids using hydantoinase from *B. thermoglucosidasius*. Nanba et al. (1996a) prepared D-N-carbamoyl-α-amino acids from the corresponding hydantoins. Grifantini et al. (1995) converted D,L-5-substituted hydantoins to D-α-amino acids using microorganisms. Durham and Weber (1995) have prepared N-carbamoylamino acids from hydantoin derivatives using hydantoinase from *A. tumefaciens.* Bommarius et al. (1995) prepared cyclic N-carbamoyl-D-amino acids. Keil et al. (1995) synthesized D-a-amino acids from

corresponding hydantoin derivatives using hydantoinase. Production of D-hydantoinase, from recombinant *E. coli,* for preparation of N-carbamoyl-D-amino was reported by Lee et al.(1996b,1997a). Sharma and Vohra (1997a,b) used D-hydantoinase from *Bacillus sp.* AR 9 for hydrolysis of hydantoin derivatives. Grifantini et al. (1997) studied hydrolysis of N-carbamoyl derivatives to D-amino acids by D-N-α-carbamoylase enzyme.

Kim et al.(1997a) synthesized D(-)N-carbamoyl derivatives from D,L-5-(p-hydroxy-phenyl)hydantoin, D,L-5-(2-methyl-thioethylene)hydantoin, D,L-5-phenylhydantoin and D,L-5-isopropylhydantoin using D-hydantoinase from *B. stearothermophilus* SD-1. Morin et al.(1997) hydrolyzed dihydrouracil, hydantoin, L-MMEH and D,L-MMEH to N-carbamoyl derivative using dihydropyrimidinase from the hulls of *Pisum sativum*. Louwrier and Knowles (1997) have prepared D-p-hydroxyphenylglycine by an enzymatic method. They have used both hydantoinase and carbamoylase enzyme. Carbamoylase enzyme was isolated from *Agrobacterium sp*. and characterized. Garcia and Azerad (1997) have hydrolyzed various D,L-hydantoins to substituted D-(-)N-phenylglycine. Bommarius et al. (1997) have synthesized (R)-tert-leucine from corresponding hydantoin derivative. Papain was used by Rai and Taneja (1998a) for conversion of a hydantoin derivative to N-carbamoyl Yamada (1998) have reported preparation of L-dopa, D-HPG, D-PG and other compound using enzyme. Kim et al.(1997b) hydrolyzed 5-substituted hydantoin to D-amino acid using enzymes.

Bommarius and Schwarm (2001) hydrolyzed D,L-(3-pyridyl)hydantoin to its N-carbamoyl derivative using a D-hydantoinase which was further converted to D-(3-pyridyl)- alanine using an N-carbamoylase. Hartley et al.(2001) used enzymes from *Agrobacterium tumefaciens* for conversion of hydroxyphenylhydantoin to hydroxyphenylglycine. Lee et al.(2001b) used an immobilized hydantoinase enzyme for the conversion of hydroxyphenylhydantoin to its N-carbamoyl derivative. Soong et al.(2001) have used D-hydantoinase from *Blastobacter sp.* A17p-4 for the hydrolysis of cyclic substrates - ureides (dihydrouacil, dihydrothymine, hydantoin etc.) and phenylhydantoin etc. The hydantoinase had more activity on hydantoin and dihydrouracil. They observed more conversion of phenylhydantoin than other substrates.

Fan and Lee (2001) used D-hydantoinase (partially purified) from adzuki bean for hydrolysis of phenylhydantoin and p-hydroxyphenylhydantoin. They found it to be more active on phenylhydantoin than p-hydroxyphenylhydantoin. They synthesized N-carbamoyl-D-phenylglycine using immobilized hydantoinase.

Tani et al. (1978) reviewed the preparation of L-tryptophan using tryptophanase. Guivarch et al. (1980) studied L-hydantoinase from *Pseudomonas sp*. and *Arthrobacter globiformi*s. Kitagawa et al. (1985) prepared N-carbamoyl-L-amino acids from 5-substituted hydantoin using hydantoinase from *Arthrobacter* DK200. Gross et al.(1987) screened microorganisms for conversion of D,L-indolylmethylhydantoin to L-tryptophan. Wagner et al.(1994) used *Arthrobacter* DSM 7329 and DSM 7330 for preparation of L-amino acids. Tarui et al. (2000) prepared (R)-2-aminocarbonylamino-5-(4-methylsulfonyloxyphenyl) valeric acid from 5-[3-[4-(Methylsulfonyloxy) phenyl]propyl]-2,4-imidazolidinedione using cells of *Arthrobacter crystallopoietes*.

Table7 cites references for the preparation of N-carbamoyl derivatives, D/ L-amino acids

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Table 7: N-carbamoyl derivative, D and L-amino acid preparation.

Source	**cells/enzyme**	**substrate**	**Product**	**refernce**
Dihydropyrimidinase	immobilized enzyme	5-ME-hydantoin	L-carbamylalanine	SNAM Progetti (1978)
Pseudomonas straita	cells	D,L-5(2thienyl)- hydantoin	N-carbamoyl-D-2-thienylglycine	Shimizu et al. (1980)
veal liver	dihydro-pyrimidinase	5-isoprpropyl- hydantoin	N-carbamoyl-D-valine	Dinelli et al. (1980)
Microbe	cells	D,L-5(ME-TH-ET) hydantoin and other 5-sub.hydantoin	N-carbamyl-D-AA	Takahashi et al. (1979)
P. putida	cells	PH, ME-TH-ET-H, ME-H, ISB-H	D(-)N-carbamoyl	Sun Wanru (1983a)
B. sphaericus	immobilized cells	D,L-HyPH	D(-)N-carbamoyl	Shiotani et al. (1991)
Bacillus brevis IFO 12333	immobilized enzyme	HyPH & others	N-carbamoyl	Powell & Power (1988)

Table 7

Peptococcus anaerobius CRDA 303 & *Clostridium*	cells	isopropylhydantoin & DHU	N-carbamoyl	Morin et al. (1991)
A. crystallopoietes AM2	cells(hydantoi-nase+carbamylase)	D,L-5-ME-hydantoin	D-alanine	Moeller et al. (1988)
Agrobacterium EO III	cells (both enzyme)	D,L-HyPH	D-P-hydroxy-phenylglycine	Ohleyer (1989)
Agrobacterium radiobacter	cells (both enzyme)	DL-isopropyl-hydantoin	D-valine	Battilotti, & Barberini (1988)
Agrobacterium sp. I-671	enzyme	D,L-HyPH	D-p-hydroxy-phenylglycine	Kim & Kim (1995b)
P. desmolyticum NCIM 2112	cells	D,L-phenylhydantoin	D(-)N-carbamoyl-phenylglycine	Gokhale et al. (1996)
B. stearothermophilus SD1	immobilized enzyme	D,L-5-HyPH	D(-)-N-carbamoyl	Lee et al.(1996a)
A. tumefaciens	enzyme	D,L-5-PH & DL-5-ME-H	N-carbamoyl-D-AA	Durham & Weber (1995)
P. sp. KBEL 101	immobilized cells	D,L-HyPH	D-HyPG	Kim et al. (1994)

Table 7

Phaseolu Vulgaris (plant)	enzyme	D,L-5methymercaptoethyl-hydantoin & other sub.	N-carbamoyl-D-methionine	Morin (1993)
rat liver	enzyme	DHU, DHT	N-carbamoyl	Kikugawa et al. (1994)
P. stutzeri ATCC	enzyme	H, DHU, DHT	N-carbamoyl	Xu & West (1994)
Pseudomonas sp. NCIM 5109	cells	D,L-PH	D-(-)-N-carbamoyl	Sudge et al. (1998)
recombinant *E. Coli*	cells	D,L-HPH & D,L-PH	D-(-)-HyPG & D-(-)-PG	Grifantini et al. (1998)
A. tumefaciens(RU-OR)	cells	D,L-HyPH	D-(-)-HyPG	Hartley et al. (1998)
lentil seed	immobilized enzyme	hydantoin derivatives	D-amino acids	Rai & Taneja (1998b)
recombinant *E. Coli*	cells	D,L-HyPH	D-(-)N-carbamoyl	Lee & Kim (1998)
B. thermocatenulatus GH-2	enzyme	D,L-PH & other hydantoin derivative	D-(-)N-carbamoyl	Park et al. (1999)
A. crystallopoietes DSM20117	enzyme	D,L-phenylhydantoin & other derivative	D-N-carbamoylPG & other N-carbamoyl	Siemann et al. (1999)
recombinant *E. Coli*	cells	D,L-HyPH	D-HyPG	Chao et al. (1999)

Table 7

recombinant *E. Coli*	cells	D,L-HyPH	D(-)N-carbamoyl	Yin et al. (2000)
recombinant *E. coli*	cells	D,L-HyPH	D-HyPG	Park et al. (2000)
recombinant *E. Coli*	cells	D,L-HPH	D-(-)N-carbamoyl	Chen et al. (1999)
Pseudomonas sp. H1	cells	D,L-HPH	N-carbamoyl-D-HyPG	Ranjan & Dave (2000)
Pseudomonas sp. NCIM 5070	cells	D,L-PH	N-carbamoylD-PG	Gokhale et al. (2000)
P. putida sp. 2262	cells	D,L-HyPH	D-HyPG	Zhang et al. (2000)
Vigna angularis	enzyme	D,L-PH	N-carbamoyl-D-PG	Arcuri et al. (2000)
Agrobacterium radiobacter	cells	DL-HyPH	D-HyPG	Lee et al. (2001a)
Arthrobacter/Flavo-bacterium HP-27	enzyme/cells(hydantoinase+carbamylase)	5-benzyl-ME-hydantoin	L-homopheny-lalanine	Nakayama & Ozawa (1990)
Flavobacterium sp.	cells(both enzyme)	D,L-5-indolyl-ME-hydantoin	L-tryptophan	Nishida et al. (1987)
Pseudomonas sp. NS671	cells	D,L-5-sustituted hydantoin	L-amino acids	Ishikawa et al. (1997)

ME=methyl; TH=thio; ET= ethyl.; AA= amino acid, IPr=isopropyl, H=hydantoin, Hy=hydroxy, P=phenyl, G= glycine, AA= amino acid .

Van Gennip et al. (1994) investigated effect of dihydropyrimidinase (DHP) and dihydropyrimidine dehydrogenase (DHPD). Deficiency and attributed some forms of epilepsy to these deficiencies. They studied the amount of dihydrouracil, dehydro-thymine, uracil, thymine etc. secretion in urine. Hayashi et al. (1996) have studied the deficiency and amount of these compounds secretion in urine. Putman et al.(1997) have reported that deficiency causes neurological disorder in children. Dihydropyrimidinase deficiency is called as dihydropyrimidinuria. Van Gennip et al. (1998) have reveiwed these deficiencies.

Hydantoins and the associated enzyme hydantoinase is clearly a very important and fruitful area of research. Biotransformation remains one of the strongest tools for conversion of hydantoins to their respective N-carbomyl derivatives and amino acids. The main theme of the current work is based on this conversion of hydantoins to amino acids.

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Scope of this thesis

Chiral compounds are useful in pharmaceuticals, food and some other fields. Alpha-amino acids are chiral compounds and used widely. D-(-)phenylglycine is one of the important alpha-amino acids used in the preparation of antibiotics. Hydrolysis of D,L-phenylhydantoin yields D-(-)phenylglycine. D.L-phenylhydantoin is prepared by chemical synthesis. D-hydantoinase enzyme hydrolyses D,L-phenylhydantoin to D-(-)-N-carbamoylphenylglycine. D-(-)-N-carbamoylphenylglycine is converted to D-(-)-phenylglycine either chemically or by carbamoylase enzyme. In this thesis we have studied hydantoinase enzyme from *Pseudomonas sp.* NCIM 2875. This hydantoinase hydrolyses a racemic mixture of D and L-phenylhydantoin to D-(-)N-carbamoyl-phenylglycine.

Objectives :

1) Optimization of medium in shake flasks for growth and hydantoinase production.

2) Optimization of conditions for maximum growth and hydantoinase production.

3) Extraction of hydantoinase enzyme from cells where cells were treated with solvents, detergents and EDTA followed by extraction with buffer.

4) Purification of hydantoinase enzyme using various methods like precipitation, chromatography etc. and study of properties of the purified hydantoinase.

5) Immobilization of cells and crude hydantoinase enzyme.

Chapter 2

Optimization of growth and hydantoinase production

Summary

Pseudomonas sp. NCIM 2875 was microorganism used in the experimental studies. Experiments were performed in shake flasks to optimize a glucose-based medium and conditions to enhance growth and hydantoinase production. A medium containing glucose, $NaNO_3$, salts and supplemented with 0.1% yeast extract and 0.1% peptone was developed. Inoculum age, pH, harvesting time etc. were optimized. 20 h. inoculum age was found to be optimal. Optimum harvesting time was 27 h. Cells were cultivated using an optimized medium and culture conditions. A maximum concentration of 12.7 g l^{-1} wet wt. cells and 4.63 units activity (micromoles of N-carbamoyl per min. per gm wet wt. cells.) were obtained

Introduction

Hydantoinase enzyme [E.C.3.5.2.2] is found to exist in plants, animals and microorganisms. Many researchers have used hydantoinase from plants and animals sources. In microorganisms it is usually intracellular. Yang and Sun (2001) have reviewed hydantoinase and its uses. Hydantoin and derivatives of hydantoin are prepared by chemical synthesis. They are hydrolyzed to N-carbamoylamino acids using either whole cells directly or hydantoinase isolated from cells. In some cases immobilization of cells or enzyme is carried to facilitate reuse of cells/enzyme. N-carbamoyl-derivative is further converted to an optically pure amino acid either by a chemical route or by using enzyme carbamoylase. In some microorganisms both enzymes (hydantoinase and carbamoylase) are present. Hydantoin and hydantoin derivatives are converted to their corresponding α-amino acids by hydantoinase and carbamoylase. It has reviewed by Ogawa and Shimizu (2000).

The screening of microorganisms is a major step in the study of hydantoinase. Screening of hydantoinase producing microorganisms has been performed by many

researchers. *Pseudomonas, Aerobacter, Agrobacterium, Actinoplanes, Nocardia* and *Streptomyces sp*. were cultivated on medium with pyrimidine to produce dihydropyrimidinase by Yamada et al. (1978a). Sun (1983b) screened several bacteria and selected *P. putida* 73104 for further studies. Morin et al. (1986b) selected *P. fluorescens* after extensive screening. *P. fluorescens* converts 5-isopropylhydantoin to N-carbamoylvaline. Gross et al. (1987) had carried out screening of microorganisms that form L-tryptophan from D,L-indolylmethylhydantoin. Lee et al. (1994) studied thermophilic cultures and selected *Bacillus sp.* SD-1 for further studies. The strain showed an optimum growth temperature of 60^0C.

Jiang et al. (1995) carried out mutation of *Pseudomonas sp*. J43 and obtained mutant M39 which has 5-FU resistance. Gokhale et al. (1996) have carried out screening and selected *P. desmolyticum* NCIM 2112 for study. It hydrolyses D,L-phenylhydantoin to its D(-)N-carbamoyl Chien and Hsu (1996) have carried out plate assay for screening of hydantoinase producing microorganisms. Sudge et al. (1998) have studied microorganisms from sea water and selected one (*Pseudomonas sp*. NCIM 5109).

Morin et al. (1990) compared the hydantoinase activities of *P. fluorescens* (strain 1.2, 1.9*)*; *P. putida* (strain 2.2.2.5, DSM 84); *Serratia liquefaciens* (strain 1.15) and *Corynebacterium pseudodiphtheriticum* (14.10.). They found hydantoinase from *Corynebacterium pseudodiphtheriticum* had more temperature stability than from other cultures. Young-Jin Ind. (1991) produced hydantoinase, by cultivating *Streptomyces sp.* Y-183, which hydrolyses D,L-phenylhydantoin and D,L-p-hydroxyphenylhydantoin. Morin et al. (1991) have studied anaerobic cultures: *Clostridium sp., C. glycolicum, C. subterminale* and *Peptococcus anaerobius. P. anaerobius* hydrolyses dihydrouracil and isopropylhydantoin. Morin (1993) also studied D-hydantoinase of legume spp. of genera: *Arachis, Cajanus, Cicer, Glycine, Lablab, Lens, Macrotyloma, Phaseolus, Pisum, Psophocarpus, Vicia* and *Vigna. Phaseolus sp., Pisum sativum* and *Vigna sp*. had more activity. Morin et al. (1995a) studied cyclic amide amidohydrolase, from *Phaseolus* and *Pisum sativum*, which hydrolyses hydantoin and derivatives of hydantoin. Rai and Taneja

(1998a) have reported that papain has hydantoinase activity. Rai and Taneja (1998b) have also reported that lentil seed contains D-hydantoinase enzyme.

Ogawa et al. (1995) studied dihydropyrimidinase activity of *Blastobacter sp*. A17p-4. Lee et al. (1997) studied D-hydantoinase production, at higher scale, from recombinant *E. coli.* Hu et al. (1998) have reported a spectroscopic method for estimation of phenylglycine in the bioconversion mixture. Van Kullenburg et al. (1999) have determined activity of dihydropyrimidinase using HPLC where they have used radioactive dihydrouracil. Li et al. (1999) have investigated hydrolysis of 5-(4-hydroxyphenyl)- hydantoin by *Pseudomonas putida* D.

D(-)phenylglycine is used as an intermediate in preparation of ampicillin. D(-)phenylglycine is prepared in three steps (fig.1). The first step is preparation of D,L-phenylhydantoin by chemical synthesis. In the second step D,L-phenylhydantoin is hydrolyzed to D(-)N-carbamoylphenylglycine by hydantoinase (cells or enzyme). In the third step D(-)N-carbamoylphenylglycine is converted to D(-)phenylglycine chemically. In this process two steps are chemical and one step is enzymatic. Such processes are called as chemo-enzymatic processes. An enzymatic step is preferred because the reaction is carried out under mild condition, it is specific and purity of product is high. In this study we have carried out work related to only one step i.e. biotransformation of D,L-phenylhydantoin to D(-)N-carbamoylphenylglycine. Screening of microorganisms was done in an earlier study[Gokhale et al. (1996)]. Some microorganisms from this study that were found to convert D,L-phenylhydantoin to D(-)-N-carbamoyl-phenylglycine, were used during the present work

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Different types of medium have been reported for production of hydantoinase. It includes complex media, semi-synthetic and synthetic media. In complex media all requirements of microorganisms are fulfilled therefore growth and activity is observed. In semi-synthetic media and synthetic medium all minimum requirements of microorganisms are provided in order to have growth and activity. In complex medium, reproducibility of data is an issue dependant upon the quality of chemicals used. It varies

batch to batch. This may affect cell yield and activity. In semi-synthetic media and synthetic media this is not problem because quality of chemicals is assured. This chapter covers optimization of growth and hydantoinase production. In this study we have optimized a glucose based medium (which contains a very small quantity of yeast extract and peptone) and cultivation parameters to enhance growth and hydantoinase activity. The entire study was carried out in shake flasks.

Materials and methods

Chemicals

Bacteriological grade yeast extract, peptone, beef extract and agar were used. Casein acid hydrolyzate, soypeptone, cottonseed meal and soybean meal were used from Hi Media., Mumbai, India. Dr. M. S. Gaikwad [P. D. Division, N.C.L. Pune] provided phenylhydantoin. KH_2PO_4, K_2HPO_4, $MgSO_4$.$7H_2O$, $MnSO_4$ .H_2O, $FeSO_4.7H_2O$, $Fe_2(NO_3)_3.9H_2O$, $CaCl_2.2H_2O$; H_3BO_3, $ZnSO_4.7H_2O$, ammonium sulphate, ammonium carbonate, urea, ammonium chloride, sodium nitrate, K-nitrate, HCl, NaOH, glucose, fructose , maltose, mannitol, lactose, xylose, sorbitol, citric acid, sucrose, p-dimethylbenzaldehyde, perchloric acid etc. chemicals (AR grade) were used from S.D. Fine Chemicals.Boisar, India. Hydantoin from Sigma Chemicals was used.

Activity determination

Biotransformation of D,L-phenylhydantoin to D(-)N-carbamoylphenylglycine is performed by the enzyme hydantoinase using either whole (wet) microbial cells or enzyme. Biotransformation is monitored by estimation of N-carbamoylphenylglycine formed (by a spectroscopic method or HPLC). It can be also monitored by the amount of alkali consumed to neutralize N-carbamoylphenylglycine. The alkali consumption method is not suitable at test tube scale cultivation. Many researchers have developed assays for hydantoinase enzyme by using a spectroscopic or HPLC method. We studied some of the methods from literature. A modified assay method was developed for

estimation of hydantoinase activity. Assay methods reported by different researchers, is cited in table 1.

Figure 1: Synthesis of D(-)phenylglycine

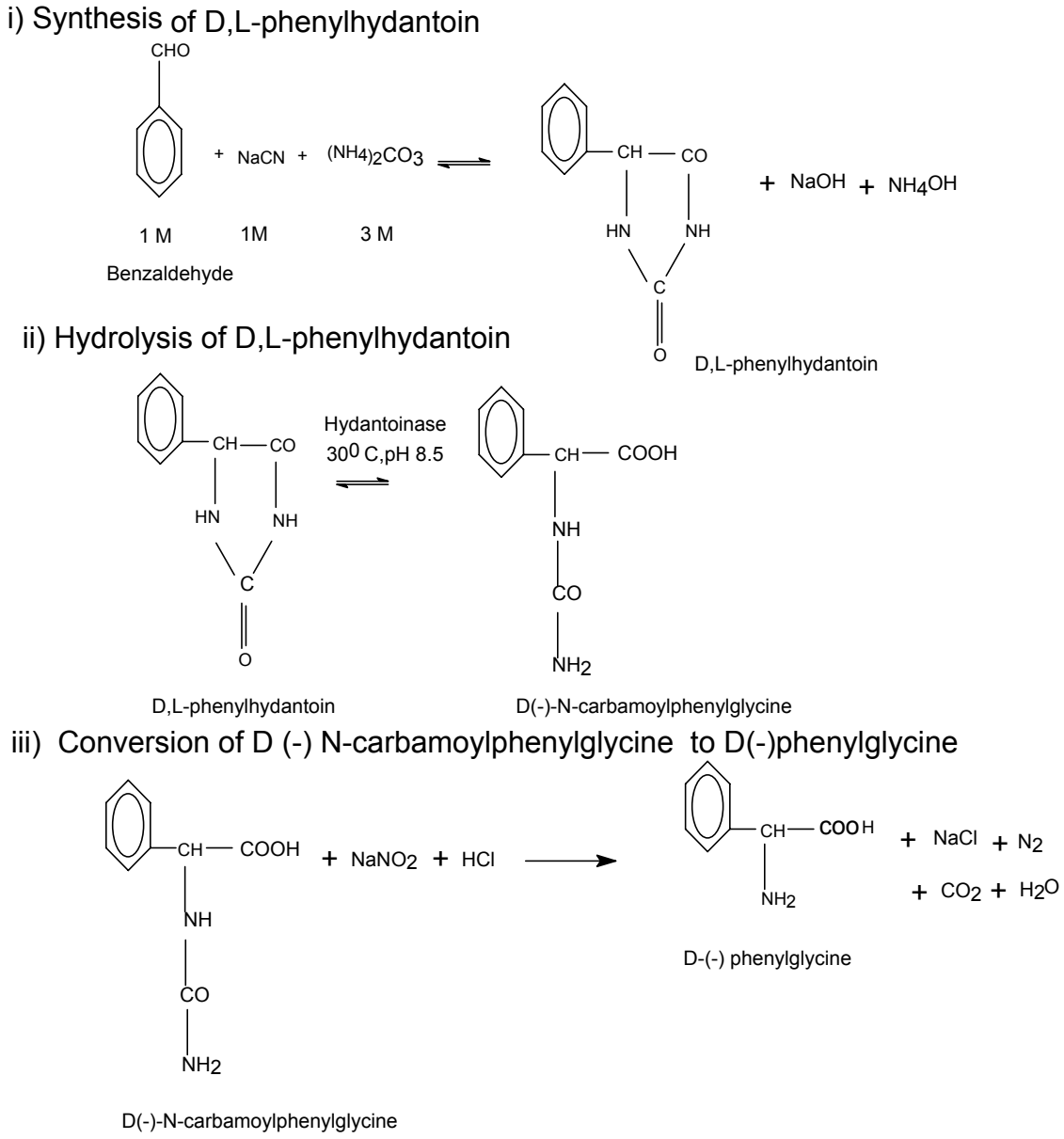

Fig. 1: Synthesis of D(-)phenylglycine from D,L-phenylhydantoin.

Table 1: Assay of hydantoinase enzyme

Substrate	**cells/enzyme**	**reaction condition**	**reaction stopped by**	**colour reagent**	**References**
200µmoles MeH/ml	0.1-0.5 mg protein/ml	pH 8.5, t= 30^0C	TCA(final conc. 40mg/ml)	2 ml mixt., 1 ml 10% PDAB in 12N HCl, o.d. at 438 nm	Cecere et al. (1975)
117µmoles MeTHEtH	enzyme	vol. 2ml, pH=8.7, t=30^0C, 20min.	0.5 ml 12%TCA	0.5 ml 10%PDAB in 6N HCl, 3 ml d.w., o.d. 420nm	Takahashi et al. (1978)
32.8µmoles substrate./ml	5 mg cells/ml	pH=9.0, t=33^0C, 2 hrs.	proc. as Yamada et al.(1978)		Shimizu et al.(1980)
100 µmoles IPrH	cells/enzyme	2ml, pH=8.0, t=30^0C, 15 min.	0.5ml 12% TCA	0.5ml10% PDAB in 6N HCl, 2ml d.w., 450nm	Morin et al. (1986b)
75µmoles MeH	extract	0.4 ml, pH=9.0, t=40^0C, 10 min.	0.1ml 10%TCA	0.1ml 10% PDAB in 6N HCl, 0.6 ml, d.w., 440nm	Weber & Durham (1996)
Reaction vol. is 30 ml: 100mg PH, 100mg cells (equivalent in 2 ml: 38µmoles PH & 6.7 mg cells)		pH =8.0, t=30^0C, 24 hrs.	procedure as Takahashi et al. (1978)		Gokhale et al. (1996)
50µmoles hydantoin	cells/enzyme	pH=9.0, t=50^0C, 30 min. for cells,	0.1 ml 12%TCA	0.1ml 10% PDAB in 6N HCl, 438nm	Garcia & Azerad (1997)

	15 min. for enzyme

MeH= Methylhydantoin ; t= temperature; PDAB= p-dimethylaminobenzaldehyde; TCA = trichloroacetic acid, MeTHEtH= methylthioethylhydantoin, IPrH= isopropylhydantoin., PH=phenylhydantoin

Methods mentioned in table 1 and some other methods available in the literature were studied and an assay method was designed with some modifications. 1 ml 0.15 M K-phosphate buffer pH 8.7 [0.15 M K_2HPO_4 was dissolved in distilled water, pH adjusted to 8.7], 1.5 ml substrate solution which contain 42.6 μmoles phenylhydantoin and 50 mg cells wet wt.(wet weight to dry weight ratio is 1:0.28) were incubated with a starting pH of 8.7 at 30^0C for 2.5 h. The reaction was terminated by adding 0.2 ml 70% $HClO_4$ and centrifuged for 5 min. 1 ml of supernatant and 1ml 0.15 M K-phosphate buffer were mixed and centrifuged. After 10 min. 2 ml PDAB (p-dimethylbenzaldehyde) reagent [2% PDAB in 2N HCl] was added to the supernatant. Optical density was measured at 438 nm. Activity was expressed as μmoles of N-carbamoylphenylglycine formed per min. per gm cells (wet weight).

Standard Graph

0.1ml- 2 ml 0.5% N-carbamoyl solution was used. Volume made to 2 ml by 0.15 M K-phosphate buffer pH 8.7. 2 ml PDAB reagent was added to it and optical density measured at 438 nm. A Standard graph, mg of N-carbamoyl vs. optical density, was plotted (fig. 2).

Fig. 2: Graph of N-carbamoyl Vs Optical density

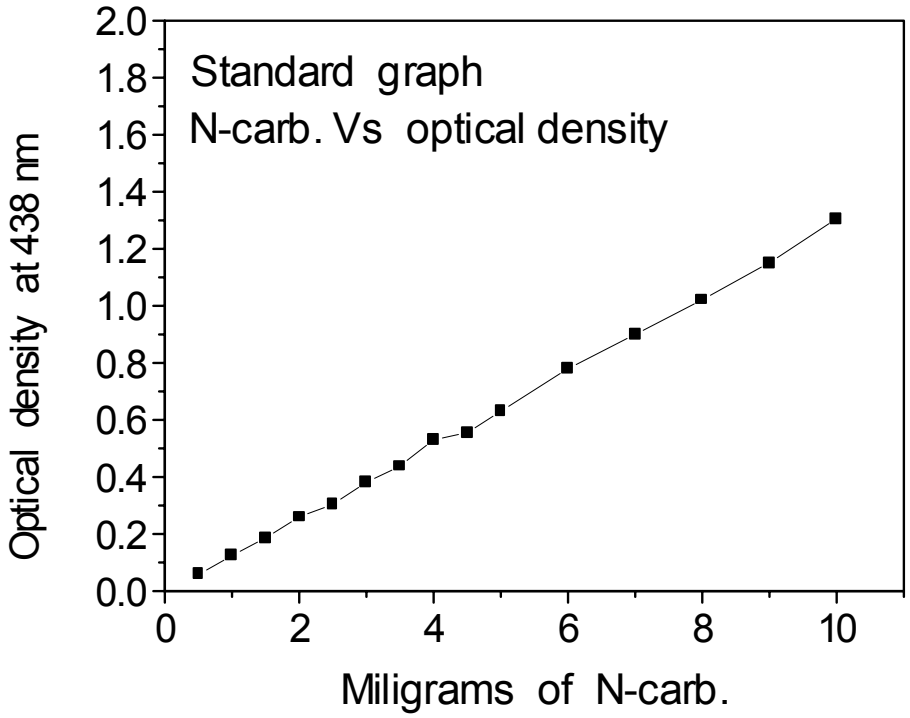

Figure 2: Standard graph of N-carrb.

Culture selection

Gokhale et al. (1996) have screened many cultures for D-hydantoinase production and selected some cultures which hydrolyzed D,L-phenylhydantoin to D(-)-N-carbamoylphenylglycine. We initiated our work using some of these cultures [*Pseudomonas sp.* NCIM 2248, 2875, 2876]. The strains were maintained on nutrient agar slants [beef extract 1, NaCl 0.5, peptone 1 and agar 2%, pH 7.0] at 4^0C. Strains were cultivated on a basal medium [Yeast extract 1, KH_2PO_4 0.15, K_2HPO_4 0.48, $MgSO_4 .7H_2O$ 0.02, $CaCl_2.2H_2O$ 0.002%, 0.1 ml stock trace element solution per 100 ml medium (Composition of stock trace element solution is: $ZnSO_4.7H_2O$ 1.1, $FeSO_4.7H_2O$ 0.1 $MnSO_4.H_2O$ 0.6, $CoSO_4.7H_2O$ 0.03, $CuSO_4$ .5 H_2O 0.004, H_3BO_3 0.006 and KI 0.0001%) , pH 7.0]. Inoculum was developed in tube and flask containing 10ml and 100 ml basal medium respectively and cultivated at 30^0C and 220 rpm for 20 h. Every time 10% inoculum was used. It was transferred to 100ml basal medium and grown at 30^0 C and 220 rpm for 24 h. Cells were centrifuged at 4^0C for 20 min. Hydantoinase activity of cells was estimated. *Pseudomonas sp.* NCIM 2875 had activity that was more than other two cultures therefore *Pseudomonas sp.* NCIM 2875 was used for further study. Aim of this study was to optimize a glucose based medium and parameters to increase hydantoinase activity.

Medium optimization

Experiments were performed to optimize medium composition for increasing growth and hydantoinase activity. Basal medium the was starting point of this study. From it, a glucose-based medium was developed. Carbon sources, nitrogen sources, salts, inoculum age, harvesting time, C/N ratio, pH, temperature, inducers etc. were optimized. Concentrations of ingredients were optimized. The entire study was carried out in shake flasks. Inoculum was developed in a 10ml tube and added to 100ml basal medium and grown at conditions as mentioned earlier. Experimental flasks were inoculated with this inoculum cultivated at 30^0C and 220 rpm for 24 h. They were harvested and centrifuged at 4^0C for 20 min. Wet weight of cells was measured and hydantoinase activity of cells determined.

Strain: *Pseudomonas sp.* NCIM 2875 used for study.

Table 2: **Basal medium**

Ingredients	**%**
Yeast extract	1.0
KH_2PO_4	0.15
K_2HPO_4	0.48
$MgSO_4.7H_2O$	0.02
$CaCl_2.2H_2O$	0.002
trace element stock solution	0.1 ml/ 100ml media
volume and pH	100ml; 7.0

pH of Basal Medium

Inoculum was developed in tubes and transferred to 100ml flasks containing basal medium with pH 7.0. It was grown at 220 rpm and 30^0C for 20 h. Basal medium having pH 7.0, 7.5 and 8.0 was prepared and inoculated. It was cultivated at 220 rpm and 30^0C for 24 h. Every time 10% inoculum was used. Flasks were harvested and cells collected by centrifugation. Hydantoinase activity of cells was determined. It is presented in table 3.

Table 3: Effect of initial pH on Enzyme activity

pH	**activity (units)**
7.0	0.41
7.5	0.43
8.0	0.54
8.5	0.44

From table 3 it was clear that pH 8 gave the best results in terms of activity, however as further experiments indicated, we obtained higher cell mass at pH 7.5. Since the

enzyme was intracellular, the need for quantifying enzyme activity vis a vis cell growth was important.

(b) Replacement of yeast extract partly by peptone:

Yeast extract in basal medium was partly replaced by peptone. 1% yeast extract in the basal medium was replaced with 0.2% yeast extract and 0.8% peptone. We have used both pH 7.5 and 8.0. Activity of cells was estimated. Total weight of cells grown at pH 7.5 was more and therefore the total activity per flask was 2.07 Units. Cell mass in flasks grown at pH 8.0 was much less therefore the total activity per flask was 1.65 units. One observation was that while selecting different carbon sources, a high sugar content resulted in a dramatic fall in pH

Carbon Sources

In an effort to replace yeast extract and peptone, which would incur a high cost on scaling up, cheaper carbon sources were experimented with. Effect of different carbon sources on growth and activity was investigated. The inoculum was developed as mentioned before. Medium composition of experimental flasks was: carbon source 1%, yeast extract 0.2, peptone 0.8, KH_2PO_4 0.15, K_2HPO_4 0.48, $CaCl_2.2H_2O$ 0.002% and trace element stock 0.1ml/100ml media, vol. 100ml, pH 8.0. Cell were harvested at 24 hrs. Cells quantity was weighed and hydantoinase activity of cells estimated. Results are presented in table 4.

Table 4: Use of carbon sources for hydantoinase production

Carbon source 1%	**Final pH**	**Cells % (wet wt.)**	**Activity : Units**
Control	8.6	0.7	1.63
Glucose	7.6	1.35	1.93
Fructose	7.6	1.25	1.83
maltose	8.1	0.81	1.75
mannitol	7.6	1.6	1.76
glycerol	7.6	1.45	1.47
lactose	8.2	0.68	1.5
xylose	7.5	1.1	1.48
Sorbitol	8.6	0.71	1.39
citric acid	8.7	1.3	1.14
Sucrose	8.6	0.74	0.99

Nitrogen sources: *organic nitrogen*

Experiments were carried out using soya peptone, casein acid hydrolyzate, soyabean meal, cottonseed meal and casein-soybean enzymatic digest in the medium using the same inoculum procedure outlined earlier. Composition of experimental flasks was: yeast extract 0.2, KH_2PO_4 0.15, K_2HPO_4 0.48, $MgSO_4.7H_2O$ 0.02, $CaCl_2.2H_2O$ 0.002%, trace element stock solution 0.1 ml/100ml media and other ingredients. Initial pH was 7.5 and 8. Cells were harvested after 24 h and wet weight determined. Hydantoinase activity of cells was estimated. Results are given in table 5.

Table 5: Use of organic nitrogen sources for hydantoinase production

Other ingredients %	**Initial pH**	**Final pH**	**cells % (wet wt.**	**Activity(units)**
peptone 0.8	7.5	8.2	1.1	2.00
peptone 0.8; glucose 1	7.5	7.5	1.65	1.45
soya peptone 0 .8	7.5	8.3	0.75	1.19
soya peptone 0.8; glucose 1	7.5	7.5	1.86	0.56
casein acid hydrolyzate 0.8; glucose 1	7.5	7.5	1.5	0.3
soyabean meal 0.8; glucose 1	7.5	7.4	1.6	0.6
cotton seed meal 0.8; glucose 1	7.5	7.4	1.5	0.9
casein-soyabean enzymat. digest 0.8; glucose 1	7.5	7.5	1.1	1.6
peptone 0.8	8	8.6	1.2	1.60
peptone 0.8; glucose 1	8	7.7	1.1	1.62
soya peptone 0.8	8	8.6	1	1.33
soya peptone 0.8; glucose 1	8	7.8	1.82	1.56
casein acid hydrolyzate 0.8; glucose 1	8	7.8	1.5	1
soyabean meal 0.8; glucose 1	8	7.8	1.7	0.7
cotton seed meal 0.8; glucose 1	8	7.8	1.2	1.3
casein-soybean enzymatic digest 0.8; glucose 1	8	7.8	1.1	1.7

Activity decreased at initial pH 7.5 when glucose was used but remained unaffected at pH 8.0. Casein-soyabean enzymatic digest with glucose had comparable activity with control at both pH. Soya peptone with glucose has comparable activity with control at pH 8.0. Casein acid hydrolyzate, soybean meal and cottonseed meal with glucose had less activity than control when grown at both the values of pH.

Nitrogen sources: *inorganic nitrogen*

Different inorganic nitrogen sources were used as major nitrogen source while yeast extract and peptone were used in small amounts, following standard inoculum procedure. Experimental media composition was: KH_2PO_4 0.15, K_2HPO_4 0.48, $MgSO_4.7H_2O$ 0.02, $CaCl_2.2H_2O$ 0.002%, trace element stock solution 0.1 ml/100ml and other ingredients. Initial pH of medium was adjusted to 7.5. Cells were harvested after 24 h and analysed. Results are shown in table 6. We have replaced $NaNO_3$ by KNO_3 and experiment was carried out. Results are given in table 6A.

Table 6: Effect of inorganic nitrogen sources on hydantoinase activity

Other Ingredients %	**Final pH**	**Cells % (wet wt.)**	**Activity (units)**
Yeast extract (YE) 0.2; peptone (pep.) 0.8	8.7	0.9	2.10
YE 0.2; pep. 0.8; glucose 1	7.4	1.4	1.54
YE 0.1; pep. 0.1; glucose 1; $(NH_4)_2SO_4$ 0.8	6.8	0.98	0.96
YE 0.1; pep.0.1; glucose 1; NH_4Cl 0.8	6.9	0.97	1.67
YE 0.1; pep. 0.1; glucose 1; $NaNO_3$ 0.75	8	0.95	2.36
YE 0.1; pep.0.1; glucose 1;$NH_4H_2PO_4$ 1.25	7.3	0.85	0.5
YE 0.1; pep. 0.1; glucose 1; urea 0.4	7.5	0.97	0.24
YE 0.1; pep. 0.1; glucose 1; urea 0.2;	6.9	1.18	0.28

$(NH_4)_2SO_4$ 0.6			
YE 0.1; pep. 0.1; glucose 1; urea 0.6; $(NH_4)_2SO_4$ 0.2	7.3	1.16	0.3
YE 0.1; pep. 0.1; glucose 1; urea 0.4; $(NH_4)_2SO_4$ 0.4	7.4	1.15	0.29
YE 0.1; pep. 0.1; glucose 1; NH_4NO_3 1	4.8	0.25	0.09
YE 0.1; pep. 0.1; glucose 1; $(NH_4)_2CO_3$ 0.75	8.7	0.18	-

Table 6 A: Comparison of $NaNO_3$ and KNO_3

Ingredients %	**Final pH**	**Cells % (wet wt.)**	**Activity (Units)**
$NaNO_3$ 0.75	8	1.02	2.45
KNO_3 1	7.8	1.0	2.38

Effect of inoculum tube age; inoculum flask age and harvesting time

The inoculum age of tube, flask and harvesting time are factors affecting the synthesis of hydantoinase activity. Therefore in the experiments mentioned below inoculum tube age, inoculum flask age and harvesting time were optimized. Experimental medium composition was: yeast extract 0.1, peptone 0.1, glucose 1, $NaNO_3$ 0.75, KH_2PO_4 0.15, K_2HPO_4 0.48, $MgSO_4.7H_2O$ 0.02, $CaCl_2.2H_2O$ 0.002% and trace element stock solution 0.1 ml/100ml. This medium is named as medium A. Initial pH of medium was adjusted to pH 7.5. Results are presented in figure 3A and 3B.

Fig. 3A: Effect of inoculum age on hydantoinase activity

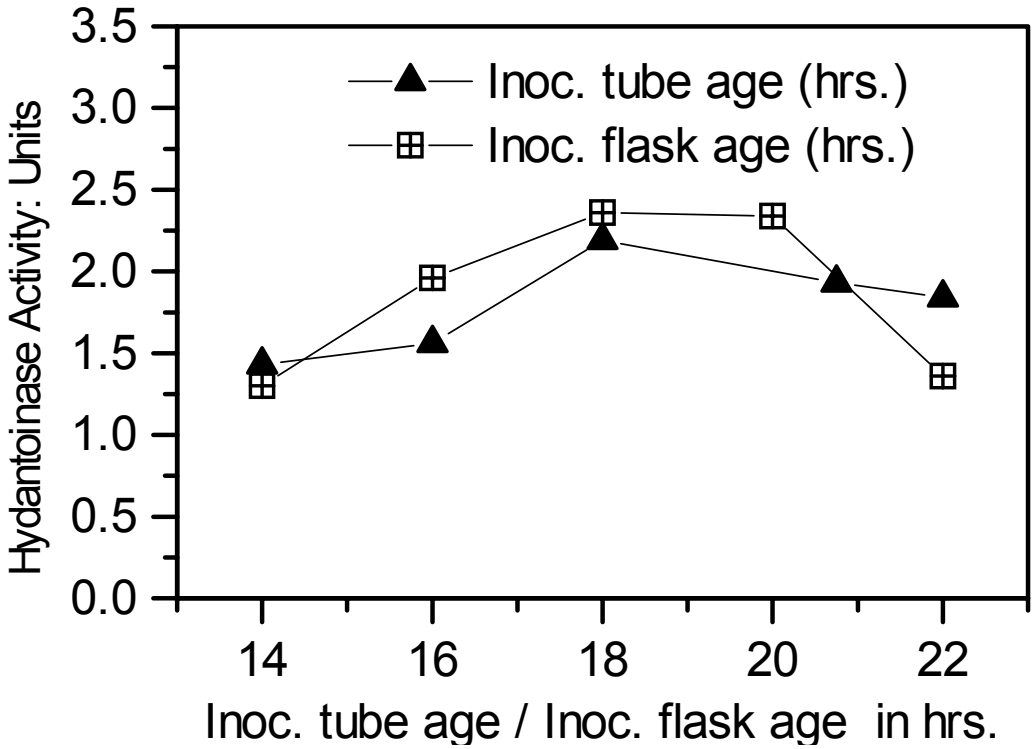

Figure 3 A: Optimum inoculum tube & inoculum flask age

Fig. 3B: Effect of harvesting time

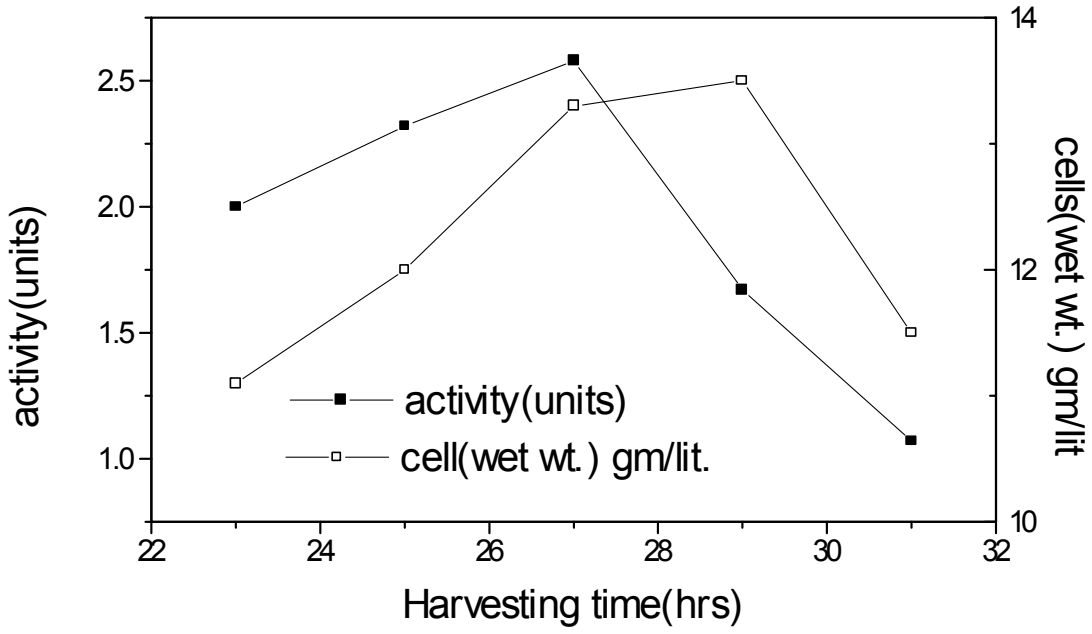

Figure 3 B: Effect of harvesting time on growth and hydantoinase activity

Effect of different inorganic salts

The effect of different inorganic salts on hydantoinase activity was investigated. Tubes and flask were inoculated with culture having an age of 20 hrs. Medium A with different concentrations of $FeSO_4.7H_2O$; $Fe(NO)_3.9H_2O$; $ZnSO_4.7H_2O$; $MgSO_4.7H_2O$; $CaCl_2.2H_2O$; H_3BO_3; $MnSO_4.H_2O$ and trace elements stock solution was used. Initial pH was 7.5. Flasks were harvested after 27 h and the hydantoinase activity determined. It was observed that hydantoinase activity was unaffected by increasing salt concentrations.

Effect of pH

Inoculum tube and flask age = 20 h

Experimental medium =Medium A

Initial pH = 7.0 -9.5

Harvesting time = 27 h

Effect of yeast extract & peptone

In control experiment medium A with initial pH 8 was used. We obtained 3.15 units activity. 3 units activity was obtained when yeast extract and peptone were omitted from medium. This indicates that yeast extract and peptone has minor effect or no effect on hydantoinase activity. Still we have used small amounts (0.1%) of yeast extract and peptone in medium to fulfil trace requirements of culture.

Effect of temperature

Medium A with initial pH 8 was used. Experiments were performed at 30^0C, 35^0C and 40^0C. At 35^0C and 40^0C negligible growth, and no activity were observed, indicating that the culture is sensitive at higher temperatures. At 30^0C a hydantoinase activity of 2.9 units was obtained.

Effect of inducers

Medium A was used with initial pH 7.5. Inducers were added to medium A to find out their effect on enzyme activity. Dihydrouracil, hydantoin and phenylhydantoin were used as inducers with different concentrations. Hydantoinase activity was determined (fig.5).

Effect of C/N ratio

Effect of Carbon: Nitrogen (C/N) ratio on growth and hydantoinase activity was studied. Medium A was used and the quantity of $NaNO_3$ was changed to adjust the C/N ratio. Initial pH was adjusted to 7.5. Results are presented in fig. 6.

Figure 4: Effect of pH

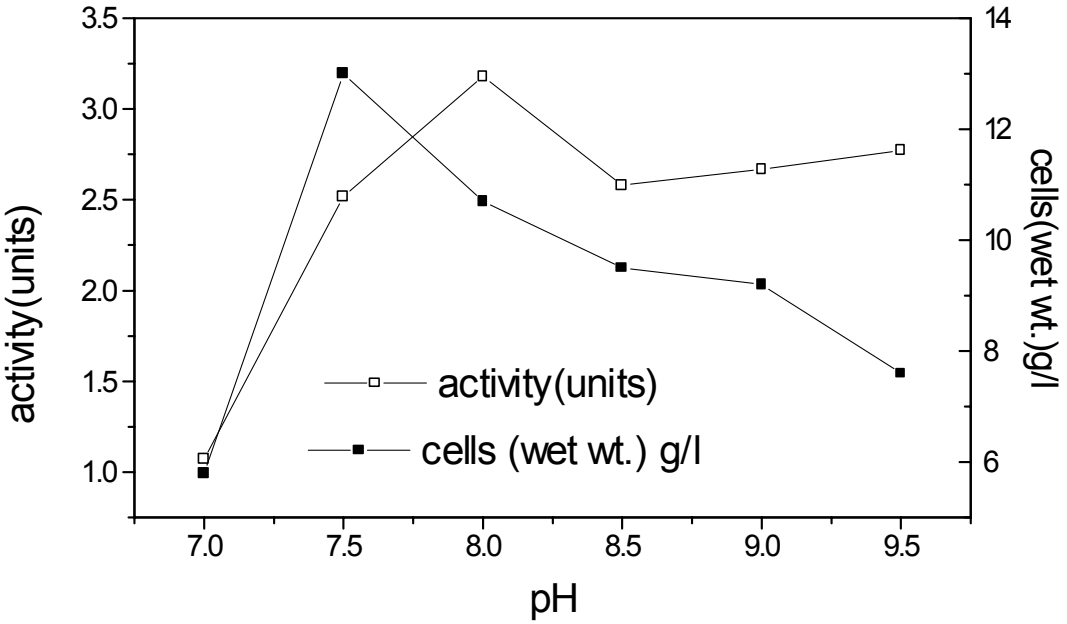

Fig. 4: Effect of pH on growth and hydantoinase activity.

Figure 5: Effect of inducers

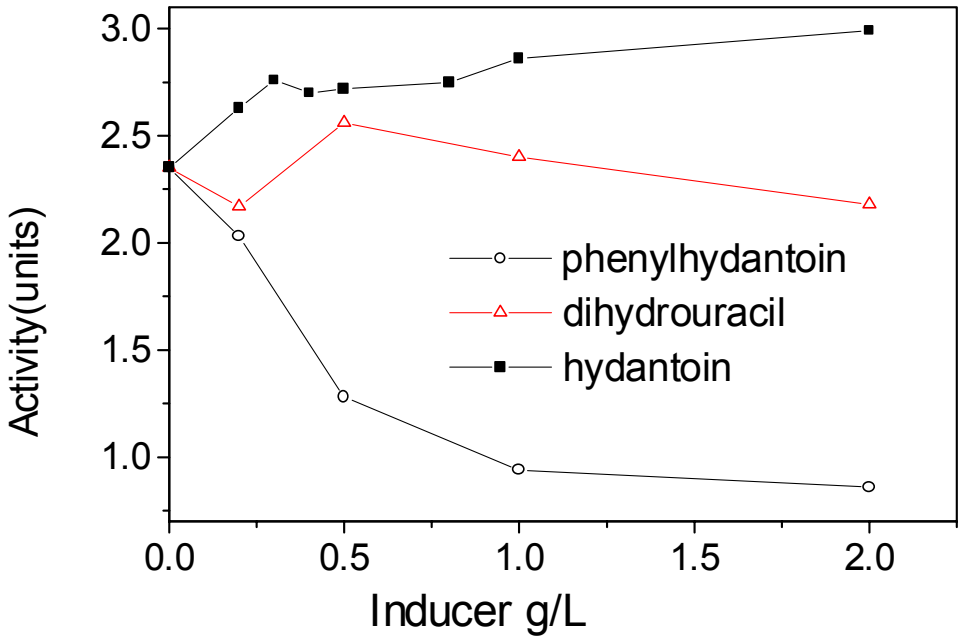

Fig. 5: Effect of inducers on hydantoinase activity.

Figure 6: Effect of C/N ratio

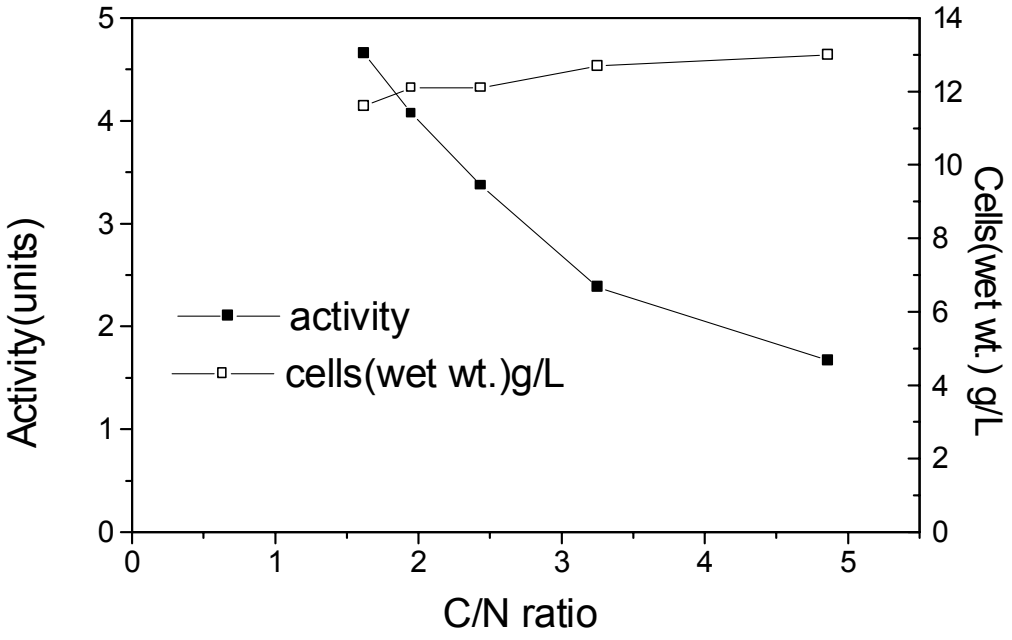

Fig. 6: Effect on C/N ratio on growth and hydantoinase activity.

Growth under optimum condition

Pseudomonas sp. 2875 was cultivated on an optimized medium under optimized culture conditions. An inoculum grown for 20 h was used. Optimized medium composition was: glucose 1, KH_2PO_4 0.15, K_2HPO_4 0.48, $NaNO_3$ 1.5, yeast extract 0.1, peptone 0.1, $MgSO_4.7H_2O$ 0.02, $CaCl_2.2H_2O$ 0.002%, trace elements 0.1 ml per 100ml and hydantoin 0.03%. Flasks were harvested at 27 h. 1.27% cells (wet weight) were obtained having a total hydantoinase activity of 4.63 units.

Comparison of NB–medium with optimized medium

Comparison of hydantoinase activities obtained by growing *Pseudomonas sp*. 2875 on NB (nutrient broth) medium and optimized medium, was done. We have obtained 3.69 and 4.46 units activities using nutrient broth and optimized medium respectively.

Discussion

Carbon sources

All studies were carried out in shake flasks. Three cultures (*Pseudomonas sp.* NCIM 2248, 2875 and 2876) were tested for hydantoinase production but it was observed that *Pseudomonas sp.* NCIM 2875 had more activity than other two cultures. Therefore *Pseudomonas sp.* NCIM 2875 was selected for further study. When basal medium with different pH 7.0, 7.5, 8.0 and 8.5 was used for hydantoinase production we observed that pH 8.0 was best (table 3). The quantity of yeast extract in the medium was reduced from 1% to 0.2% and at the same time 0.8% peptone was introduced in basal medium. Increased in enzyme activity was observed because of the addition of peptone.

Cultivation of *Pseudomonas sp* was conducted using glucose, fructose, maltose, mannitol, glycerol, lactose, xylose, sorbitol, citric acid and sucrose as carbon sources. Experiments were carried out at pH 8, because of high pH drop encountered during growth Results presented in table 4. indicated respectively 30.06 and 39.26% less activities were obtained than the control when citric acid and sucrose were used as carbon source. Glycerol, lactose, xylose and sorbitol gave 9.81, 7.97, 9.2 and 14.72 % less activities than control respectively. With glucose, fructose, maltose and mannitol the hydantoinase activity increased by 18.4, 12.26, 7.36 and 7.97% respectively than the control. Glucose flasks showed more hydantoinase activity than all used carbon sources. Cell yield was nearly doubled by glucose. Maximum cell yield was observed with mannitol. From the results we decided to use glucose as carbon source for further work.

Cotoras and Wagner (1984) have carried out growth of *Agrobacterium radiobacter* BH 20 using different carbon sources. They have used *Agrobacterium radiobacter* BH 20 in preparation of L-amino acids. Hydantoinase activity of *Agrobacterium radiobacter* BH 20 was significantly increased by fructose but in our case we obtained little increase. By using fructose they obtained 4.5 times more activity than glucose but we had similar activities on both substrate. Using glycerol and sucrose Cotoras and Wagner (1984) had obtained 17 and 20% activities but we had 76.16 and 51.29 % activities respectively as compared with glucose. Morin et al. (1986b) have grown *Pseudomonas fluorescens* DSM 84 and used it in the preparation of N-carbamoyl-valine. They observed 76.92, 41.66 and 39.74 % less hydantoinase activities than control using glucose, glycerol and fructose respectively but we had 18.4 and 12.26% more activities than control using glucose and fructose respectively and by using glycerol 9.81% less activity than control. Morin et al. (1986b) observed 7.05, 11.53 and 5.12 % less activities using sorbitol, maltose and molasses are comparable with control respectively. Weber and Durham (1996) had carried out growth of *Agrobacterium tumefaciens* using glucose and maltose. They had used cell extracts to hydrolyze phenylhydantoin.

Garcia and Azerad (1997) grew hydantoinase-producing microorganisms on media containing 1% sachharose. Achary et al. (1997) carried out growth of *Agrobacterium radiobacter* NRRL B 11291 using different carbon sources. *Agrobacterium radiobacter* NRRL B 11291 produces hydantoinase which hydrolyses p-hydroxyphenylhydantoin. *Agrobacterium radiobacter* NRRl B11291 had little more activity than control when grown on fructose while we have also got similar trend. Acharya et al. (1997) obtained significant increase in activity than control using sucrose, glucose, maltose, mannitol, lactose and glycerol. Siemann et al. (1999) cultivated *Arthrobacter crystallpoietes* DSM 20117 on media containing sodium lactate and citric acid. It produced D-hydantoinase which converted D,L-phenylhydantoin to its N-carbamoyl Lee et al. (1997) used glycerol as carbon source for D-hydantoinase production from recombinant *E. coli.*

Nitrogen sources

Results obtained using organic nitrogen in medium are cited in table 5. When peptone was used with glucose as carbon source, enzyme activity at pH 7.5 was lower than that of the control but at pH 8.0 activity was comparable with control. When soya peptone was used with glucose at pH 7.5 activity decreased by 61.37% but at pH 8.0 it was found to be comparable with control. When peptone was replaced by casein acid hydrolysate, soybean meal and cottonseed meal enzyme activities were less than those of the control. When casein-soybean enzymatic digest was used in above media, activities were comparable with those of control at both pH values. Peptone and soy peptone were found to be good nitrogen sources. Siemann et al. (1999) had grown *Arthrobacter crystallpoietes* DSM 20117 on media containing 0.1% yeast extract. Morin et al. (1986a) observed more activity on yeast extract and less on peptone, which is contrary to our results.

Different inorganic nitrogen sources [$(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, $NH_4H_2PO_4$, NH_4NO_3, $(NH_4)_2CO_3$, KNO_3 and urea] were used in the medium. Yeast extract and peptone were used in small amounts (0.1%). Results are presented table 6 and 6A. No

activity was observed for ammonium carbonate. Poor activities were obtained using ammonium nitrate, urea, urea + ammonium sulphate, and ammonium dihydrogen phosphate. Less activity was obtained with ammonium sulphate. Activity comparable with control was observed with ammonium chloride. More activities were achieved by using $NaNO_3$ and KNO_3 but cell yield was less than control. $NaNO_3$ and KNO_3 are good nitrogen sources. $NaNO_3$ has little more activity than KNO_3. Therefore we have selected $NaNO_3$ as nitrogen source. Thus yeast extract and peptone can be replaced by inorganic nitrogen sources. It was decided, however, to use yeast extract and peptone in small amounts (0.1%) in order to supplement trace requirements. Morin et al. (1986b) have used $NaNO_3$, NH_4NO_3 and $(NH_4)_3PO_4$ as nitrogen sources but they did not obtain growth while we found growth and activity on $NaNO_3$. We observed poor growth and negligible activity on NH_4NO_3. Achary et al. (1997) have cultivated *Agrobacterium radiobacter* NRRL B 11291 on different nitrogen sources (NH_4NO_3, KNO_3, $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$ and NH_4CH_3COO) They observed more activity on NH_4CH_3COO. All other nitrogen sources, except KNO_3, had activity in range of 10-12.5 U/ml. Contrary to our observation they obtained more activity using NH_4Cl than $NaNO_3$. Using NH_4NO_3 we had poor activity but they had more activity. Achary et al. (1997) obtained good activity with $(NH_4)_2SO_4$ while we had less activity than control. Cotoras and Wagner (1984) observed good activity on $(NH_4)_2SO_4$. West (1997) obtained 21 fold increase in dihydropyrimidinase activity of *Burkholderia cepacia* ATCC 25416 by replacing ammonium sulfate with pyrimidine or dihydropyrimidine bases.

Inoculum age and harvesting time

Optimization of inoculum age of tube inoculum and flasks inoculum was done (Fig.3A). The optimum time was found to be 18-20 h for tube and flask inoculum. Harvesting time was optimized (fig. 3B). Optimum time of harvesting is 27 h. When Weber and Durham (1996) grew *Agrobacterium tumefaciens* ATCC 55632 in fermenter, they reported an optimum harvesting time of 18-24 h. They used a cell extract of *Agrobacterium tumefaciens* ATCC 55632 for hydrolysis of

phenylhydantoin. An inoculum with an age of 42 and 45 h, was used by Siemann et al.(1999). Siemann et al. (1999) have used 27 h harvesting time for fermenter.

Salt concentrations

Effect of different concentrations of $MgSO_4 7H_2O$; $FeSO_4.7H_2O$; $Fe(NO_3)_3.9H_2O$; $CaCl_2.2H_2O$; $ZnSO_4.7H_2O$; H_3BO_3 ; $MnSO_4.H_2O$ and trace elements stock solution on hydantoinase activity were studied. We did not observe any discernable effect of salt concentrations on enzyme activity. Cotoras and Wagner (1984) carried out growth of *Agrobacterium radiobacter* BH 20 and found 0.001% $MnSO_4.H_2O$ was an essential constituent of the medium. Syldatk et al. (1987) reported that Mn^{+2} was required for hydanoinase activity of *Arthrobacter sp.* BH 20. Yungjin Ind., Korea (1992) observed an increase in activity for K_2HPO_4. Weber and Durham (1996) used Ca and Mg in their medium for growth of *Agrobacterium tumefaciens* ATCC 55632. Achary et al. (1997) have observed increase in hydantoinase activity by Mn (0.005%). Our observations show no discernable effect of Manganese concentration on hydantoinase activity. Achary et al. (1997) observed that Fe, Zn and Co concentrations had no effect hydantoinase activity an observation supported by our data. Siemann et al. (1999) grew *Arthrobacter crystallpoietes* DSM 20117 on medium containing Ca, Mg, Zn and Mn.

pH

Effect of pH on cell growth and hydantoinase activity was investigated. Maximum activity was observed at pH 8.0 and cell yield at pH 7.5 (fig.4). Although activity at pH 7.5 was less than pH 8.0, we have used pH 7.5 because cell yield is more than that at pH 8.0. Durham and Weber (1995) have used pH 7.2 for growth of *Agrobacterium tumefaciens* 47C. Siemann et al. (1999) used pH 7.0 for growth of *Arthrobacter crystallpoietes* DSM 20117.

Temperature

Pseudomonas sp. NCIM 2875 was cultivated at 30^0, 35^0 and 40^0C. We observed negligible growth and enzyme activity at 35^0C. There was no activity and almost no growth at 40^0C . Temperature 30^0C was found to be best for growth and hydantoinase activity. Gokhale et al. (1996); Garcia and Azerad (1997) and Siemann et al. (1999) have used 30^0C temperature for growth. Weber and Durham (1996) have grown *Agrobacterium tumefaciens* ATCC 55632 at 28^0C. D-hydantoinase activity of *Bacillus stearothermophilus* SD-1 is stable at 55^0C. It forms N-carbamoyl from hydantoin derivatives [Kim et al.(1997c)].

Yeast extract and peptone

Pseudomonas sp. NCIM 2875 was cultivated on medium with and without yeast extract and peptone. Hydantoinase activities were compared. It was observed that when yeast extract and peptone were omitted from medium 4.76% less activity was obtained. It seems that requirement of yeast extract and peptone is restricted to small amount that may supply essential co-factors. Therefore only small amounts of yeast extract and peptone (0.1%) were used in the medium to avoid co-factor deficiency. Weber and Durham (1996) grew *Agrobacterium tumefaciens* ATCC 55632 in a medium containing yeast extract and peptone in the range 0-1%. Siemann et al.(1999) used 0.1% yeast extract in their medium.

Inducers

Phenylhydantoin, dihydrouracil and hydantoin were tested as inducers in the medium. No significant increase in enzyme activity was achieved using inducers. 17-27% increase in activity was obtained by using 0.03-0.2% hydantoin as inducer (fig.

5). 0.03% hydantoin was used as inducer in the medium. Yamada et al. (1978) have reported 3-fold increase in activity by using 0.1% uracil as inducer. Morin et al. (1986b) and Siemann et al. (1999) have used 0.1% hydantoin as inducer but Morin et al. (1999) did not find increase in activity. *Agrobacterium tumefaciens* ATCC 55632 required 0.1-1% hydantoin as inducer in medium [Weber and Durham (1996)]. Cotoras and Wagner (1984) observed that 0.025% 5-indolylmethylhydantoin acted as an inducer for their culture. Syldatk et al. (1987) found that D,L-5-indolylmethylhydantoin acted as an inducer whereby increased hydantoinase activity was obtained for *Arthrobacter sp.* BH 20. Young-Jin Pharm. Industry, S. Korea (1991) reported that 0.3% dihydrouracil was essential as inducer for *Streptomyces sp*. Y-183 but in case of our culture we found negligible increase in activity by dihydrouracil. Meyer and Runser (1993) studied the effect of 2,4-thiouracil as inducer on enzyme activity of *Agrobacterium sp.* IP I-671. They found that hydantoinase activity increased by 40 fold and activity of N-carbamoylamino acid aminohydrolase by 15 fold. Morin et al. (1995b) observed that 0.005% thymine acted as an inducer and increased enzyme activity of *P. putida* DSM 84 by 80 fold in continuous culture. Garcia and Azerad (1997) used 2-thiouracil as inducer. Li and Hu (1999) found 30 times increase in activity using methylthioethylhydantoin as inducer when they grew *P. putida 9801*.

C/N ratio

Optimization of C/N ratio was studied. Maximum activity was achieved at a C/N ratio 1.62. At this ratio the concentration of $NaNO_3$ in the medium was 1.5%. Cell yield decreased (fig.6). *Pseudomonas sp*. 2875 was grown on an optimized medium and under optimized conditions, when 4.63 units hydantoinase activity and cell yield 1.27% (wet wt.) was achieved. Ratio of wet weight of cells to dry weight cells was found to be approx. 1:0.28. When activity was calculated on basis of per mg of dry weight cells then hydantoinase activity was 0.0165units (micromoles of N-carbamoylphenylglycine formed per min. per mg dry weight cells)

Table 7: Comparison of activities

Culture	**activity (units)**	**Temp. ^{0}C**	**References**
Agrobacterium tumefaciens CIP67.1	0.0138 units*	50	Garcia and Azerad (1997)
Pseudomonas sp. ATCC 43648	0.200 units*	50	
Pseudomonas fluorescens CIP69.13	0.0152 units*	50	
Arthrobacter crystallpoietes DSM 20117 .	17.9 U/g (dry wt. cells)**	50	Siemann et al. (1999)
Pseudomonas sp. NCIM 2875	0.0165 units*	30	present study
Pseudomonas sp. NCIM 2875	16.53U/g (dry wt. cells)**	30	present study

*units = micromoles of N-carbamoylphenylglycine per min. per mg dry weight cells.

** units = U/g (dry wt. cells)

From table 7 it was clear that our culture had more activity than *Agrobacterium tumefaciens* CIP 67.1 and *Pseudomonas fluorescens* CIP 69.13 but less than that of *Pseudomonas sp.* ATCC 43648. We obtained a cell yield 3.55 g/l (dry wt.) while Siemann et al. (1999) obtained 2.8 g/l for their culture. We obtained less activity as compared to the results of Siemann et al. (1999). Shimuzu et al. (1980) carried out biotransformation of hydantoin derivatives using cells of *Pseudomonas straita* IFO12996. This strain converted 2-thienylhydantoin at the maximum reported rate. However the rate for phenylhydantoin conversion was less. Bioconversion of phenylhydantoin was carried out by Garcia & Azerad (1997), Durham Weber (1995), Keil et al. (1995) and Lee et al. (1994) using hydantoinase enzyme at a higher temperature. Bioconversion of phenylhydantoin and other compounds was reported by Yamada (1998).

Pseudomonas sp. NCIM 2875 was cultivated on nutrient broth (NB) medium and optimized medium. We observed 20% more activity for the optimized medium. This glucose based optimized medium with small supplementation with yeast extract

and peptone can be called as semi-synthetic. In our study we observed that yeast extract and peptone could be omitted from the medium but this adversely affected growth and enzyme production. 0.1% yeast extract and peptone in the medium was able to fulfill trace requirements. Table 8 cites media used by various researchers for production of hydantoinase. In this discussion we have classified media into three categories.

i**)Complex**: Medium containing complex ingredients as major carbon and nitrogen source.

ii) **Semi-synthetic**: Medium containing mainly synthetic ingredients with small quantities of complex ingredients.

iii) **Synthetic**: Medium containing only synthetic ingredients.

Table 8

Medium type	**medium composition**	**references**
complex	polypeptone, yeast extract, meat extract, peptone, salt-media	Lee et al.(1994)
complex	nutrient broth	Gokhale et al. (1996)
complex	peptone 1, yeast extract 1%, $(NH_4)_2SO_4$ 0.5% , salt-media	Weber & Durham (1996)
complex	nutrient broth	Sudge et al.(1998)
semi-synthetic	glucose, yeast extract, salts-media	Olivieri et al.(1981)
semi-synthetic	fructose, yeast extract, ammonium sulphate, inducer, salt-media	Cotoras & Wagner (1984)
semi-synthetic	glucose, yeast extract, ammonium sulphate, salts-media	Yokozeki et al. (1987a)
semi-synthetic	yeast extract, peptone, amm. sulphate, succinate, glucose, maltose, inducer, salt-media	Weber & Durham (1996)

semi-synthetic	peptone, yeast extract, sachharose, inducer, salt-media	Garcia & Azerad (1997)
semi-synthetic	yeast extract, carbon sources, salts-media	Achary et al. (1997)
semi-synthetic	sodium lactate, citric acid, yeast extract, amm. sulphate, inducer, salt-media	Siemann et al.(1999)
semi-synthetic	glucose, $NaNO_3$, 0.1% yeast extract, 0.1% peptone, inducer, salt-media.	present study
synthetic	glucose, inorganic nitrogen sources, salts-media	Achary et al. (1997)

Besides the above media compositions some more examples are cited in table 9.

Table 9

Source	**medium and growth conditions**	**references**
P. striata IFO 12996	meat extract, glycerol-medium, 0.1% uracil 30^0C, 18 hrs.	Yamada et al. (1979)
P. striata IFO12996	meat extract, YE, pep, uracil-medium, 33^0C, 24 hrs.	Yamada et al. (1978a)
P. putida 73104	YE-lactic acid-medium with 5-Me-H inducer 0.2%, 28^0C, 13.5 hrs.	Sun (1983b)
Arthrobacter DK200	YE, Pep, glucose, N-carbamoyl tryptophan-medium 30^0C, 20 hrs.	Kitagawa et al.(1985)
Bacillus brevis IFO 12333	YE-salts medium	Powell & Power (1988)
Agrobacterium EO III	complete medium, 35^0C	Ohleyer (1989)
P. putida DSM 84	Yeast extract, glycerol, salts-medium	Chevalier et al.(1989a)
Flavobacterium HP-27	glucose-CSL-medium, 26^0C, 48 hrs.	Nakayama & Ozawa(1990)

Agrobacterium sp. IP-I 671	sucrose, peptone, yeast extract, salts-medium 30^0C, 20 hrs.	Runser et al.(1990)
Peptococcus anaerobius CRDA 303	opt. pH=6.5-9.5, opt. temp. 60^0C; pH stability =5-9.5, temp.55^0C; storage stability 4^0C 14 days in aerobic condition.	Morin et al.(1991)
Pseudomonas sp. KBEL101	glucose, yeast extract, salts-medium, pH 5.5, temp. 30^0C	Kim et al.(1994)
Agrobacterium sp. I 671	sucrose, peptone, yeast extract, salts, inducer medium, pH 7.0 & 30^0C for 14 h (in fermenter)	Kim & Kim (1995b)
P. desmolyticum NCIM 2112	nutrient broth, 18-20 h	Gokhale et al.(1996)
recombinant *E. coli*	glucose/glycerol, yeast extract, citric acid, $(NH_4)_2HPO_4$, salts-medium.	Lee et al.(1997)
Pseudomonas sp. NCIM 5109 (halophilic)	nutrient broth with 2% NaCl opt. pH 6.5-9, temp.25^0C	Sudge et al. (1998)
P. putida D	YE-glycerol-medium, 5-Me-Th-Et-H inducer, 28^0C, 18 h	Li et al. (1999)
Pseudomonas sp. NCIM 5070 ATCC55940	molasses and CSL, 16-20 h	Gokhale et al. (2000)

opt.= optimum; temp.= temperature; YE = yeast extract; pep = peptone; Me-H= methylhydantoin; Me-Th-Et-H=methylthioethylhydantoin.

Complex, semi-synthetic and synthetic media were used to produce hydantoinase by several researchers. In complex medium nutrient broth, yeast extract, meat extract, peptone, polypeptone etc. are used. In semi-synthetic medium researchers have used glucose, sachharose, sodium lactate, citric acid etc. as carbon sources in the range of 0.075-2%.

Conclusion

A glucose based medium was optimized.. Growth parameters like pH, temp. inoculum age etc. were studied to achieve maximum growth and hydantoinase production. Medium composition was glucose 1, $NaNO_3$ 1.5, yeast extract 0.1, peptone 0.1, hydantoin 0.03% and other ingredients. Optimum pH and temp. were 7.5 and 30^0C respectively. Maximum cell yield and hydantoinase activity were 12.7 g l^{-1} (wet wt.) and 4.63 units respectively. This activity was higher than activity obtained by cultivating cells on nutrient broth (complex medium). Also this activity was higher than the basal medium. The complex medium was replaced by a glucose based medium supplemented with 0.1% yeast extract and peptone. Quantities of yeast extract and peptone were low. By minimizing the concentration of peptone and yeast extract, and including glucose and inorganic nitrogen sources the cost of the medium was substantially decreased.

Chapter 3

Extraction of hydantoinase

Summary

Cells of *Pseudomonas sp.* NCIM 2875 were exposed to different concentrations of solvents, detergents and EDTA. Hydantoinase enzyme was extracted from treated cells into buffer. Extractions were performed using various contact times and buffer extraction times. 11.37% extraction was obtained with EDTA treatment. 24.05 and 24.1% extractions were achieved with tween-80 and sodium dodecyl sulphate treatment respectively. Among the solvents used, ethanol and ethyl acetate treatment have shown maximum extractions i.e. 31.42 and 33.63% respectively.

Introduction

Enzymes are either extracellular or intracellular. Cells can also be used to carry out reactions. When enzymes are bound to cell wall, reactions are faster however when they are substrate has to intracellular . But when they are not bound to cell wall, substrate has to penetrate cell wall, thus reaction rate is slower. In some cases substrate is large therefore it cannot penetrate the cells, in such cases it is necessary to extract the enzyme. This is done by various methods such as certain chemicals to increase pore size to facilitate penetration by substrate. When enzyme is taken out of the cells and used, the reaction rate is faster. Different methods have been used to break cells [Vadehra et al. (1965); Coakley et al. (1977), Darbyshire (1981), Felix (1982); Hustedt et al. (1984); Atkinson et al. (1987); Brummer and Gunzar (1987); Naglak et al. (1990); Schutte and Kula (1990,1993); Price (1992); Xiu et al. (1994)]. Patil and D'souza (1997) have studied permeabilization of *Haloarcula vallismortis*, a halophilic archaebacteria, using enzymatic, physical and chemical methods where they have estimated activity of glyceraldehyde-3-phosphate dehydrogenase.

Cells are disrupted by three methods i.e. physical, chemical and enzymatic. In physical method use of ball mill, ultra-sonication, pressure etc. is done to break cells (table 1A). In chemical method solvents, detergents etc. are used for rupturing or permeabilization of cells (table 1B). In enzymatic method use of lysozyme, chitinase etc. is done. In some cases two methods are combined to break the cells. Some researchers have coupled extraction with enzyme separation method to increase cell disruption.

Table 1A: Physical methods

Disruption of	**Methods**	**Reference**
Microbial cells (b-galactosidase)	Biotex X-25 app., thermal shock & dyno-mill	Poznanski et al. (1979)
Selenastrum capricornutum (chlorophyll)	pyrex grinder & cell homoginizer	Rehnberg et al. (1981)
Streptomyces albogriseolus (glucose isomerase)	Pressure disruption, grinding, ultrasonication and autolysis	Gavristov et al. (1981)
Bacillus subtilis 21/3 (hexamethylene diamine-degrading enzyme)	grinding in sand, ultrasonication, freezing and thawing, homogenization and electric field	Roi et al. (1982)
E. coli	ballistic, ultrasound, hydroextrusion and cryoextrusion.	Mikhaleva (1983)
microorganisms	Netzsch LME 20 mill and Manton Gaulin M3 homogenizer	Schuette et al. (1984)
Bacillus amyloliquefaciens	ultra-sonication, autolysis and ball milling.	Cumming et al. (1985)
Candida tropicalis pK 233	Freeze-blast method	Omori et al. (1989)

microorganisms	dynomill	Xiu and Su (1994)
yeast cells	high pressure CO_2	McLean and Schaschke (1996)
yeast cells	hydrodynamic cavitation	Save et al. (1994)
Lactobacillus casei sp. casei (aminopeptidase)	microfluidizer	Choi et al. (1997)
Saccharomyces cerevisiae (trehalose)	microwave heating	Liu et al. (2000)
E. coli ATCC 11105 (penicillin G acylase)	sonication	Kheirolomoom et al. (2001)

Table 1B: Chemical methods

Disruption of	**Chemicals**	**References**
Saccharomyces cerevisiae (superoxide dismutase)	alcoholic solvent e.g. isopropanol	Tan et al. (1996)
Kluyveromyces lactis (lactase)	chloroform-ethanol	Lou and Chen (1999)
Alcaligenes eutrophus	surfactant-chelant.	Chen et al. (1999)
bacteria	Alkaline solution	Yokoyama and Inoue (2000)
E. coli ATCC 11105 (penicillin G acylase)	toluene/EtOH, guanidine-HCl, guanidine-HCl/EDTA, lysozyme and lysozyme/EDTA	Kheirolomoom et al. (2001)

Downs (1983) has isolated polysaccharides by disrupting cells of *Pseudomonas* NCIB 11592 by using Na dodecyl sulfate followed by protease treatment. Tokuyama Soda Co., Ltd., Japan (1984) carried out disruption cells of *Streptomyces avellaneus* R20 suspended in solvents (alcohol, ketones) and buffer using Dynomill. They have used water-soluble organic solvents. Harrison et al. (1991) pretreated cells with different chemicals e.g. anionic detergents, EDTA, pH treatment; lysozyme before homogenization to get more disruption. Liu et al. (1996) have disrupted cells of *Corynebacterium crenatum* 6282 by giving lysozyme treatment to cells before sonication. Nandakumar et al. (2000) found that cell disruption increases by giving tween-80 treatment before sonication or grinding cells.

Xiu and Su (1993) have isolated alcohol dehydrogenase from *Saccharomyces cerevisiae* by disrupting cells by Dyno-mill and aqueous two-phase system simultaneously. Barthomeuf et al. (1994) have extracted tannase by disrupting cells of *Aspergillus niger* LCF 8 followed by extraction with reverse micellar. Su and Feng (1999) have isolated alcohol dehydrogenase by combining disruption of cells by ball mill and aqueous two-phase system.

The methods like sonication, dynomilling etc. are energy intensive, costly, immobile and very skillful. Still they are used in majority of the cases because other methods have some disadvantages and also not studied in detail. In some cases chemical and enzymatic methods are used successfully. In chemical method solvent immflammation and hazardness limits the use. Enzymatic method is costly. In chemical method if mild conditions are optimized and hazardness is reduced then it will be very useful for breaking of cells. Fenton (1982) has studied release of beta-galactosidase from yeast cells using different solvents. Vadehra et al. (1965) have isolated enzyme from bacterial cells using solvents for cell-rupture. Extraction by chemical methods is cheaper, easy and can be reproduced anywhere. Therefore we have attempted this approach for our study. Literature regarding the use of solvents and detergents for disruption or permeabilization of hydantoinase producing cells is

cited in discussion. This chapter covers work carried out for extraction of hydantoinase from cells. We have used various solvents and detergents to rupture or permeabilize the cells of *Pseudomonas sp.* 2875 followed by extraction with buffer where hydantoinase is extracted from ruptured or permeabilized cells into buffer.

Materials and Methods

Chemicals

Distilled solvents and AR grade chemicals were used. Other chemicals are same as mentioned in materials and methods of chapter 2.

Strain

Pseudomonas sp. NCIM no.2875, obtained from NCIM (Pune), was used for study. It was maintained on nutrient agar [beef extract 1, NaCl 0.5, Peptone 1 and agar 2 %, pH 7.0].

Growth

We have used basal medium [yeast extract 1, KH_2PO_4 0.15, K_2HPO_4 0.48, $MgSO_4.7H_2O$ 0.02, $CaCl_2.2H_2O$ 0.002% and trace element stock solution 0.1 ml/100ml, pH 7.0] for inoculum development.

10% inoculum was used. The composition of trace element stock solution is mentioned in chapter 2. 10 ml basal medium was inoculated and cultivation carried out at 30^0C and 220 rpm for 20 h. 100 ml basal medium was inoculated with this and growth carried out at above conditions. 100 ml experimental medium [Composition: glucose 1, yeast extract 0.1, peptone 0.1, $NaNO_3$ 1.5, KH_2PO_4 0.15; K_2HPO_4 0. 48; $MgSO_4.7H_2O$ 0.02; $CaCl_2.2H_2O$ 0.002 %; trace element stock solution 0.1 ml/100ml and hydantoin 0.03%, pH 7.5.] was inoculated with this inoculum and above conditions used for growth. It was harvested at 27 h and cells were collected by

centrifugation for 20 min. Weight of wet cells was measured and hydantoinase activity of cells estimated. Cells were used for extraction experiments.

Hydantoinase activity of cells

Hydantoinase activity of cells was estimated by method used in chapter 2.

Hydantoinase activity of enzyme

1 ml enzyme and 1.5 ml substrate solution which contain 42.6 μmoles phenylhydantoin were used in assay. Further procedure is as like activity of cells. Hydantoinase activity is expressed in units (μmoles of N-carbamoylphenylglycine formed per min. per enzyme released from equivalent gm wet weight cells).

Extractions

Different solvents, detergents and EDTA (ethylenediaminetetraacetic acid) were used for cell rupture or permeabilization followed by buffer extraction where buffer extracts hydantoinase enzyme from treated cells of *Pseudomonas sp.* NCIM 2875. Concentrations, contact time and buffer extraction time were optimized. Solvents tested for cell treatment were: methanol, ethanol, isopropanol, butanol, isoamyl alcohol, acetone, ethyl acetate, toluene, xylene, hexane, cyclohexane, dichloromethane, ethylene dichloride, dimethyl sulfoxide etc. The detergents studied for cell disruption were sodium dodecyl sulphate, triton X-100 and tween-80. Use of ethylenediaminetatraacetic acid (EDTA) was also tried for cell disruption study.

A] Solvents

1) Toluene

a) Use of cell slurry

Experiments were performed using 10% cell slurry. Extraction was carried out using toluene concentrations 1-7.5 % at 220 rpm for 3, 4.5, 6 and 7.5 h. Supernatant was separated by centrifugation and hydantoinase activity determined.

b) Use of cells (not slurry)

0.5 g cells (wet) were weighed. Toluene was added to have final concentration 5, 15, 20 and 35% w/w [weight (wet wt.) of cells + weight of toluene = total weight. w/w % of toluene = weight of toluene*100 /total weight]. 0.75-6 h contact time was studied. 5 ml 0.15 M K-phosphate buffer pH 8.7 was added to it, mixed and incubated at 30^0C, 220 rpm for 10 and 15 h. Supernatant was separated by centrifugation and analysed.

2) Acetone

0.5 g cells (wet) were weighed. Acetone was added to get final concentration 10-80% w/w [Concentration of acetone = weight of acetone *100 /{weight (wet) of cells (0.5g) + weight of acetone}]. 15 min., 1 h and 3 h. contact time was used. 5 ml 0.15 M K-phosphate buffer pH 8.7 was added and kept at 30^0C and 220 rpm for fixed time. Supernatant was separated by centrifugation and hydantoinase activity estimated. The same procedure was followed for methanol, ethanol, isopropanol and butanol and ethyl acetate with some changes.

3) Ethanol

a) Effect of ethanol concentrations

In addition to above procedure extraction was carried out using 1, 5 and 15% ethanol. Extractions were not obtained by using 1, 5 and 40-80% ethanol (table 2A).

Table 2 A

Ethanol % w/w	**Extraction % (15 min. contact time)**	**Extraction % (1 h contact time)**	**Extraction % (3 h contact time)**
10	-	-	16.31
15	7.43	8.87	9.35
20	3.69	6.31	-

b) Effect of ethanol contact time

Procedure as mentioned in (2) was used with change in solvent concentration and contact time. We have used 10, 12.5 % ethanol and 2, 3, 4, 5 and 6 h solvent contact time (table 2B).

Table 2B

Solvent contact time (h)	**Extraction % (10% ethanol)**	**Extraction % (12.5% ethanol)**
2	12.59	16.85
3	16.06	12.86
4	18.7	11.52
5	14.62	14.63

6	14.98	14.5

c) Effect of buffer extraction time

Procedure like acetone was used with change in solvent concentration, contact time and buffer extraction time. We have used 10% ethanol; 4 & 6 h contact time; and 15, 20 & 25 h buffer extraction time (table 2C).

Table 2C

Buffer extraction time (h)	Extraction % (4 h Contact time)	Extraction % (6 h Contact time)
15	25.99	30.53
20	29.95	31.42
25	12.33	21.54

4) Ethyl acetate

a) Optimization extraction condition

Effect of ethyl acetate concentration, contact time, and buffer extraction time on extraction of was studied. Results are presented in figure 1A-C and table 3

Fig. 1A: Effect of ethyl acetate concentration and contact time

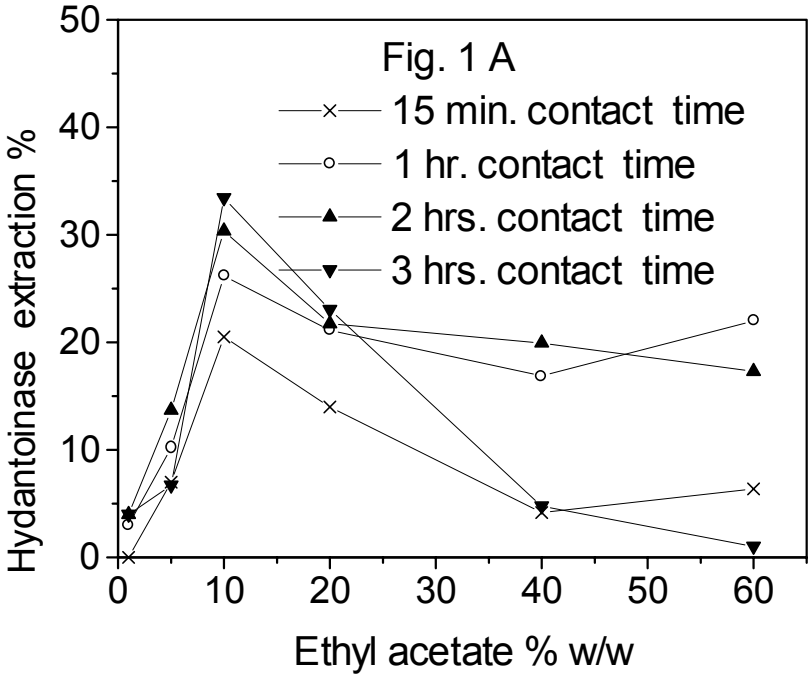

Fig.1 A: Effect of different concentrations of ethyl acetate and contact time on extraction of hydantoinase enzyme.

Fig. 1B: Effect of ethyl acetate concentration and buffer extraction time

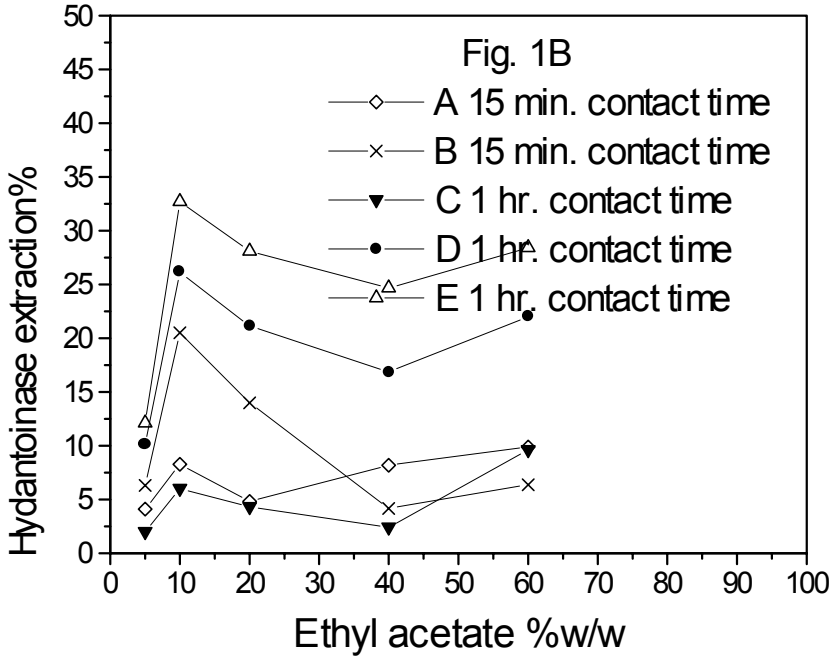

Fig. 1B: Effect of ethyl acetate concentration and buffer extraction time on hydantoinase extraction:

A- 15 min. solvent contact time with 5 h buffer extraction.

B- 15 min. solvent contact time with 10 h buffer extraction time.

C- 1 h solvent contact time with 5 h buffer extraction time.

D- 1 h solvent contact time with 10 h buffer extraction.

E- 1h solvent contact time with 15 h buffer extraction

Fig. 1C: Effect of ethyl acetate concentration and buffer extraction time

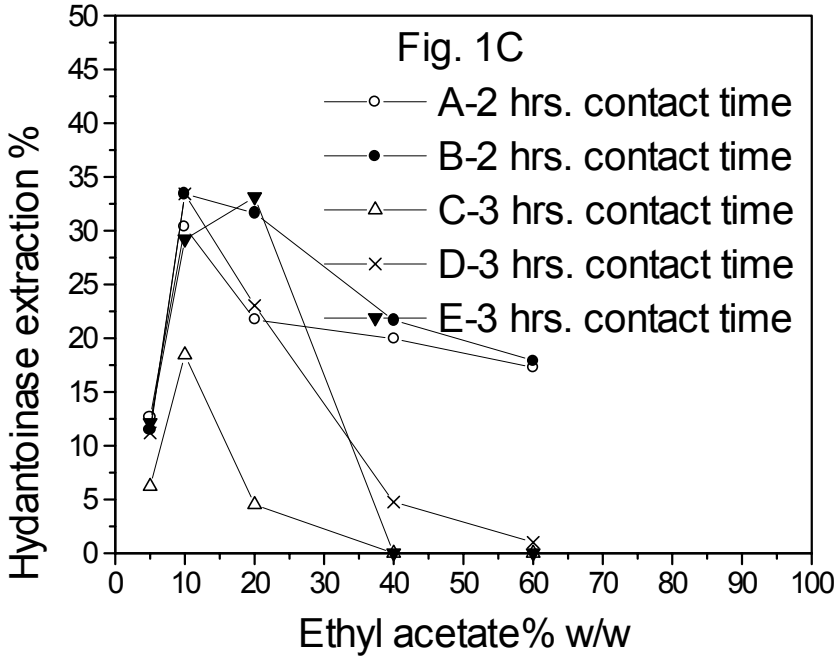

Fig. 1C: Effect of ethyl acetate concentration and buffer extraction time on hydantoinase extraction:

A- 2 h solvent contact time with 10 h buffer extraction time.

B- 2 h solvent contact time with 15 h buffer extraction time.

C- 3 h solvent contact time with 5 h buffer extraction time.

D- 3 h solvent contact time with 10 h buffer extraction time.

E- 3 h solvent contact time with 15 h buffer extraction time.

Table 3

Ethyl acetate %	**Solvent contact**	**Enzyme**	**Enzyme extraction**

	time (h)	extraction % (15 h buffer extraction time)	% (20 h buffer extraction time)
10	1	32.7	26.04
20	1	26.31	28.69
10	2	33.63	25.16
20	2	31.63	28.1

b) Solvent removal

0.5 g cells (wet) were exposed (1 and 2 h contact time) to 60% ethyl acetate. 2 ml buffer was added and mixed well. Supernatant and cells were separated by centrifugation. Hydantoinase activity of supernatant determined, no hydantoinase activity observed. Cells were subjected to buffer extraction for 15 hrs. 20.15 and 20.76% extractions were obtained using 1 and 2 h solvent contact time respectively. Solvent removal is not useful in increasing extraction.

5) Isoamyl alcohol, methyl isobutyl ketone, ethylene dichloride, diethyl ether and 1,4-dioxan

Studies were performed using varying concentrations of above solvents and contact time with 15 hrs. buffer extraction. The results where maximum extractions were obtained are presented in figure 2

6) Xylene, dichloromethane, chloroform, hexane, cyclohexane, petroleun ether and dimethyl sulfoxide

Extractions were carried out using above solvents with different contact time and buffer extraction time. Results are shown in figure 3, 4 and 5.

Figure 2: Use of solvents for extraction of hydantoinase

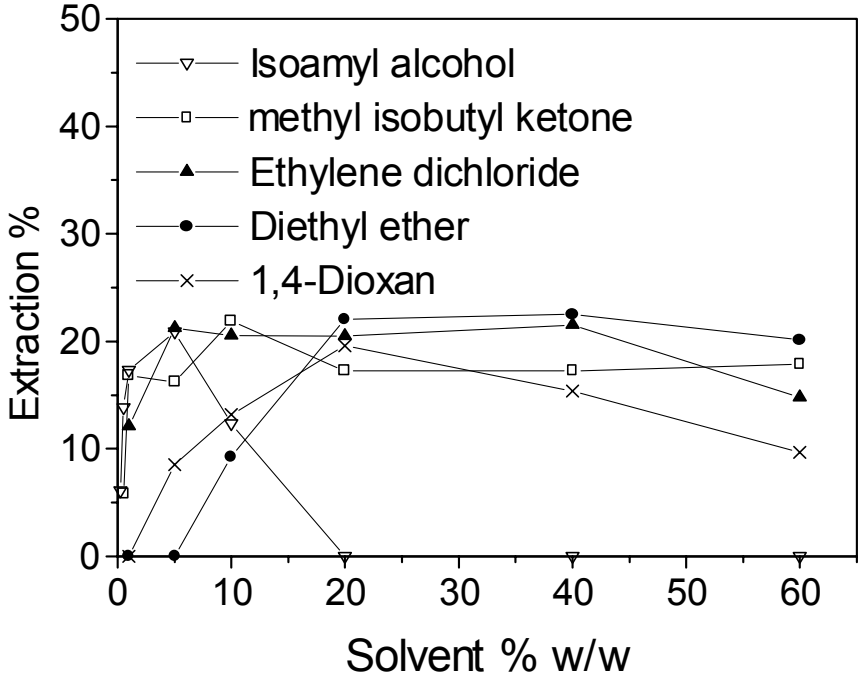

Fig. 2: Extraction of hydantoinase from cells, use of solvents for cell treatment followed extraction by buffer for 15 h.

Isoamyl alcohol & Methyl isobutyl ketone: 15 min. contact time.

Ethylene dichloride & diethyl ether: 1 h contact time.

1,4-Dioxan: 6 h contact time.

Fig.3: Effect of xylene on extraction

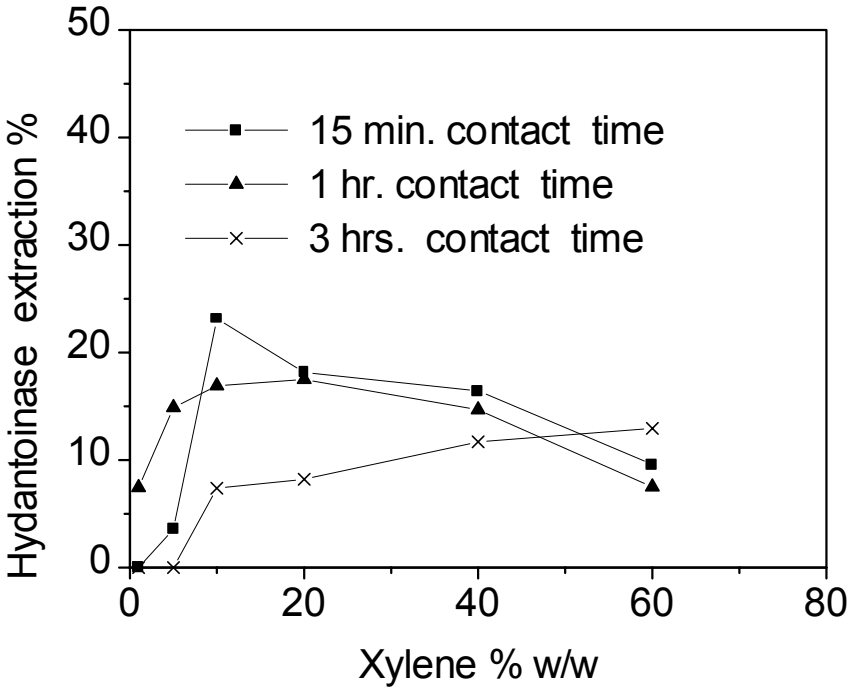

Fig. 3: Effect of xylene % w/w and contact time on extraction of hydantoinase (15 h buffer extraction time).

Fig. 4: Effect of dichloromethane on extraction

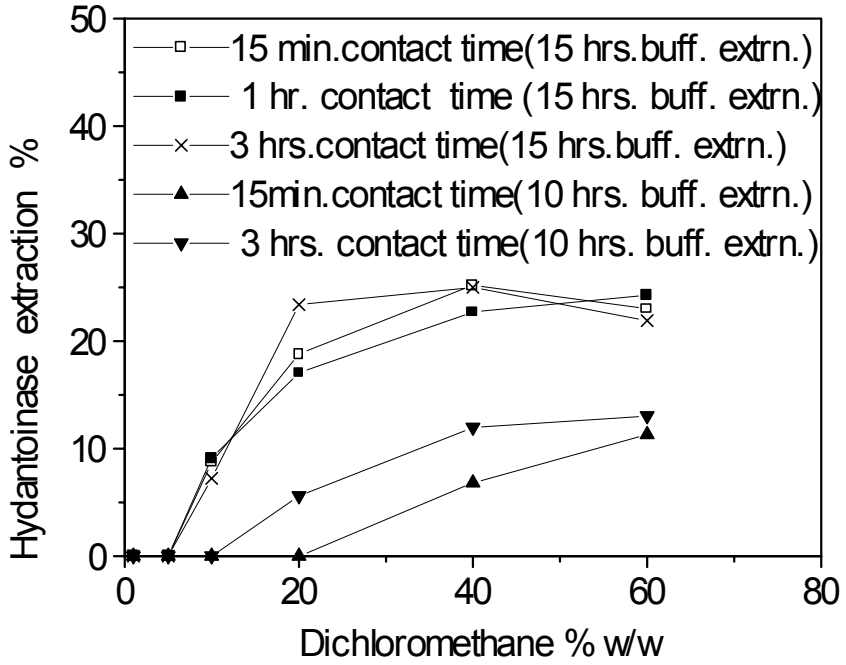

Fig. 4: Effect of dichloromethane % w/w and contact time on extraction of hydantoinase (15 h buffer extraction time).

Fig. 5: Effect of chloroform on extraction

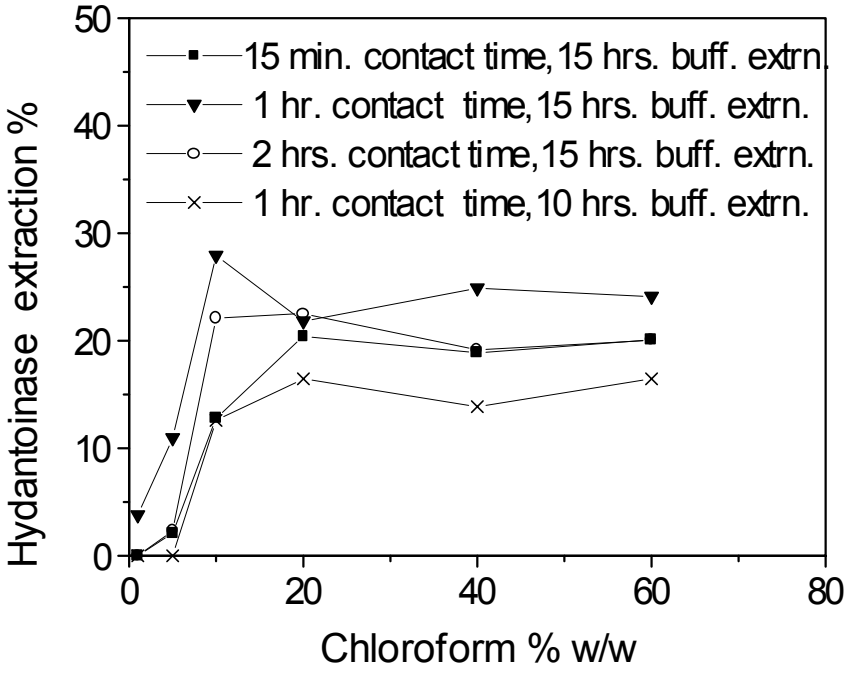

Fig. 5: Effect of chloroform % w/w, contact time, buffer extraction time on extraction of hydantoinase.

B] Detergents

Concentrated solutions of detergents were prepared and added to the cells to get particular concentrations of detergents for extraction of hydantoinase.

1) Sodium dodecyl sulphate (SDS)

0.005, 0.01, 0.02, 0.04 and 0.08 g SDS were used for extraction. It is 0.95, 1.81,, 3.33, 5.71 and 8.88% w/w respectively. Extractions were carried out using 15 min., 1 hr., 2 h, 3 and 4 h contact time. We have used 2.5, 5, 10 and 15 h buffer extraction time. Extractions were also carried out by adding SDS and buffer at same time to the cells with 15 h extraction time. Results are presented in figure 6, 7 and 9.

Fig. 6: Effect of SDS (5 h buffer extraction time)

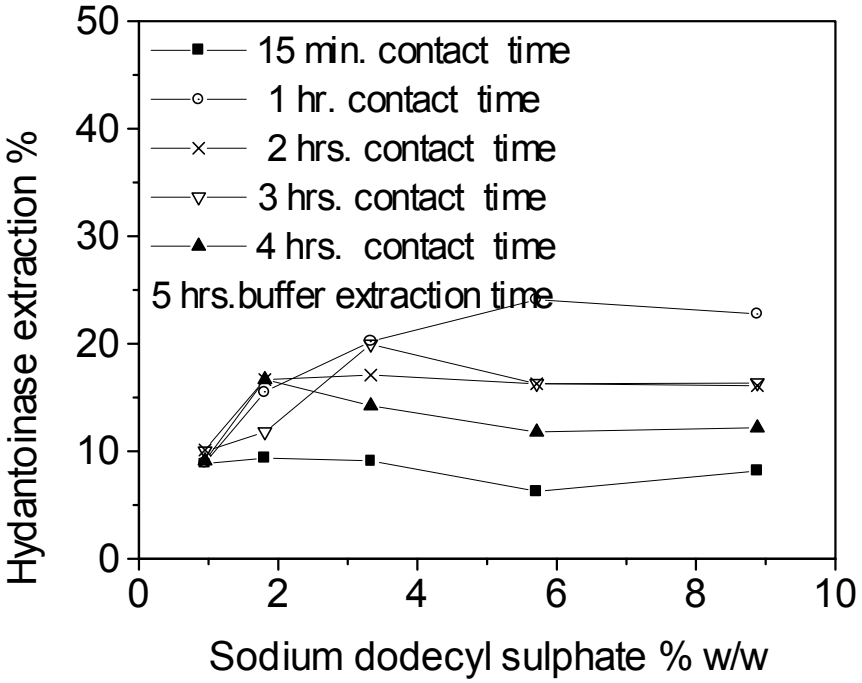

Fig. 6: Effect of SDS % and contact time with 5 h buffer extraction time on extraction of hydantoinase enzyme.

Fig. 7: Effect of SDS (10 hrs. buffer extraction time)

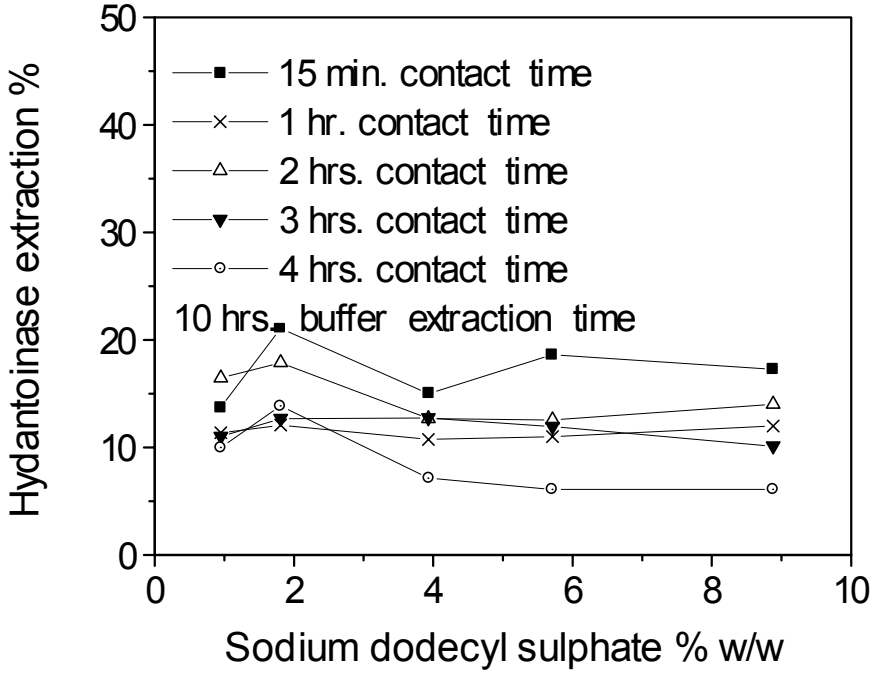

Fig. 7: Effect of SDS % and contact time with 10 h buffer extraction time on extraction of hydantoinase enzyme.

2) Triton X-100

0.008, 0.016, 0.032, and 0.064 g triton X-100 which corresponds to 1.45, 2.66, 4.57 and 7.11 % w/w respectively were used for cell disruption. 1 and 2 h contact time was used with 10 and 15 h buffer extraction time. Extraction was also performed using 4 hrs. contact time with 15 h buffer extraction time. Extractions were performed by adding triton X-100 and buffer at same time to the cells using 15 h extraction time.

3) Tween-80

01, 0.02, 0.04, 0.08, 0.1, 0.2, and 0.4 g tween-80 and buffer were added to the cells at same time with 15 h extraction time. In another set of experiment we have used 0.05, 0.1, 0.2 and 0.4 g tween-80 with 1, 2 and 4 h contact time, and 15 h buffer extraction time. We have used 10 h buffer extraction time also for 2 h contact time. 0.05, 0.1, 0.2 and 0.4 g tween-80 is 6.45, 12.19, 17.54 and 22.47 % w/w respectively. Results are presented in figure 8 and 9

Comparative results, where maximum extractions were observed, are plotted in figure 10.

C] Ethylenediaminetetraacetic acid (EDTA)

0.01, 0.02, 0.04 and 0.08 g EDTA was used for disruption of cells. It corresponds to 1.63, 2.77, 4.25 and 5.79% w/w respectively. We have used 15 min., 1 h, 2, 4 and 6 h contact time for extraction. Extractions were carried out using 10 and 15 h buffer extraction time with 15 min., 1h and 2 h contact time. Some experiments were done using 15 h buffer extraction time, and 4 and 6 h contact time. In another set of experiment EDTA and buffer were added to the cells at the same time with 15 h extraction time.

Figure 8: Effect of tween-80

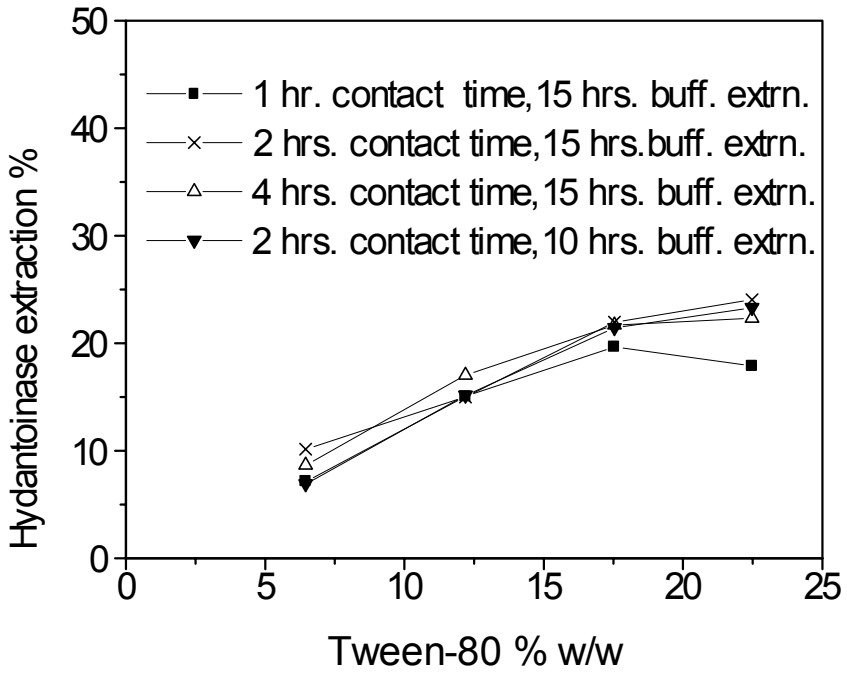

Fig. 8: Effect of tween-80, contact time and buffer extraction time on extraction of hydantoinase

Figure 9: Effect of detergents on extraction

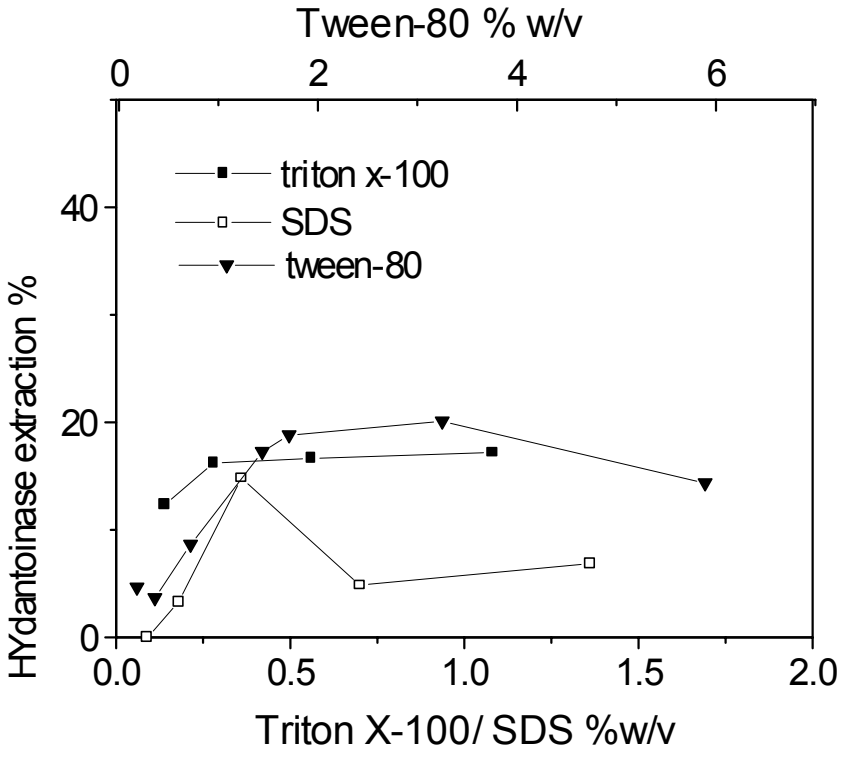

Figure 9: Effect of SDS, triton X-100 and tween-80 on extraction of hydantoinase (with addition of detergent and buffer at same time and 15 h buffer extraction time)

Figure 10: Use of detergents for extraction

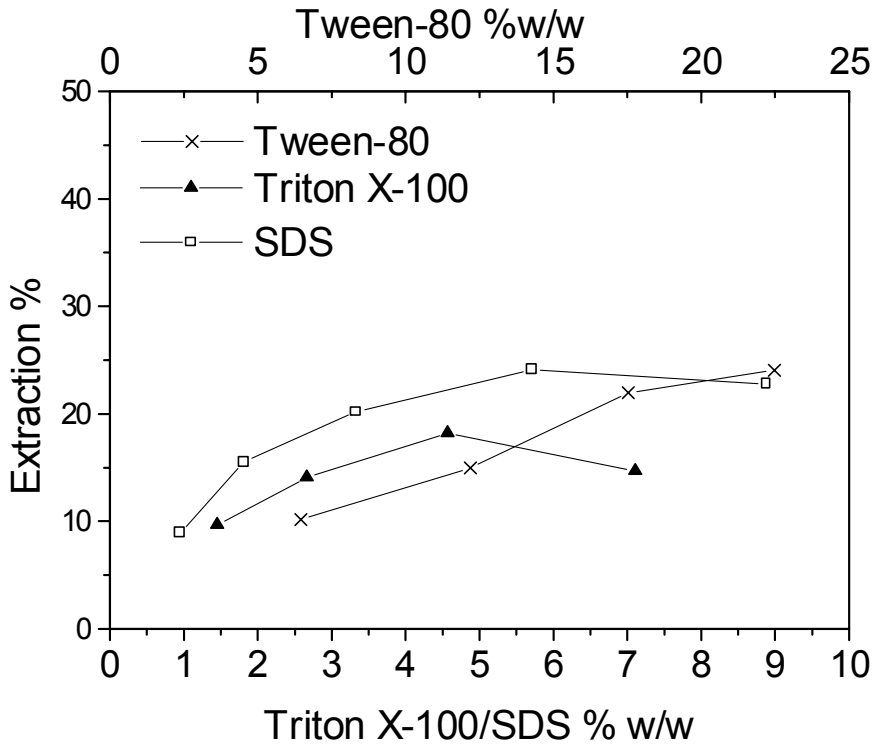

Fig. 10: Use of detergents in extraction of hydantoinase enzyme.

Triton X-100: 4 h contact time & 15 h buffer extraction time.

SDS (sodium dodecyl sulphate): 1 h Contact time & 5 h buffer extraction time.

Tween-80: 2 h contact time & 15 h buffer extraction time.

Results

Studies on extraction of enzyme was done using solvents, detergents and EDTA for cell rupture or cell-permeabilization. Buffer was used for extraction of hydantoinase enzyme from treated cells into buffer. 8.12% and 8.7 % extraction of hydantoinase enzyme obtained when cells slurry was treated with 1% toluene (7.5 h) and 5 % toluene (4.5 h) respectively. A maximum of 14.32 % extraction when cells were treated with 5 % toluene with 6 h contact time followed by 15 h buffer extraction time. Acetone, isopropanol and butanol were not effective agents for extraction. In case of methanol 14.96% extraction was achieved when 40% methanol was used with 15 min. solvent contact time and 10 h buffer extraction time. Upon increasing the solvent contact time no activity was observed (deactivation).

When 10% ethanol was used with 3 h contact time and 10 h buffer extraction time we had 16.31% extraction (table 2A). From results shown in table 2B it is clear that we have obtained more extraction by using 10% ethanol than 12.5% ethanol for cell treatment. Using 10% ethanol with 4 hrs. contact for cell treatment, 18.7 % hydantoinase extraction observed where buffer extraction time was 10 hrs.(table 2B). Optimum extraction of 31.42 % was achieved when 10% ethanol with 6 h contact time was used for cell rupture or permeabilization with buffer extraction time 20 h (table 2C). Extraction of hydantoinase enzyme increased by increasing buffer extraction time but by further increase in time we observed decrease in extraction due to denaturation.

Low conc. Of ethyl acetate gave incomplete enzyme extraction. 33.42 % extraction was obtained by using 10% ethyl acetate (3 hrs. contact time, 10 hrs. buffer extraction time) for cell rupture or permeabilization. At higher concentrations denaturation effect is more therefore lower results were obtained (Fig. 1A). Extractions were increased with increase in buffer extraction time using 1 hr. Contact time, and for certain concentrations (5, 10 & 20%) of ethyl acetate with 15 min.

contact time (fig. 1B). Similar trend was observed with 20% ethyl acetate with 2 and 3 h contact time. When 2 & 3 h contact time and 10% ethyl acetate was used for cell treatment we have obtained maximum extraction for 15 h & 10 h buffer extraction time respectively (fig.1C). We have achieved maximum 33.63% extraction of hydantoinase by using 10% ethyl acetate for cell treatment, 2 h contact time and 15 h buffer extraction time (table 3). Solvent removal strategy is not useful in enhancing extraction.

Extractions were not observed by using 20-60% isoamyl alcohol for cell treatment. 20.9 & 21.89 % extractions were obtained with 5% isoamyl alcohol & 10% methyl isobutyl ketone respectively with 15 min. contact time and 15 h buffer extraction time. Using 1 h Contact time and 15 h buffer extraction we had 21.54 and 22.51 % extractions with 40% ethylene dichloride and 40% diethyl ether respectively. When cells were exposed to 1,4-dioxan for 6 h and buffer extraction for 15 h we had maximum 19.57 % extraction. Comparative results are shown in figure 2.

Results obtained with xylene, dichloromethane and chloroform are shown in figure 3-5. Extraction of hydantoinase enzyme was increased with increase in buffer extraction time in case of dichoromethane and chloroform. In case of xylene it was decreased at higher concentration of xylene (contact time 15 min. & 1 h) due to denaturation whereas for 3 hrs. contact time slow increase in extraction was observed may be due to slightly more enzyme release rate than denaturation. Extraction rate at higher concentration is dependent on rate of enzyme release and denaturation. 8.4 and 15.11% extractions were observed using cyclohexane and hexane for cell treatment respectively. We found 6.25 and 12.8% extraction using dimethyl sulfoxide and petroleum ether for cell treatment respectively.

When 0.95% SDS and 5 h buffer extraction time was used, we found that extraction of hydantoinase enzyme is unaffected by contact time. Lower results were obtained with buffer extraction 2.5 and 15 h buffer extraction time. More extraction

was observed by 5 h buffer extraction time (fig. 6) than 10 h buffer extraction time (fig. 7). Maximum 24.1% extraction was obtained by 5.71% w/w SDS with 1 h contact time and 5 h buffer extraction time.

Maximum 18.2 % extraction was achieved by using 4.57% triton X-100, 4 h contact time and 15 h buffer extraction time. As concentrations of tween-80 increased extractions of hydantoinase enzyme also increased for all contact time except incase of 1 hr. contact time and 22.47% concentration. We have observed slightly more extraction for 15 h buffer extraction time than 10 h buffer extraction time. Maximum 24.05% extraction of hydantoinase was achieved using 22.47 % tween-80 with 2 h contact time and 15 h buffer extraction time (fig. 8). Maximum 11.37 % extraction was obtained by using 4.25 %w/w EDTA with 4 h contact time and 15 h buffer extraction time. When EDTA and buffer were added at the same time to cells no extraction was observed whereas with detergents less extractions were observed (fig. 9). Results obtained by detergent treatment are compared in figure 10.

Summary of hydantoinase extraction

Solvent/detergent/ EDTA	**concentration % w/w**	**contact time**	**Buffer extraction time (h)**	**extraction of hydantoinase %**
toluene	5	6 h	15	14.32
xylene	10	15 min.	15	23.15
hexane	60	4.5 h	15	15.11
cyclohexane	60	3 h	15	8.4
petroleum ether	60	6	15	12.8
acetone	10-80	15 min., 1 h, 3 h	10	-
MIBK	10	15 min.	15	21.89
methanol	40	15 min.	10	14.96
ethanol	10	6 h	20	**31.42**
isopropanol	10-80	15 min., 1 h, 3 h	10	-
butanol	10-80	15 min., 1 h, 3 h	10	-
isoamyl alcohol	5	15 min.	15	20.9
ethyl acetate	10	2 h	15	**33.63**
diethyl ether	40	1 h	15	22.51
1,4-dioxan	20	6	15	19.57
DMSO	60	1 h	15	6.25
dichloromethane	40	15 min.	15	25.22
ethylene dichloride	40	1 h	15	21.54
chloroform	10	1 h	15	27.23
SDS	5.71	1 h	5	24.1
triton X-100	4.57	4 h	15	18.2
tween-80	22.47	2 h	15	24.05

EDTA	4.25	4 h	15	11.37

Discussion

Many researchers have reported use of solvents, detergents and other chemicals to rupture or permeabilize cells and extraction of intracellular enzymes [Lazarus et al. (1966), Weitzman (1973), Fenton (1982)]. By using 5% toluene treatment, 6 h contact time and 15 h buffer extraction time we have obtained 14.32% extraction of hydantoinase. Fenton (1982) have used toluene to release beta - galactosidase from yeast and obtained 15% release by using 80% toluene, 90 min. contact time and 15 h buffer extraction time. Fenton (1982) has calculated solvent % using value of dry weight of cells. We have calculated using wet weight of cells. Lazarus et al. (1966) have isolated hexokinase from yeast using toluene. Weitzman (1973) have used toluenized bacterial cells in his study of enzyme regulation. Serrano et al. (1973) have carried out permeabilization of yeast using toluene with ethanol. Vadehra et al. (1965) carried out extraction of protease from using different methods where they used toluene in one of the methods. Toluene treated cells were used in assay by Achary et al. (1997) while estimating enzyme activity. Kheirolomoom et al. (2001) have treated cells of *E. coli* with different chemicals &/ lysozyme to isolate penicillin G acylase. They have used toluene/ethanol, guanidine-HCl, guanidine-HCl/EDTA, lysozyme and lysozyme/EDTA. They have reported isolation of penicillin G acylase by sonication of cells.

We have calculated solvent % using values of wet weight of cells: 5, 10, 20, 40, 60 and 80 % w/w. If we calculate solvent % using values of dry weight of cells then solvent % are: 15.9, 28.6, 47.5, 70.5, 84.3 and 93.4 % respectively.. By using 10 % xylene for cell rupture or permeabilization we have achieved 23.15 % extraction of hydantoinase enzyme. 8.4, 12.8 and 15.11% extraction of hydantoinase enzyme was obtained using cyclohexane, petroleum ether and hexane, for cell rupture or permeabilization respectively. No extraction was achieved with acetone. Fenton

(1982) have used acetone to release beta-galactosidase from yeast and he obtained 20% release using 80% acetone with 90 min. solvent contact time and 15 h buffer extraction time. We observed 21.89% extraction of hydantoinase enzyme by using 10% w/w (28.6% on dry wt. basis) methyl isobutyl ketone (MIBK) for cell rupture or permeabilization with 15 min. solvent contact time and 15 h buffer extraction time. Fenton (1982) has carried out isolation of beta-galactosidase from yeast cells using MIBK. He obtained 15 % release by using 80 % MIBK with 1.5 h solvent contact time and 15 h buffer extraction time.

By using 40% methanol (70.5% on dry weight basis) for cell rupture or permeabilization, 15 min. contact time and 10 h buffer extraction time we have obtained 14.96% extraction of hydantoinase. Fenton (1982) has used methanol for release of beta-galactosidase from yeast. By using 80% methanol with 90 min. contact time and 15 h buffer extraction time he achieved 90% release. By using 10% (28.6% on dry wt. basis) ethanol for cell rupture or permeabilization with 6 h contact time and 20 h buffer extraction we have achieved 31.42% extraction of hydantoinase enzyme. Fenton (1982) has achieved 90% release of beta-galactosidase from yeast using 80% ethanol, 90 min. solvent contact time and 15 h buffer extraction time. Sugimoto (1974) has carried out autolysis of yeast using ethanol and NaCl.

We did not obtain extraction using isopropanol for cell rupture. Tan et al. (1996) have extracted superoxide dismutase from *Sacchromyces cerevisiae* using 90% isopropanol (120 min. contact time). Fenton (1982) has achieved 85% extraction of beta-galactosidase using 80% isopropanol. We did not obtain extraction using butanol. Fenton (1982) have obtained 2% extraction of beta-galactosidase by using 80% butanol. We have obtained maximum 20.9 % extraction of hydantoinase by 5 % isoamyl alcohol with 15 min. contact time and 15 h buffer extraction time. Isolation of s-RNA from *E. coli* was carried out by Rammler et al. (1965). They have used 10% isoamyl alcohol, bentonite, polyvinyl sulphate and 0.001 M ethylenediaminetetracetic acid. 0.2% DNA and 3.5% protein was released.

By using 10% ethyl acetate (47.5% on dry weight basis) for cell rupture or permeabilization with 2 h solvent contact time and 15 h buffer extraction time, we have achieved maximum 33.63% extraction of hydantoinase enzyme. Peppler (1979) has reported use of ethyl acetate for preparation of autolysed dried yeast at high temperature i.e. 45^0C. He has also reported use ethyl acetate for isolation of various enzymes from yeast. Ethyl acetate treatment of yeast cells for permeabilization and enzyme release is reported by Wiseman and Jones (1971), Arnold (1972a,b) and Naglak et al. (1990). Ohleyer (1989) has reported that hydantoinase activity could be recovered by permeabilization of cells with organic solvent.

By using 20% 1,4-dioxan for cell rupture or permeabilization we have obtained 19.57% extraction of hydantoinase enzyme. Kim and Kim (1993) have studied conversion of p-hydroxyphenylhydantoin in presence of 10 % 1,4-dioxan. They found increase in hydantoinase activity by 1.5 times in presence of 1,4-dioxan. We had only 6.25% extraction when 60% dimethyl sulfoxide used for cell treatment. Kim & Kim (1993) have studied effect of DMSO on hydantoinase activity. They observed two fold increase in activity by using 5 % DMSO. They have used bacterial strain KBEL 101 for conversion of p-hydroxyphenylhydantoin where they have used DMSO. Yin et al. (2000) have carried out conversion of p-hydroxyphenthydantoin to its N-carb. They have permeabilized cells of *E. coli* by 1.5% DMSO. They observed increase in rate of hydrolysis by 80% with permeabilized cells. By using 40% diethyl ether for cell rupture or permeabilization with 1 hr. contact time we have achieved 22.51 % extraction of hydantoinase enzyme. Vosberg and Hoffmann-Berling (1971) have studied DNA synthesis using ether treated *E. coli* cells. 5% protein and 3% nucleic acid was released from cells. Durwald and Hoffmann-Berling (1971); Mirelman et al.(1977); and Mirelman and Nuchamowitz (1979) have studied biosynthesis using ether treated bacterial cells.

When 10% chloroform was used for cell treatment we obtained 27.23 % extraction of hydantoinase. Linz (1948) has used chloroform for treatment of colony of gram negative bacteria and removed antigen. Bernheim (1972) has studied

swelling of cells *Pseudomonas aeruginosa* in presence of chloroform and salts. Cell swelling (LiCl influx) and K-efflux are influenced by chloroform [Bernheim (1974)]. Ames et al. (1984) have used chloroform to release periplasmic protein from cells of gram negative bacteria.

When cells treated with 22.47 % tween-80 we have obtained 24.05 % extraction of hydantoinase enzyme. Brown and Richards (1964) have studied effect of tween-80 on cells of *Pseudomonas aeruginosa*. They found that cells became more susceptible by tween-80 treatment. Nandkumar et al. (2000) observed more cell lysis by tween-80 treatment before mechanical treatment. They found that cell lysis is influenced by surfactants used. Chen et al. (1999) have disrupted cells of *Alcaligenes eutropus* by using surfactant-chelant solution.

18.2% extraction was obtained by 4.57%w/w, which is 0.56 % w/v, triton X-100 treatment to cells. 90-95% pullulanase was released from *Aerobacter aerogenes* using 0.4% triton X-100 [Wallenfels et al.(1966)]. Dunnill (1975) and Buckland et al. (1976) have studied isolation of cholesterol oxidase from *Nocardia rhodocrous* using 0.5% triton X-100. Dunnill (1975) has obtained 53% extraction. 10% intracellular protein was released by 0.5% triton X-100 treatment to *E. coli* [Naglak and Wang (1990)].

Tanaka et al. (1993) have studied lysis of *Bacillus subtilis* cells using 50mM/100 mM triton X-100 to release glucose-6-phosphate. Novella et al. (1994) recovered 8 % penicillin acylase from *E. coli* by using 0.5% w/v triton X-100. Lee et al. (1997) have used triton X-100 treated cells in assay of hydantoinase enzyme. They have used 1% triton X-100. Balk et al. (1998) have isolated ATP from cells by 0.1-5% w/w surfactant treatment to cells and ATP was used for bioluminescence test for detection of microorganisms in aq. dispersions. Dunnill (1975) obtained more extraction when triton X-100 is used with pepsin than triton X-100 alone.

We have achieved 24.1 % hydantoinase extraction using 5.71% w/w sodium dodecyl sulphate (SDS) for cell rupture or permeabilization. Woldringh (1970) have used 0.05% SDS for lysis of *E. coli* and observed that 35% RNA, 24% protein and 22% DNA was released. Wallenfels et al. (1966) observed that 90-95% pullulanase has released from *Aerobacter aerogenes* using 0.1% w/v SDS treatment. Burton et al. (1998) have disrupted bacterial cells using detergents and found increase in hydantoinase activity. They have concluded that hydantoinase is membrane bound.

When cells were treated to 4.25% w/w (0.673% w/v) ethylenediaminetetraacetic acid (EDTA), we have obtained 11.37% hydantoinase extraction. Bactericidal effect of EDTA on *Pseudomonas aeruginosa* and *Alcaligenes faecalis* was studied by Gray and Wilkinson (1965a). Gray and Wilkinson (1965b) have investigated effect of EDTA on cell wall of *P. aeruginosa, A. faecalis, E. coli* and *Proteis mirabilis*. Leive (1968) has studied permeability changes occurred in *E. coli, Aerobacter* and *Salmonalla sp*. by EDTA treatment. Bactericidal effect of EDTA is more on *P. alcaligenes* than *P. aeruginosa* therefore more intracellular solute is released from *P. alcaligenes* [Key et al. (1970)]. Robert et al. (1970) have studied effect EDTA treatment on *P. aeruginosa.* They found release of intracellular material from cells due to leakage of cell wall caused by EDTA. Falconer et al. (1997) have treated cells of *E. coli* with 3 mM EDTA. They observed that 11% protein was released from stationary phase cells by 90 min. treatment and 21.3% protein released from logarithmic phase cells.

Conclusion

Extractions were not obtained using acetone, isopropanol and butanol treatment. 8.4, 6.25 and 11.37% hydantoinase extractions were obtained by cyclohexane, DMSO and EDTA treatment respectively. We observed 19.57, 12.8, 14.32, 14.96, 15.11 and 18.2% extractions with 1,4-dioxan, petroleum ether, toluene, methanol, hexane and triton X-100 treatment respectively. 21.54, 22.51, 20.9, 21.89, 23.15, 24.05, 24.1, 25.22 and 27.23 % extractions were obtained when cells were treated with ethylene dichloride, diethyl ether, isoamyl alcohol, MIBK, xylene, tween-80, sodium dodecyl sulphate, methylene dichloride and chloroform respectively. 31.42 and 33.63% extractions were observed with ethanol and ethyl acetate treatment respectively. We have achieved maximum 33.63% extraction of hydantoinase enzyme by ethyl acetate treatment to cells of *Pseudomonas sp*. NCIM 2875. There is still more scope for using some more cell rupturing or permeabilising agents, combination of these agents to get more extraction. There is further scope for reusing treated cell for further extraction.

Chapter 4

Purification and properties of hydantoinase enzyme

Summary

Hydantoinase enzyme from *Pseudomonas sp.* NCIM 2875 was purified using various techniques such as precipitation using solvents or inorganic salts, chromatographic techniques like ion exchange chromatography and hydrophobic interaction chromatography and aqueous two phase system etc. Solvents like methanol, ethanol, isopropanol and acetone were able to precipitate of the enzyme from solution but hydantoinase activity was not detected in the precipitated protein. When ammonium sulphate was used for precipitation, maximum enzyme activity was recovered in the precipitated protein but specific activity was not increased much. Purification was not achieved by using aqueous two-phase system. Specific activity was increased only 2.7 times when ion exchange chromatography was used. But by using hydrophobic interaction chromatography (macro prep t-butyl HIC support) 235 fold purification was achieved. The enzyme was further purified to a single band on SDS-PAGE and properties of purified hydantoinase were investigated. Optimum pH and temperature of hydantoinase were 8.5 and 30^0C respectively. No metal ions were required for enhancing the hydantoinase activity and stability. The molecular weight of the hydantoinase enzyme protein was found to be 49K by SDS-PAGE whereas gel filtration showed its molecular weight as 193K. This may be because the hydantoinase protein may be tetramar of the enzyme subunits. Kinetic parameters mainly Km and Vm values were determined. Km value is much higher than hydantoinases reported from other cultures.

Introduction

Different techniques are used for enzyme concentration and purification. Salting out using inorganic salts, precipitation by solvents and water-soluble polymers, aqueous two-phase system, various chromatographic techniques etc. are some of the methods commonly used for this purpose.

Either a single or combinations of techniques are used for purification of enzymes. Salts such as NaCl, Na_2SO_4, $(NH_4)_2SO_4$ etc. are used for precipitation of enzymes. Solvents such as methanol, ethanol, isopropanol, acetone etc. precipitate the enzyme from its solution. Water soluble polymers e.g. polyethyleneglycol, polyethyleneimine, DEAE-dextran, protamine sulphate etc. are sometimes used to precipitate enzyme. Some researchers have used aqueous two-phase system for purification of enzymes. But purification of enzymes by ion exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, gel chromatography, expanded bed adsorption chromatography etc are more successful.

Maguire and Dudley (1978) have carried out partial purification of dihydropyrimidinase from calf and rat liver. Yungjin Co., Japan (1992) has reported purification of hydantoinase enzyme from *Streptomyces* Y-183 using chromatographic techniques. Runser and Meyer (1993) purified hydantoinase enzyme from *Agrobacterium sp.* and studied its properties. Xu and West (1994) have used ultrafiltration and ion exchange chromatography for purification of dihydropyrimidinase from *Pseudomonas stutzeri* ATCC 17588 and studied its properties. Purification of L-hydantoinase from *A. aurescens* DSM 3745 is reported by May et al. (1996). Nanba et al. (1998) purified N-carbamyl-D-amino acid amidohydrolase from recombinant *E. coli* and studied its properties. Purification of D-hydantoinase from *Arthrobacter crystallopoietes* DSM 20117 is investigated by Siemann et al. (1999) and studied its properties. Some more examples of hydantoinase purification are cited in Table 1.

In the present work purification of hydantoinase enzyme from *Pseudomonas sp.* 2875 was tried by using techniques like precipitation, aqueous two-phase system, ion exchange chromatography and hydrophobic interaction chromatography etc. and studied the properties of the purified enzyme.

Table 1: Purification of hydantoinase enzyme

Source	**Purification steps**	**References**
P. straita D-hydantoinase	protamine sulfate treatment, (NH4)2SO4 fractionation, DEAE-cellulose & hydroxyl-apatite chromatogs. and Sephadex G-200	Takahashi et al. (1978)
Pseudomonas striata IFO 12996	protamine-H2SO4,(NH4)2SO4 pptn., dialysis, DEAE-Sephadex A-50,amm. sulphate fractionation, hydroxyapatite chromatography, Sephadex G-200	Yamada et al. (1979)
Arthrobacter DK-200 L-hydantoinase	protamine sulphate, (NH4)2SO4 fractionation, column chromatography	Kitagawa et al. (1985)
P. fluorescens DSM 84 D-hydantoinase	hydrophobic interaction chromatog on phenyl-Sepharose or chromatography on Sepharose 4B, gel filtration on Sephacryl S-400, and preparative electrophoresis.	Morin et al. (1986a)
Agrobacterium sp. IP I-671 D-hydantoinase	$(NH4)_2SO4$ fractionation and ion-exchange chromatography.	Runser & Ohleyer (1990)

Table 1 (continued)

Rat liver dihydropyrimidinase	ammonium sulfate fractionation, DEAE-Sepharose CL-6B, carboxymethyl-Sepharose CL-6B, hydroxylapatite and Sephacryl S-300 chromatography	Kikugawa et al. (1994)
Bacillus stearothermophilus SD-1 D-hydantoinase	(NH4)2SO4 fractionation, anion-exchange chromatog., heat treatment, hydrophobic interaction chromatog. & preparative gel electrophoresis.	Lee et al. (1995)
Bacillus stearothermophilus SD-1 D-hydantoinase	immunoaffinity chromatography	Lee et al. (1997)
B. thermocatenu-latus GH-2 D-hydantoinase	immunoaffinity chromatography	Park et al. (1998)
recombinant *E. coli* hydantoinase L-N-carbamoylase	One chromatog. step (amylose resin) followed by metal affinity chromatog.	Pietzsch et al. (2000)
recombinant *E. coli* D-hydantoinase	HIC, anion-exchange chromatog. and gel permeation chromatography	Abendroth et al. (2000)
Pseudomonas putida 9801 dihydropyrimidi-nase	Ammonium sulfate fractionation, column chromat.(DEAE-cellulose and hydroxylapatite) and gel filtration (Sephadex G-200).	Huang et al. (2000)
Pseudomonas 2262 D-hydantoinase	Thermal treatment, ammonium sulfate fractionation and Q-Sepharose fast flow, Phenyl-Sepharose fast flow Superose 12 column chromatography	Shi et al. (2001b)

Materials and methods

Chemicals

Bacteriological grade yeast extract, peptone, beef extract and agar from Hi-media (Bombay) were used. Phenylhydantoin was provided by Dr. M.S.Gaikwad [P.D. Division, NCL, Pune]. AR grade PDAB (p-dimethylaminobenzaldehyde), perchloric acid, phosphoric acid, ammonium sulphate and other chemicals were from SD Fine chemicals Bombay. Distilled solvents (laboratory grade) were used in the experiments. Coomassie Brilliant Blue G-250 (Alrich Chem.), ion exchange resins (Indion Co., India and Sigma Chemicals), phenyl-sepharose (Pharmacia) and macro prep t-butyl-HIC from Bio-Rad were used. Protein molecular weight markers were obtained from Sigma Co.

Strain

Pseudomonas sp. NCIM no. 2875 was used in the studies. Strain was maintained on nutrient agar [beef extract 10, NaCl 5, Peptone 10 and agar 20 g /L, pH 7.0].

Growth of culture and extraction of hydantoinase enzyme

Cultivation of *Pseudomonas sp.* NCIM 2875 and hydantoinase activity of cells was estimated as mentioned in chapter 3.

In order to extract the enzyme from cells, 1-gram ethyl acetate was added to 10 grams of the cells (10% on basis of wet wt. cells) and kept it for one hour. 100 ml of 0.15M potassium phosphate buffer (pH 8.7) was added to it and mixed at 400 rpm for 10 min. It was incubated on shaker for 15 h at 30^0C. Supernatant was separated and analysed for hydantoinase activity and protein Concentration.

Protein estimation

a) Lowry method [Lowry et al. (1951)]

Reagent A: % Na_2CO_3 in 0.1 N NaOH

Reagent B1: 1 % $CuSO_4.5H_2O$

Reagent B2: 2 % Na-K–tartarate

Reagent C: 100 ml reagent A + 1 ml reagent B1 + 1 ml reagent B2 (prepared freshly).

Folin reagent (2N): diluted 1:1 (prepared freshly).

BSA: 1 mg/ml

Assay: 1 ml sample or standard solution and 5 ml reagent C were mixed well and incubated at room temperature for 10 minutes. 0.5 ml Folin reagent (diluted) was added to it and incubated at room temperature for 30 min. Optical density was measured at 500 nm.

While using Lowry method if precipitate was found Biuret method was used.

b) **Biuret method [Gornall et al. (1949)]**

Biuret reagent: 0.15 gm $CuSO_4.5H_2O$ and 0.6 gm Na-K-tartarate was dissolved in 50 ml distilled water. 30 ml 10 % NaOH solution was added to it. 0.1 gram KI was dissolved in it and volume was made to 100 ml. Solution was stored at room temperature. Solution of bovine serum albumin (BSA) (10 mg/ml) was prepared and used as standard.

Assay: 1 ml sample or standard solution and 4 ml Biuret reagent were mixed well and incubated at room temperature for 30 minutes. Optical density was measured at 550 nm.

While carrying hydantoinase purification in some samples there was very less protein so a sensitive protein estimation method (Coomassie blue method) was also used.

c) Coomassie Blue method [Spector (1978); Sedmark and Grossberg (1977)]

Coomassie blue reagent: 100 mg Coomassie brilliant blue G-250 was dissolved in 50 ml 95% ethanol and mixed with 100 ml 85% phosphoric acid. Solution was diluted to 1 liter and filtered.

Assay: 1.5 ml sample containing 50 micrograms protein and 1.5 ml Coomassie blue reagent were mixed. Optical density was measured at 595 nm after 2-30 min.

Precipitation of protein: (for protein estimation)

Protein was precipitated from the samples to be analysed. For this 1 ml enzyme and 0.1 ml perchloric acid mixed well and incubated at room temperature for

5 minutes. It was centrifuged for 5 min. Pellet was dissolved in 1 ml of 0.1 N NaOH and protein analysis was done by Biuret or Lowry method.

Purification of Hydantoinase

Techniques like aqueous two-phase system, precipitation, ion exchange chromatography and hydrophobic interaction chromatography (HIC) were used for purification of hydantoinase enzyme from *Pseudomonas sp.* NCIM 2875

I] Aqueous two-phase system:

Hydantoinase purification was attempted by using polyethyleneglycol (PEG) ranging from molecular weights of 400 to 6000 and two salts i.e.K_2HPO_4 and ammonium sulphate.

Method:

PEG having molecular weight 400, 1540, 4000 and 6000 were used. Required quantities of PEG, K_2HPO_4 and water were weighed. Enzyme solution was added to it and mixed well. In case of PEG-ammonium sulphate system, required ammonium sulphate, water and enzyme were added to get a solution. Then weighed quantity of PEG was added to it and mixed well. It was incubated at 20^0C for 1.5 hours and centrifuged for 5 minutes. Separated phases were analysed for protein and hydantoinase activity.

Results are presented in table 2-9.

Table 2: PEG 1540- K_2HPO_4 system

PEG %	**K_2HPO_4 %**	**protein % (in PEG phase)**	**activity % (in PEG phase)**
18	8	20.42	7.14
6	15	36.65	16.65
6	25	72.31	35.44
6	30	66.83	33.32
5	30	48.43	23.46
11	30	56.59	27.55

Table 3: PEG 4000-K_2HPO_4 system

PEG %	K_2HPO_4 %	protein % (in PEG phase)	activity % (in PEG phase)
4.87	23.37	31.25	17.58
7.93	15.3	46.25	20
8.7	12.6	42.83	18.98
16	8	52.33	32.65
16	6	42.63	18.36

Table 4: PEG 6000-K_2HPO_4 system

PEG %	K_2HPO_4 %	protein % (in PEG phase)	activity % (in PEG phase)
6.4	11.9	9.17	-
7.04	14.37	11.09	-
12	8	34.55	35.71
14	6.8	28.86	22.43
14	5	33.31	31.7

Table 5: PEG 400-$(NH_4)_2SO_4$ system

PEG %	$(NH_4)_2SO_4$ %	protein % (in PEG phase)	activity % (in PEG phase)
16	19	25.35	17.34
18	19	19.06	10.20
14	25	50.11	32.1
16	25	52.11	39.44
18	25	n. d.	33.94
8	30	20.12	14.28
16	30	45.89	24.04
20	30	38.57	19.56

Table 6: PEG 1540-$(NH_4)_2SO_4$ system

PEG %	$(NH_4)_2SO_4$ %	protein % (in PEG phase)	activity % (in PEG phase)
8	17	49.97	31.52
8	25	35.48	23.91
12	17	53.97	35.86
12	25	n. d.	28.16
12	30	48.76	26.08
16	25	52.01	32.6
16	30	32.49	16.8
16	17	n. d.	20.16

Table 7: PEG 4000-$(NH_4)_2SO_4$ system

PEG %	$(NH_4)_2SO_4$ %	protein % (in PEG phase)	activity % (in PEG phase)
4	19	26.66	14.22
4	25	27.59	15.97
4	30	32.19	17.77
8	14	58.26	33.61
8	19	40.84	23.91
8	25	n. d.	20.42
8	30	n. d.	22.58
16	19	56.6	28.25
16	25	n. d.	26.46
16	30	53.04	25.20

Table 8: PEG 6000-$(NH_4)_2SO_4$ system

PEG %	$(NH_4)_2SO_4$ %	protein % (in PEG phase)	activity % (in PEG phase)
4	19	15.48	14.26
4	25	n. d.	17.77
4	30	18.68	16.26
8	19	n. d	16.17
8	25	n. d.	19.35
8	30	n. d.	26.82
16	19	33.58	19.32
16	25	n. d.	20.16
16	30	38.41	28.45

n. d. = Not determined.

Table 9: Use of aqueous two-phase system (summary)

PEG mol. wt.	PEG %	salt %	protein % (in PEG phase)	hydantoinase activity % (in PEG phase)
400	14.2	19.2 % K_2HPO_4	10.23	6.6
1540	6	25 % K_2HPO_4	72.31	35.44
4000	16	8 % K_2HPO_4	52.33	32.65
6000	12	8 % K_2HPO_4	34.55	35.71
400	16	25 % $(NH_4)_2SO_4$	52.11	39.44
1540	12	17 % $(NH_4)_2SO_4$	53.27	35.86
4000	8	14 % $(NH_4)_2SO_4$	58.96	33.61
6000	16	30 % $(NH_4)_2SO_4$	38.41	28.45

II] Precipitation of hydantoinase enzyme by solvents and polyethyleneglycol (PEG)

Various solvents such as acetone, methanol, ethanol, isopropanol, 1,4-dioxan, dimethylformamide and dimethylsulfoxide were used to precipitate hydantoinase enzyme from solution. Precipitation of protein was obtained for all solvents but there was no hydantoinase activity. With polyethyleneglycol (PEG) having molecular weight of 400 and 1540 hydantoinase protein was precipitated but there was no activity. With Polyethyleneglycol having molecular weight of 4000 and 6000 protein was not precipitated.

III] Precipitation of hydantoinase by $(NH_4)_2SO_4$

Ammonium sulphate is widely used for precipitation of enzymes because it stabilizes the enzymes beside precipitation. In the present work also ammonium sulphate was used for precipitation of the hydantoinase enzyme.

Method:

Desired amount of ammonium sulphate (AR grade) was dissolved in 5-ml hydantoinase enzyme solution and kept at 4^0C for one hour. The precipitated protein was centrifuged for 5 minutes. Supernatant was discarded and pellet was dissolved in 0.15 M K-PO_4 buffer having pH 8.7 and analysed for protein and hydantoinase activity. Results are shown in table 10.

Table 10: Precipitation of hydantoinase by $(NH_4)_2SO_4$

$(NH_4)_2SO_4$ g	**$(NH_4)_2SO_4$ %**	**protein %**	**activity %**
2	40	38.67	54.05
2.5	50	59.77	82.42
3	60	73.42	90.53
3.6	72	74.37	93.23

IV] Ion Exchange Chromatography

There are many reports of use of ion exchange chromatography for the purification of hydantoinase enzyme. [Takahashi et al. (1978), Yamada et al. (1979), Runser and Ohleyer (1990), Runser et al. (1990), Kim and Kim (1995), Lee et al. (1995), Siemann et al. (1999), Huang et al. (2000) Abendroth et al. (2000) etc.]. In the present work different anion exchange supports have been attempted for purification of hydantoinase enzyme.

Method:

A glass column with sintered glass at bottom was packed with anion exchange matrix and equilibrated with 0.03M K-PO_4 buffer pH 8.5. Temperature of the column was maintained at 4^0C. 5 ml hydantoinase enzyme solution was loaded to the column at a flow rate of 0.2 ml/min. Washing of column was performed with the same buffer. Matrices, which adsorbed more than 60% hydantoinase enzyme were eluted by 0.2-3M NaCl in 0.03M potassium phosphate buffer pH 8.5 at a flow rate of 0.4ml/minute. All fractions were collected and analysed for hydantoinase activity (table 11A).

Table 11 A: Purification of hydantoinase by ion exchange chromatography

Matrix	**activity %**		
	adsorbed	**washed**	**retained**
Dowex1B*50	64.19	13.58	50.61
Amberlite IRA 400	66.66	28.39	38.27
Tulsion A 2*MP	75.3	41.97	33.33
Indion polystyrene beads	69.13	17.28	51.85
Indion 284	100	61.72	38.28
Indion 925	29.12	15.13	13.99
Amberlite IRA 900	91.48	8.27	83.21 *
Indion 870	66.99	1.45	65.44 *
Indion 244	100	29.62	70.38 *

*Elution was carried out with 0.2-3 M NaCl. Protein was eluted but there was no activity.

4 g Amberlite IRA 900 was packed in column and elution was carried out with 0.2-1 M ammonium sulphate in 0.03M potassium phosphate buffer pH 8.5. Results are presented in table 11B.

Table 11 B: Purification of hydantoinase by Amberlite IRA 900

Adsorption %		**washing %**		**retained %**		**elution by $(NH_4)_2SO_4$**		
Protein	**activity**	**protein**	**activity**	**protein**	**activity**	**protein %**	**activity %**	**molarity ***
76.4	85.22	32.13	13.63	44.27	71.59	26.86	69.31**	0.2
						1.75	-	0.5
						0.52	-	1

* Molarity of $(NH_4)_2SO_4$

** Specific activity is 0.096 while control enzyme has sp. activity 0.036.

V] Hydrophobic Interaction Chromatography

Use of hydrophobic interaction chromatography (HIC) for purification of enzymes is reported by many authors [Westhoff and Kamp (1997); Mcguire et al. (1996)); Soubeyrand & Manjunath (1998); Song et al. (2000) etc.]. Butyl-sepharose, octyl-sepharose, thiopropyl-sepharose, macro prep butyl-HIC, macro prep methyl-HIC, phenyl-sepharose, phenyl-agarose etc are used as matrices for hydrophobic interaction chromatography. Purification of hydantoinase enzyme using various matrices are cited in table 12

Table 12: Use of HIC in purification of hydantoinase enzyme

Source	**HIC matrix**	**References**
Bovine liver	phenyl-sepharose	Brooks et al. (1983)
P. fluorescens DSM 84	phenyl-Sepharose	Morin et al. (1986a)
A. crystallopoietes DSM 20117	phenyl-sepharose	Siemann et al. (1999)
Pseudomonas 2262	phenyl-Sepharose	Shi et al. (2001)

Method:

Purification of hydantoinase enzyme was carried out using hydrophobic interaction chromatography (HIC) with phenyl-sepharose and macro prep t-butyl-HIC as HIC-matrices. Purification work was carried out by the procedure mentioned by Builder (1993). HIC-matrix was packed in a glass column and washing was carried out with distilled water and buffer. Temperature of the column was maintained at 4^0C. Column was equilibrated with 1M ammonium sulphate in 0.03 M K-phosphate buffer of pH 7.6. Enzyme solution containing 1M ammonium sulphate was loaded on it. Washings were carried out with 1M ammonium sulphate in 0.03M K-phosphate buffer having pH 7.6.

Elution was carried out with 1-0 M ammonium sulphate in 0.03 M K-phosphate buffer of pH 8.2; followed by 0.03M, plain buffer of pH 8.2, pH 8.5, pH 9.0 and 0.001M K-phosphate buffer having pH 9; distilled water; 25% glycerol; triton X-100; 1-4 M urea and 0.5% SDS. Hydantoinase enzyme was adsorbed on phenyl-sepharose matrix but enzyme could not be eluted using all possible eluants.

When phenyl sepharose was replaced with macro prep t-butyl-HIC and eluted with 2M urea followed by buffer washings, hydantoinase enzyme was observed in buffer washing. Purity of hydantoinase was checked using SDS-PAGE and it was found that it was a pure hydantoinase enzyme.

Hydantoinase was purified on higher scale as below:

Pseudomonas sp. NCIM 2875 was cultivated in shake flasks. Cells were collected by centrifugation. 200 grams cells were mixed with 28 ml ethyl acetate and incubated at room temperature for 1 hour. 2 liter 0.15M K-phosphate buffer of pH 8.7 was added to it and mixed well. It was incubated at 28^0C and 220 rpm for 15 h. Supernatant was separated by centrifugation and volume made to 2 liter. Supernatant was saturated with ammonium sulphate and kept at 4^0C for 2 h. Precipitate was collected by centrifugation and dissolved in 300 ml 0.15M K-phosphate buffer of pH 8.7. Ammonium sulphate was added in enzyme solution to get 1M concentration and kept at 4^0C for 4 hours. Supernatant was collected by centrifugation and used for HIC.

A glass column having 50 cm height and 1.5 cm diameter was packed with macro prep t-butyl-HIC matrix to get a bed volume of 50 ml. Temperature of the column was maintained at 4^0C. The matrix was washed with distilled water followed by 0.03M K-phosphate buffer of pH 7.6 (buffer-1) and finally equilibrated with 1M ammonium sulphate containg buffer-1. Enzyme was loaded on column at a flow rate of 0.5 ml/min. Washings were carried out with buffer-1 with flow rate of 3 ml/minute till absorbance was 0 at 280 nm. Washing was carried out by 1- 0 M ammonium sulphate in 0.03 M K-phosphate buffer pH 8.2; 0.03, 0.001 M K-phosphate buffer pH 8.2-9 and distilled water at 4^0C.

Elution was carried out at 28^0C by 2M urea in 0.03 M K-phosphate buffer of pH 8.8 followed by 0.03M K-phosphate buffer having pH 8.8 at a flow rate 1 ml/min. Eluted fractions were collected in 3 M ammonium sulphate solution in 0.03 M K-PO_4 buffer pH 8.7. Purity of hydantoinase enzyme was checked by electrophoresis using SDS-PAGE. Results are summarized in table 13. Purified enzyme was used for determining molecular weight and studying kinetic parameters.

Table 13: Purification of hydantoinase using t-butyl-HIC

Steps	**volume (ml)**	**protein (mgs)**	**activity (units)**	**specific activity**	**fold purification**	**recovery (%)**
Original enzyme	2000	4260	232	0.054	-	-
ammonium sulphate precipitate	445	3132.8	182.45	0.057	1.05	78.64
1 M ammonium sulphate	460	2796.8	170.2	0.061	1.12	73.36
t-butyl-HIC	60	4.02	51	12.69	235	21.98

VI] Properties of Hydantoinase

A] Molecular Weight Determination

1. SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

SDS-PAGE: Purified hydantoinase was passed through PD-10 column to remove salts and salt free fraction was concentrated using ultrafiltration (10000 MW cut off) and used for SDS-PAGE as reported by Laemmli (1970). Bovine serum albumin (66 kDa), porcine heart fumarase (48.5 kDa), carbonic anhydrase (29 kDa), beta-lactoglobulin from bovine milk (18.4 kDa) and alpha-lactoalbumin from bovine milk (14.2kDa) were used as standard protein molecular weight markers. Gel electrophoresis (vertical slab gel) was carried at 200V and 10^0C for 4 hours. Silver staining was done as reported by Blum et al. (1987). SDS-PAGE Gel photograph is shown in fig 1. Figure 2 shows the graph of Rf values Vs molecular weight.

Fig. 1: SDS-PAGE

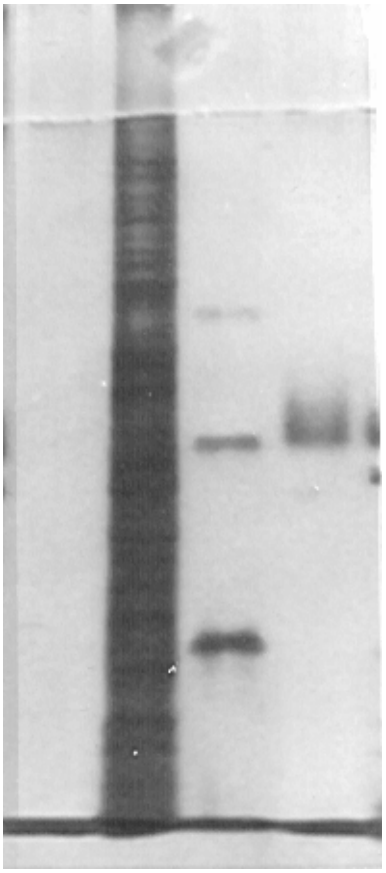

Fig. 1: SDS-PAGE: lane 1-crude hydantoinase, lane 2-molecular weight markers and lane 3-pure hydantoinase.

Fig. 2: Rf values Vs molecular weight

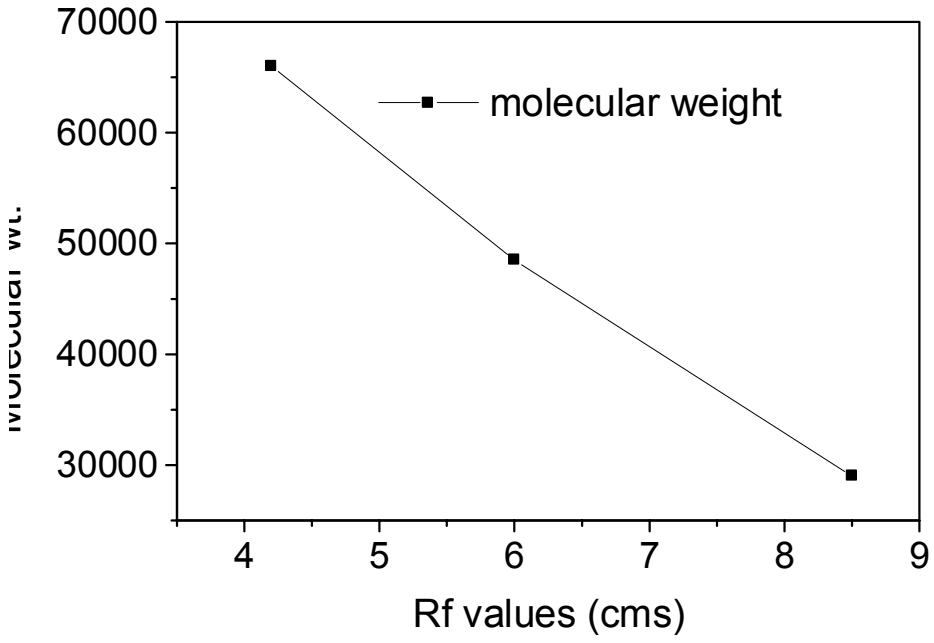

Fig. 2: Rf values were plotted against molecular weight. Molecular weight of pure hydantoinase enzyme was determined from its Rf value.

2) Gel filtration

Molecular weight was also determined using gel filtration chromatography. Sephadex G-200 and Biogel P-300 were used as gel matrices. Procedure given in Pharmacia catalogue was used for Sephadex G-200 and that given in Bio-Rad catalogue was used for Biogel P-300. Pre-swollen matrices were packed in a jacketed glass column having length of 50 cm and diameter of 1.1 cm. and equilibrated with 0.03 M K-phosphate buffer pH of 7.6 (buffer-1). Enzyme was loaded on column. Washing was done with buffer-1 and fractions were collected. Optical density was measured at 280 nm and hydantoinase activity was determined. Standard proteins [apoferritin (443 kDa), beta-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa) and cytochrome-C (12.5 kDa)] were used for molecular weight determination. Void volume was determined using blue dextran (2mg/ml). Ratio of eluted volume to void volume was used to determine molecular weight. Molecular weight was found to be 192100 and 195248 Dalton by Sephadex G-200 and Biogel P-300 matrices respectively. Therefore average molecular weight by gel filtration is 193674.

B] Optimum pH and temperature

1) Optimum pH: pH of hydantoinase enzyme was adjusted between pH 5-10.5 using acetate, phosphate and carbonate buffer. Activity of enzyme was determined at different pH values ranging from 5-10.5 at 30^0C (fig.3).

2) Optimum Temperature: pH of hydantoinase enzyme was adjusted to 8.5 and activity was determined at different temperatures ranging from 20^0C and 45^0C (fig. 4).

C] Temperature and pH stability

1) pH stability: pH of hydantoinase enzyme was adjusted between pH 5 to 10.5 and it was incubated at 30^0C for 2.5 h. After that hydantoinase activity was determined (fig. 3).

2) Temperature stability: pH of hydantoinase was adjusted to 8.5. It was incubated at 20, 25, 30, 35, 40, and 45^0C for 2.5 hours and hydantoinase activity was determined (fig.4).

3) Storage stability: pH of hydantoinase was adjusted to 8.5 and was kept at 4^0C. Activity was estimated with time interval.

Fig. 3: Optimum pH

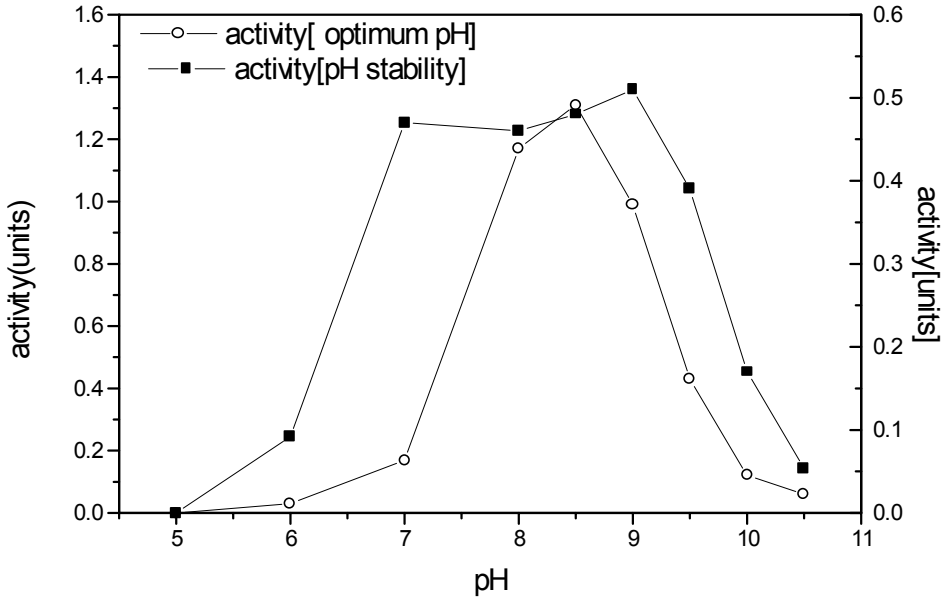

Fig. 3: Optimum pH (control: 1.2 U/ml) and pH stability (Control: 0.525U/ml) of hydantoinase.

Fig. 4: Optimum temperature

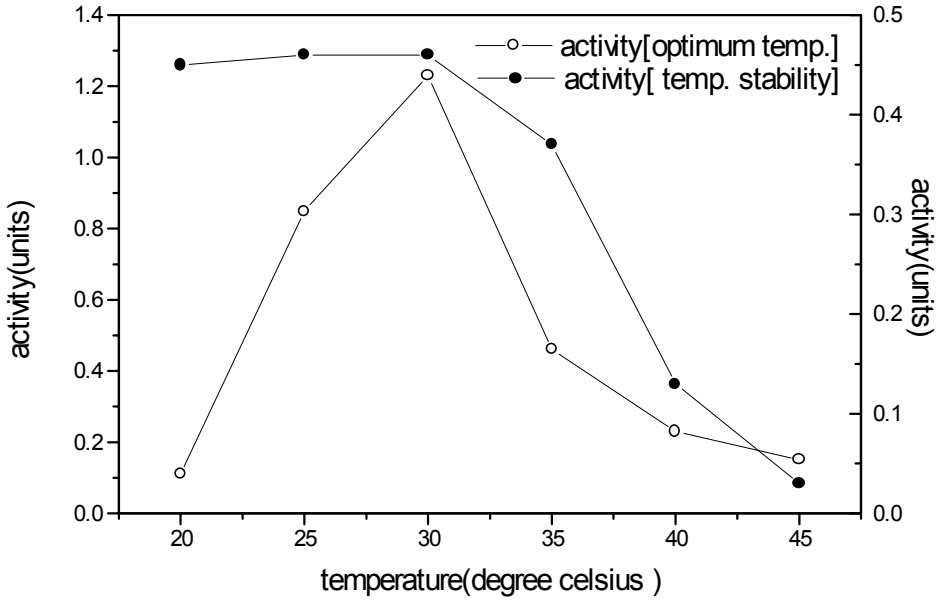

Fig. 4: Optimum temperature (control: 1.2U/ml) and temperature stability (control: 0.525U/ml) of hydantoinase.

D] Determination of kinetic parameters

Different concentrations (10.65, 14.2, 17.75, 21.3 mM) of phenylhydantoin were used and reaction was carried out at 8.7 pH and 30^0C temperature. Samples were withdrawn at 2, 3, 4, 5 and 6 hours, and analysed for N-carbamoyl acid. Graph of N-carbamoyl acid Vs time was plotted for each substrate concentration. Initial velocity (V) was determined from the slope at 0 hours and a graph of 1/S against 1/V was plotted (fig. 5), where S is substrate concentration and V is initial velocity.

Figure 5: Km and Vmax determination

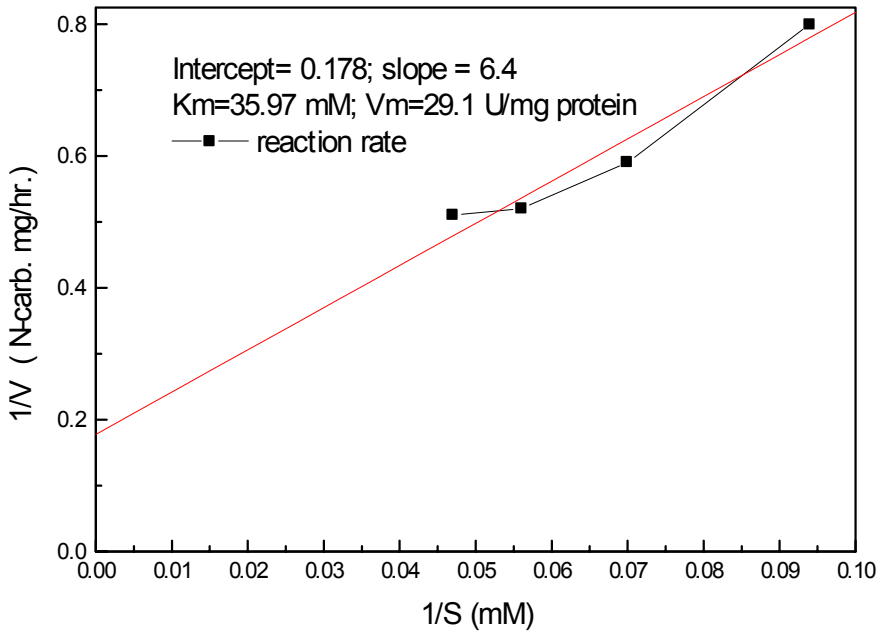

Fig 5: Km and Vmax determination: Km=35.97mM; Vmax= 29.1 U/mg protein

E**] Substrate specificity**

Assay of hydantoinase enzyme was carried out using different substrates such as dihydrouracil, hydantoin, phenylhydantoin and p-hydroxyphenylhydantoin etc. Activity for each substrate under identical condition was estimated (table14).

Table 14: Substrate specificity

Substrate	**Relative Activity %**
Dihydrouracil	289.65
Hydantoin	206.89
Phenylhydantoin	100
p-hydroxyphenylhydantoin	18.62

F] Effect of metal ions:

Mg, Fe, Mn, Zn, Cu, Ca, Ag and Al metal ions with 0.1mM and 10 mM concentration were added in assay mixture. Hydantoinase activity was determined (table 15). Activity of control was 1.45U/ml.

Table 15: Effect of 0.1mM and 10 mM metal ions on hydantoinase activity

Metal ions	**relative activity % (0.1 mM metal ions)**	**relative activity % (10 mM metal ions)**
Mg	88.26	84.13
Fe	109.64	104.12
Mn	110.33	82.06
Zn	102.06	51.72
Cu	68.96	51.03

Ca	98.61	77.23
Ag	8.27	-
Al	84.82	16.55

Discussion

Aqueous two-phase system using PEG of molecular weights ranging from 400 to 6000 and two salts namely K_2HPO_4 and ammonium sulphate were used. Though specific activity and enzyme recovery in both systems were not high, it was observed that in PEG-K_2HPO_4 system higher molecular weight PEG was better whereas reverse was the trend in the case of PEG-ammonium sulphate (table 2-9).

When ammonium sulphate was used for precipitation of hydantoinase enzyme maximum activity was recovered, but specific activity was not increased much (Table 10). From the results obtained using ion exchange chromatography (anion exchange resins) it was clear that Amberlite IRA 900 was better matrix than Indion 870 and Indion 244 (table 11A). When NaCl was used for elution, no activity was found although protein was eluted. Whereas on using ammonium sulphate for elution of protein bound to Amberlte IRA 900 matrix, activity was observed in eluted fraction. But there was only 2.7 fold increase in specific activity (table 11B).

Hydantoinase enzyme was purified using HIC. Hydantoinase enzyme adsorbed on phenyl-sepharose was too strong to elute it. Siemann et al. (1999) have purified hydantoinase from *Arthrobacter crystallopoietes* by using HIC (phenyl-sepharose). Brooks et al. (1983) have purified dihydropyrimidine amidohydrolase (which converts dihydrouracil, dihydrothymine) from bovine liver using HIC (Phenyl- sepharose). Morin et al. (1986a) have reported purification of hydantoinase (which converts dihydrouracil, isopropylhydantoin) from *Pseudomonas fluorescence* by HIC (phenyl-sepharose). Lee et al. (1995) have studied purification of hydantoinase from *Bacillus stearothermophilus* SD-1 using HIC as one of the steps.

Abendroth et al. (2000) used HIC in fluidized bed mode for purification of hydantoinase.

In the present work hydantoinase enzyme was purified using macro prep t-butyl-HIC support. Single band was obtained on SDS-PAGE (fig.1). Molecular weight was calculated on basis of Rf values and it is 49330 (fig.2). Molecular weight was also determined by gel filtration with Sephadex G-200 and Biogel P-300 as matrices. Molecular weights were found to be 192100 and 195248 respectively. Therefore average molecular weight of native enzyme by gel filtration is 193674. Since there was a single band on SDS-PAGE, it may be a tetramer with each subunits having molecular weight of 49330.

Siemann et al. (1999) purified hydantoinase from *Arthrobacter crystallopoietes* DSM 20117. They found molecular weights of subunit and native enzyme were 60 kDa and 257 kDa respectively. Park et al. (1999) purified hydantoinase from *B. thermocatenulantus* GH-2 by immunoaffinity chromatography and reported molecular wt. of subunit and native enzyme as 56 kDa and 230 kDa respectively. Maguire et al. (1978) partially purified dihydropyrimidinase from calf and rat liver and determined molecular wt. of subunits as 59000 and 62000 respectively and that of native enzyme as 252000 and 266000 respectively. Hydantoinase acting on phenylhydantoin in above cases is tetramer with molecular wt. of subunit ranging in 56-62 kDa and native ranging in 230-275 kDa. The molecular weight of the enzyme in the present work was lower than those results. Fan and Lee (2001) have reported hydantoinase having lower molecular weight of 52.5 kDa from adzuki. Molecular wteight of hydantoinase from *Pseudomonas fluorescens* strain DSM 84 are 60000 and 230000 (subunit and native) respectively. Molecular wt. of hydantoinase from *Pseudomonas straita* and *P. stutzeri* ATCC 17588 are 190000 and 115000 respectively. Molecular weight of dihydropyrimidinase from *P. putida* is 156000 Huang et al. (2000).

Optimum pH and temperature of hydantoinase studied in the present work are 8.5 and 30^0C respectively (fig.3 & 4). Optimum pH and temperature of hydantoinase from *A. crystallopoietes* DSM 20117 are 8 and 50^0C respectively [Siemann et al.

(1999)]. Park et al. (1999) have reported optimum pH 7.5 and temp. 65^0C of the hydantoinase from *B. thermocatenulantus* GH-2. Partially purified hydantoinase from adzuki bean has optimum pH 8.5 and temperature 60^0C Fan and Lee (2001). Optimum pH and temperature of hydantoinase extract from *A. tumefaciens* ATCC 55632 are 10 and 70^0C respectively Weber and Durham (1996). pH stability of the enzyme from present studies was in range 7.0 to 9.0. Enzyme is stable at temperatures below 30^0C. Half-life is 216 hours at 4^0C. Half-life of hydantoinase from *A. crystallopoietes* DSM 20117 in phosphate buffers is 400 hrs. Its half life is 6 hours at 50^0C [Siemann et al. (1999)]. Park et al. (1999) observed 45 min. half life of the hydantoinase from *B. thermocatenulantus* GH-2 at 80^0C. Fan and Lee (2001) found that hydantoinase had retained 76% activity at 40^0C in 6 days. Weber and Durham (1996) observed that 90% activity was retained at 9 pH and 25^0C in 24 hours.

Km and Vmax values of hydantoinase from NCIM 2875 were 35.97mM and 29.1U/mg protein respectively. Siemann et al. (1999) have estimated Km and Vmax of their hydantoinase and, are 8 mM and 217 U/mg respectively. Fan and Lee (2001) have estimated Km (13.3 mM) and Vmax (1422.9 micromoles/min. gm protein) of the hydantoinase from adzuki beans. Km values of present enzyme is higher than these two references. Vmax is lower than Siemann et al. (1999) and higher than Fan and Lee (2001).

Hydantoinase in the present work was nearly 3 times more active on dihydrouracil and 2 times on hydantoin, but was less active on p-hydroxyphenylhydantoin (table 14). Hydantoinase reported by Siemann et al. (1999) was less active against dihydrouracil and p-hydroxyphenylhydantoin but 5 times active on hydantoin. The hydantoinase used by Weber and Durham (1996) was more active against dihydrouracil and methylhydantoin as compared to phenylhydantoin.

Though some people have reported enhancememnt of activity in presence of metal ions. Hydantoinase reported by Park et al. (1999) required Mn for activity. But in the present work no metal ions enhanced activity except the Ag ions were inhibitory (table 15).

All these results corroborate with the findings of the present work.

Conclusion

Purification of hydantoinase enzyme was attempted using different methods such as aqueous two-phase system, ammonium sulphate precipitation, ion exchange chromatography and hydrophobic interaction chromatography (HIC) etc. With aqueous two-phase system, enzyme recovery as well as specific enzyme activity were poor whereas by precipitation with ammonium sulphate enzyme recovery was good but specific activity was not improved much. Though enzyme activity was good by when Amberlite IRA 900 matrix was used for ion exchange chromatography specific activity was increased by only 2.7 times.

When phenyl-sepharose was as matrix for HIC enzyme was adsorbed on phenyl-sepharose but it was not possible to elute the enzyme. Using macro prep t-butyl matrix as HIC support obtained 235-fold purification and 21.98 % recovery of hydantoinase was obtained.

Molecular weights of native hydantoinase and each subunit were determined and were 193674 and 49330 respectively. Some properties such as optimum pH, optimum temperature, pH stability, temperature stability, Km, Vmax, substrate specificity and metal ion effect were studied. Hydantoinase was stable in the pH range of 7.0 to 9.0 and temperature below 30^0C. Kinetic parameter namely Km and Vm values were 35.97 mM and 29.1 U/mg protein respectively.

Chapter 5

Immobilization of cells and hydantoinase enzyme

Summary

Cells of *Pseudomonas sp.* NCIM 2875 containing hydantoinase enzyme were immobilized in calcium alginate and used for biotransformation of phenylhydantoin to N-carbamoyl acid. Similarly, crude hydantoinase enzyme extracted from the cells was immobilized on various matrices and bioconversion of phenylhydantoin to N-carbamoyl acid and reusability of the immobilized enzyme was studied. Under identical experimental conditions, free cells converted 81.2% of phenylhydantoin as against 47.6% conversion for immobilized cells. But it was possible to reuse the immobilized cells for 5 times. With hydantoinase immobilized on phenyl-sepharose 38.18% conversion was obtained as against 52.41% with crude enzyme. This immobilized enzyme could be reused for five times. Hydantoinase enzyme immobilized in 6% polyacrylamide gel converted 32.14% hydantoin to N-carbamoyl acid and was reused consecutively for 7 times.

Introduction

Cells or enzymes either in free or immobilized form are used for biotransformations. Very often these are expensive commodities in the bioconversion reactions and their recovery and reuse is required for economic benefits. Free enzymes being soluble in water cannot be recovered for reuse.

Cells can be recovered by centrifugation for reuse, but centrifugation after each use is not economical on large scale. But the cells and enzymes on immobilization can be reused easily. In this case large number of recycles of immobilized cells/enzyme are desired to lower the cost of the process.

Cells and enzyme are immobilized by various methods. Entrapment of cells is one of the most common methods for cell immobilization [Abott (1977), Brodelium and Vandamme (1987)]. Cells are entrapped in gels like polyacrylamide gel, calcium alginate, cellulose triacetate or collagen etc. Cell immobilization in calcium alginate is extensively reported in literature [Chevalier et al.(1989a,b); Ryu and Lee (1997); Chen et al. (1999); McCabe et al.(2001); Carvalho et al.(2002) etc.].

Martin et al. (1998) immobilized Hydantoinase and carbamoylase on chitosan and chitin. Several researchers have studied immobilization of hydantoinase [table1]. Here, immobilization of *Pseudomonas sp.* NCIM 2875 cells and hydantoinase extracted from the cells is reported and immobilized hydantoinase and cells were used for biotransformation of phenylhydantoin to its N-carbamoyl acid. Cells were immobilized in calcium alginate gel and hydantoinase enzyme, extracted from *Pseudomonas sp*. NCIM 2875 was immobilized in polyacrylamide gel. Immobilization of hydantoinase enzyme using celite, amberlite, macro prep DEAE-support, macro prep butyl-HIC and phenyl-sepharose was also tried.

Table 1: Immobilization of hydantoinase

Source	**Cells / hydantoinase enzyme**	**immobilization system**	**references**
P. putida	cells	calcium alginate	Chevalier et al. (1989a)
P. putida DSM 84	cells	calcium alginate	Chevalier et al. (1989b)
B. sphaericus	cells	In polysaccharide beads byinclusion & cross-linked with glutaraldehyde	Shiotani et al. (1991)
Pseudomonas sp. KBEL 101	cells	polyacrylamide gel	Kim et al. (1994)
Bacillus stearother-mophilus SD-1	enzyme	DEAE-cellulose resin	Lee et al. (1996)
Bacillus sp. KNK108 & recombinant *E. coli*	enzyme (hydantoinase & decarbamoylase)	duolite A568	Nanba et al.(1996)
Pseudomonas, Bacillus, or *Arthrobacter*	enzyme	immobilized on porous Mg-Al-silicate & treated with gluteraldehyde-cong. crosslinking agent	Kim & Kim (1997)
lentil seeds	enzyme	DEAE-cellulose	Rai & Taneja (1998)
recombinant *E. coli*	cells	calcium alginate	Chen et al.(1999)
Arthrobacter aurescens	enzymes (hydantoinase and L-N-carbamoylase)	Eupergit C, Eupergit C 250 L, and EAH-Sepharose	Ragnitz et al. (2001)

Materials and Methods

Chemicals

Phenylhydantoin was provided by Dr. M.S.Gaikwad (P.D. Division, N.C.L., Pune). Sodium alginate (Loba Chem.,Bombay); celite 545 (SD Fine Chem., Bombay); amberlite IRA 900 (Sigma Chem.) and all other chemicals (AR grade) were from SD Fine Chem (Bombay), were used.

Strain:

Pseudomonas sp. NCIM no. 2875 was used. It was maintained on nutrient agar [beef extract 10, NaCl 5, peptone 10 and agar 20 g /L, pH 7.0].

Growth of culture and estimation of hydantoinase activity:

Growth of *Pseudomonas sp.* NCIM 2875 and hydantoinase activity of cells, enzyme and protein estimation were carried out as mentioned in chapter 3. Extraction of hydantoinase enzyme was performed as cited in chapter 4.

Immobilization of cells

2, 4 and 6% sodium alginate was prepared. 5 ml of the sodium-alginate solution was mixed with 0.6 gram cells (wet wt.) and mixture was added drop-wise in 0.2M cold $CaCl_2$ solution with a syringe. Beads formed were kept at 4^0C for 2 hours and washed twice with distilled water. Biotransformation reaction was carried out by using 0.05gram phenylhydantoin, beads containing immobilized cell (obtained from 5 ml sodium alginate solution) and 10 ml tris-HCl buffer (0.15 M, pH 9). Reaction mixture was incubated on a shaker at 30^0C temperature and 100 rpm for 4 hours. After the reaction, immobilized cells were recovered by decanting the reaction product and residual substrate, rinsed with distilled water and reused for next biotransformation reaction as maintained above. These reuse experiments were carried out till there was an appreciable loss in the activity of the cells. The results are shown in figure 1.

Immobilization of Hydantoinase

Celite, Amberlite IRA 900 and Macro prep DEAE- support

1 gram each of celite, amberlite IRA 900 and Macro prep DEAE supports were soaked in phosphate buffer. 5-ml hydantoinase enzyme was added to it and kept stirring at 100 rpm and 30^0C for 15 hours. Supernatant was decanted and matrices were washed with buffer and used for biotransformation of 0.5% phenylhydantoin (in 10-ml volume) to N-carbamoyl acid in 10-hour reaction time. Results are shown in table 3.

HIC matrices:

Phenyl-sepharose, macros prep butyl-HIC and macro prep methyl-HIC were used for immobilization of hydantoinase. For this 1 gram matrix was equilibrated with 1M $(NH_4)_2SO_4$ in 0.15 M K-phosphate buffer of pH 8.7 and hydantoinase enzyme saturated with 1M $(NH_4)_2SO_4$ was added to the matrix. It was stirred at 100 rpm and 30^0C for 1 hour. Supernatant was removed and matrix was washed twice with buffer and used for biotransformation of 0.5% phenylhydantoin in 10-ml volume at 100 rpm for 4 hours. Results are presented in table 4.

Polyacrylamide Gel

40% acrylamide-bisacrylamide solution was prepared by dissolving 38-gram acrylamide and 2 gram bisacrylamide in distilled water. 0.89, 1.25 and 1.68 ml of this solution was added to 5ml enzyme, 0.2 ml of 10% ammonium persulphate and 0.2 ml TEMED (N,N,N',N'-tetramethylene-ethylenediamine) to get polyacrylamide concentrations of 6, 8 and 10% respectively and mixed well at 30 C. After 15 minute gel was formed that was washed thrice with distilled water and was cut into pieces. Biotransformation was carried out using enzyme immobilized in the polyacrylamide gel pieces and reusability of the immobilized enzyme was studied. Results are presented in table 5.

Results

(figure 1)

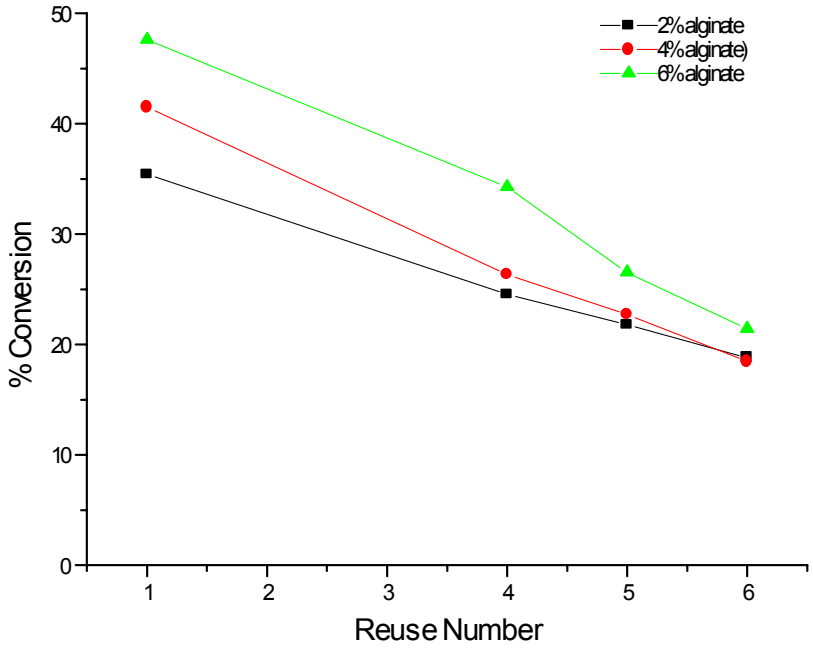

Fig. 1: Cells were immobilized in alginate and used for biotransformation of phenylhydantoin. 2, 4 and 6 % alginate was used for immobilized. Study of reuse of immobilized cells is presented in above graph.

Table 2: Hydrolysis of phenylhydantoin by immobilized hydantoinase enzyme.

Matrix	**protein loaded %**	**activity retained %**	**hydrolysis %**
celite	60.24	72	17.24
amberlite IRA 900	23.84	86.25	5.45
macro prep DEAE-support	47.69	53.73	21.81

Control = 52.1% (for celite and amberlite) and 54.54% (for macro prep DEAE-support).

Table 3: Hydrolysis of phenylhydantoin by immobilized hydantoinase.

Matrix	**protein loaded %**	**activity retained %**	**hydrolysis %**					
			I	**II**	**III**	**IV**	**V**	**VI**
phenyl-sepharose	3.125	81.53	37.14	38.18	27.14	29.45	14.18	1.45
butyl-HIC	10.76	94.6	21.81	7.87	5.32	1.09	n.d.	n.d.

n. d. = not determined

Control = 52.41 and 50.19% hydrolysis.

Table 4: Immobilization of hydantoinase in polyacrylamide gel: relative hydrolysis of phenylhydantoin

Gel %	**relative hydrolysis %**							
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
6	24	23.93	29.34	32.14	27.26	29.13	26.57	20.93
8	26.37	25.78	26.57	27.26	25.12	29.13	25.12	20.73
10	20.4	29.58	25.12	30.76	29.13	29.13	25.12	22.38

Discussion

From figure 1 it is clear that the cells immobilized in calcium alginate converted phenylhydantoin significantly and few number of reuses were possible. As compared to free cells (81.2%) conversions were lower which may be due to mass transfer limitation and poor solubility of the substrate. It was also observed that higher concentration of the alginate was better for the immobilization as that might be restricting the leaching out of the cells. With each recycle percentage conversion was decreased which indicated slow leaching of cells from beads.

Chevalier et al. (1989a,b) immobilized cells of *P. putida* DSM 84 in calcium alginate and used for biotransformation of dihydrouracil and reported 40-45% conversion of dihydrouracil. They found swelling and breaking of beads after 96 hours. Kim et al. (1994) have immobilized cells of *Pseudomonas sp.* KBEL 101 in polyacrylamide gel and used for biotransformation of hydroxyphenylhydantoin and found 50 hours half life which on supplying nitrogen source to the cells, was increased to 25 days. Chen et al. (1999) studied biotransformation of hydroxyphenylhydantoin using cells of recombinant *E. coli* immobilized in calcium alginate and observed more than 95% conversion even after 3 cycle. Durham and Weber (1995) used hypol for immobilization of cells of *A. tumefaciens* and used for biotransformation of methylhydantoin and observed 78% conversion even after 8 cycle.

With hydantoinase immobilized on celite, amberlite IRA900 and macro prep DEAE-support, biotransformation was poor whereas by using macro prep methyl-HIC biotransformation was not at all observed that might be due to binding of active sites of enzyme by methyl-HIC. Hydantoinase immobilized on butyl-HIC showed some conversion but could not be reused. Lower conversion by butyl-HIC-immobilized hydantoinase may be due to binding of the active sites of enzyme to the matrix. Hydantoinase immobilized on phenyl-sepharose had shown good conversion but after 5 recycle it declined to 1.45 % which may be due to washing out of

hydantoinase from the matrix (table 4). Fan and Lee (2001) have immobilized hydantoinase from Adzuki beans. They treated hydantoinase entrapped calcium alginate beads with polygluteraldehyde and used it for biotransformation of phenylhydantoin and observed more than 95% conversion even after 5 repeated batches.

In the present work, hydantoinase was immobilized by entrapment in polyacrylamide gel and was used repeatedly for 8 times. Slow decline in the conversion was observed. There was no effect of gel concentration on bioconversion (table 5). Slight leaching out of the cells was observed in 7 and 8^{th} use. Increasing enzyme loading may increase bioconversion.

Conclusion

Cells of *Pseudomonas sp.* NCIM 2875 immobilized in calcium alginate in the form of beads converted maximum of 47.6% phenylhydantoin to N-carbamoyl acid as compared to 81.2% by free cells. Percentage conversion of phenylhydantoin was decreased to half after 6^{th} reuse. Hydantoinase enzyme immobilized on celite, amberlite IRA900 and macro prep DEAE-support showed negligible conversion. Whereas hydantoinase immobilized on phenyl-sepharose could be reused for 3 cycle only. Hydantoinase immobilized in polyacrylamide gel could be reused successfully for 8 times.

Chapter 6

General conclusions and future work

Growth and Hydantoinase production

In this thesis we studied hydantoinase enzyme from *Pseudomonas sp.* NCIM 2875. Optimization of growth and hydantoinase production was carried out in shake flasks. Medium optimization and parameters for enhancing growth and hydantoinase production were studied. When yeast extract was partly replaced by peptone increase in enzyme activity was observed. Various carbon sources e.g. glucose, fructose, sucrose, maltose, mannitol, glycerol etc. were used in the basal medium. Maximum activity was obtained with glucose and cell yield also was nearly double that of the control, therefore glucose was used in further study.

Both organic and inorganic nitrogen sources were used in the medium to find out their effect on growth and hydantoinase activity. Use of soy-peptone, casein acid hydrolysate, soybean meal, cottonseed meal and casein-soybean enzymatic digest was done in the medium. Activity obtained using soy-peptone at pH 8.0 was comparable with control. When casein-soybean enzymatic digest was used in the medium, activity comparable with control at pH 7.5 and 8.0 was obtained. $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, $NH_4H_2PO_4$, $(NH_4)_2CO_3$, $NaNO_3$, KNO_3, and urea were used in the medium as nitrogen source. Maximum activity was achieved by using $NaNO_3$. Activity obtained by using $NaNO_3$ was slightly more than KNO_3. Less cell yield than control was obtained with $NaNO_3$ and KNO_3. In further studies $NaNO_3$ was used as nitrogen source.

Optimization of inoculum age was performed in tube and flask. Inocula having different age were used for study. An inoculum age of 18 -20 h was found to be optimum for tube inoculum and flask inoculum. Optimization of harvesting time was carried out. It was observed that 27 h is optimum for harvesting. Effect of different salts with different concentrations was investigated. Salt concentrations had no significant effect on hydantoinase activity.

Effect of inducers (of hydantoinase enzyme) was studied. Dihydrouracil, hydantoin, and phenylhydantoin were used as inducers with different concentrations. It was observed that hydantoin was the best inducer. Effect of different C/N ratios on growth and hydantoinase activity was investigated. Maximum hydantoinase activity was obtained by using C/N ratio 1.62 where $NaNO_3$ concentration was 1.5%. However, cell yield decreased at this C/N ratio. In this medium yeast extract and peptone was present. In absence of yeast extract and peptone less activity than that in control was observed. This indicates that though yeast extract and peptone did not have a pronounced effect on activity, it was required in the medium to provide the co-factors necessary for growth. Only 0.1% of yeast extract and peptone was used in the medium.

Optimization of other conditions e.g. pH and temperature was carried out. Experiments were performed using different pH. A maximum activity at pH 8.0 was obtained than at pH 7.5, however maximum cell yield was observed at pH 7.5. Effect of various cultivation temperatures i.e. 30, 35 and 40^0C on growth and hydantoinase activity was studied. At 30^0C more activity and growth was obtained than other temperatures used.

The final composition of optimized growth medium was: Glucose 1, $NaNO_3$ 1.5, KH_2Po_4 0.15, K_2HPO_4 0.48, yeast extract 0.1, peptone 0.1, $MgSO_4.7H_2O$ 0.02, $CaCl_2.2H_2O$ 0.002, hydantoin 0.03% and trace element 0.1 ml/100ml , pH 7.5. Cells were cultivated using this medium. Inoculum (grown in tubes and flasks) having 20 hrs. age was used. Cells were harvested at 27 hrs.. Using basal medium for cultivation 0.43 unit hydantoinase activity was obtained but the optimized medium yielded 4.63 units hydantoinase activity. One of the objectives of this study was to arrive at a medium which would be simple and cost effective. This was achieved.

Extraction of Hydantoinase Enzyme

Extraction of hydantoinase enzyme was carried out from cells of *Pseudomonas sp.* NCIM 2875. Different solvents, detergents and EDTA were used to rupture or permeabilize cells of *Pseudomonas sp*. NCIM 2875. These cells were further subjected to buffer extraction where hydantoinase enzyme was extracted from cells into buffer. Methanol, ethanol, isopropanol, butanol, isoamyl alcohol, acetone, methyl isobutyl ketone, ethyl acetate, 1,4-dioxan, toluene, xylene, hexane, cyclohexane, ethylene dichloride, dichloromethane, dimethyl sulfoxide, diethyl ether, petroleum ether, sodium dodecyl sulphate, triton X-100, tween-80 and EDTA were used for rupturing or permeabilizing cells. Experiments were performed using 0.25-80% w/w solvents, 0.95-22.47% w/w detergents and 1.63-5.79%w/w EDTA. Contact time in the range of 15 min. to 9 hrs. was used. Buffer extraction was carried out for 3-20 hrs. Experiments were conducted at 30^0C. In some experiments extractions were carried out using solvents/ detergents/EDTA and buffer simultaneously.

When acetone, isopropanol and butanol were used for extraction from cell poor results were obtained. When toluene, methanol, dimethyl sulfoxide, hexane, cyclohexane, petroleum ether, 1,4-dioxan and triton X-100 and ethylenediaminetetraacetic acid (EDTA) were used for cell rupture or permeabilization, enzyme recoveries below 20%. 20.9, 23.15, 21.89 and 25.22% extractions were obtained with 5% isoamyl alcohol, 10% xylene, 10% methyl isobutyl ketone and 40% dichloromethane respectively using 15 min. contact time and 15 h buffer extraction time. 21.54, 27.23 and 22.51 % extractions were obtained with 40% ethylene dicloride, 10% chloroform and 40% diethyl ether respectively in 1 h contact time and 15 h buffer extraction. 24.05 % extraction was achieved by 22.47% tween-80 in 2 h contact time and 15 h buffer extraction time. 31.42 % extraction was obtained by using 10% ethanol with 6 h contact time and 20 h buffer

extraction time. Maximum 33.63% extraction was achieved when 10% ethyl acetate was used for cell rupture with 2 h contact time and 15 h buffer extraction time.

Purification of Hydantoinase

Purification of hydantoinase enzyme was performed by using various methods. Solvent precipitation, ammonium sulphate precipitation, aqueous two-phase system, ion exchange chromatography and hydrophobic interaction chromatography (HIC) were used for purification of hydantoinase. Properties of purified hydantoinase were studied. Precipitation of enzyme was carried out using different solvents i.e. acetone, methanol, ethanol, isopropanol, 1,4-dioxan, dimethyl formamide and dimethyl sulfoxide. Though precipitation occurred recovery of enzyme activity was nil. Purification study was carried out using aqueous two-phase system. Polyethylene glycol-salt system was used where K_2HPO_4 and ammonium sulphate were used as salts. Different concentrations of polyethylene glycol (PEG), K_2HPO_4 and ammonuim sulphate were tested.

A maximum of 35.71% activity was observed in PEG phase when 12% PEG (molecular weight 6000) and 8% K_2HPO_4 were used. Maximum 39.44% activity was obtained in PEG phase when 16% PEG (molecular weight 400) and 25% ammonium sulphate were used. Studies on aqueous two-phase system did not give very encouraging results. Precipitation of enzyme was carried out using 20-72% ammonium sulphate. Maximum 93.23% precipitation (activity basis) was observed by using 72% ammonium sulphate. Maximium enzyme was recovered. but specific activity did not show a significant increase.

Purification of hydantoinase enzyme was attempted using ion exchange resins. Dowex 1*B50, Amberlite IRA 400, Tulsion A 2*MP, Indion polystyrene beads, Indion 284, Indion 925, Amberlite IRA 900, Indion 870 and Indion 244 were tested as matrices. Enzyme loading on Amberlite IRA 900, Indion 870 and Indion 244 was appreciable, however the maximum enzyme loading was observed on Amberlite

IRA900. Elution was carried out using NaCl. Enzyme was eluted but there was not activity. When Amberlite IRA 900 was used for enzyme recovery elution was carried out with ammonium sulphate. Specific activity increased 3 times.

Hydrophobic interaction chromatography (HIC) was used for purification of hydantoinase enzyme. Phenyl-sepharose and macro prep t-butyl support were used as HIC matrices. Hydantoinase enzyme was adsorbed on phenyl-sepharose but could not be eluted. Hydantoinase was adsorbed on macro prep t-butyl support. It was eluted in washing followed after elution by urea. Purity of eluate was checked and was found to contain pure hydantoinase.

Purity of hydantoinase enzyme was checked by SDS-PAGE. Single band was observed. Properties of purified hydantoinase were investigated. Molecular weight was determined on basis of Rf values obtained in SDS-PAGE. Molecular weight was 49,330. Molecular weight was also determined by gel filtration. Sephadex G-200 and biogel P-300 gel were used as matrices. Molecular weights of hydantoinase enzyme were 192100 and 195248 by using Sephadex G-200 and Biogel P-300 respectively. Average molecular weight by gel filtration was 193,674. Therefore hydantoinase enzyme exists as a homo-tetramer. Other properties of purified hydantoinase were studied. Optimum pH and temperature were 8.5 and 30^0C respectively. Hydantoinase was stable in pH range 7-9.0 and at temperatures below 30^0C. Halflife of hydantoinase at 4^0C and pH 8.5 was 216 hrs. Km and Vmax of hydantoinase enzyme were 35.97 mM and 29.1 U/mg protein respectively. Substrate specificity of hydantoinase was determined. It was less active against p-hydroxyphenylhydantoin, nearly 3 times active against dihydrouracil and 2 times active against hydantoin as compared to phenylhydantoin. Effect of metal ion on hydantoinase activity was investigated. Ag inhibited hydantoinase activity. Fe increased activity slightly. Enzyme ctivity was inhibited by 10 mM of Mg, Mn, Zn, Cu, Ca and Al.

Immobilization

Immobilization of whole cells and crude hydantoinase was carried out. Cells of *Pseudomonas sp.* NCIM 2875 were immobilized in calcium alginate beads and used for biotransformation of phenylhydantoin. Maximum conversion was 47.6% when immobilized cells were used whereas conversion using free cells was 81.7%. The immobilized beads were used 5 times. Conversion efficiency decreased to below half. Hydantoinase enzyme was immobilized on Celite, Amberlite IRA 900, macro prep DEAE-support etc. Conversion efficiency of enzyme immobilized on Celite, Amberlite IRA 900 and macro prep DEAE-support was poor. Hydantoinase enzyme was immobilized on phenyl-Sepharose and macro prep t-butyl HIC-support. Good conversion was observed with enzyme was immobilized on phenyl-Sepharose but conversion went down to 1.45% after 5 recycle. Less conversion was observed using enzyme immobilized on macro prep t-butyl HIC-support. Hydantoinase enzyme was entrapped in polyacrylamide gel and used for bioconversion. Conversion efficiency was low and after 7 recycles conversion efficiency started declining.

Future work

The work carried out in this thesis is mentioned above. Further scope of work includes Growth and hydantoinase production in fermenter. This will increase growth and hydantoinase production. Batch, fed batch and continuous culture studies require be done in order to increase the growth and hydantoinase production. Simultaneous work on N-Carbamoylase needs to be done. This can ensure complete conversion of hydantoin to the corresponding amino-acid.

We have carried out extraction of hydantoinase enzyme by using solvents, detergents and EDTA for cell rupture. Combination of solvents, detergents and other chemicals will increase extraction efficiency. Techniques like solid shear (Bead Mill) need to be examined as an alternate approach to enzyme recovery. There is further scope for purification of hydantoinase using other approaches such as Foam flotation, Affinity chromatography and membrane separation. Immobilization of free hydantoinase on different matrices may improve efficiency. Entrapment of hydantoinase in polyacrylamide gel could be further studied in detail. Some of the above mentioned work will be undertaken for detail study in near future.

Chapter 7

References

Abdel-Fatah, M.K. (1977) 'Studies on the asparaginolytic enzymes of streptomyces II. Purification and characterization of L-asparaginase from *Streptomyces longsporusflavus* (F-15) strain.' *Egypt. J. Microbiol.* vol. Date 1996, **31(3),** 303-312 [C.A. 128: 280061].

Abendroth, J.; Chatterjee, S.; Schomburg, D. (2000) 'Purification of a D-hydantoinase using a laboratory-scale streamline phenyl column as the initial step.' *J. Chromatogr., B: Biomed. Sci. Appl.,* **737(1 + 2),** 187-194.

Abott, Bernard J.(1977) 'Immobilized cells' in '*Annual reports on fermentation processes',* **vol. 1,** Ed. by Perlman, D. and Tsao, G. T., p. 205-233, Academic Press New York.

Achary, A.; Hariharan, K.A.; Bandhyopadhaya, S.; Ramachandran, R . and Jayaraman, K. (1997) Application of numerical modelling for the development of optimized complex medium for D- hydantoinase production from *Agrobacterium radiobacter* NRRL B 11291'. *Biotechnology Bioengineering* **55(1),** 148- 154.

Aida, K.; Chibata, I.; Nakayama, K.; Takinami, K.; Yamada, H. [Eds.],(1986) In: *Biotechnology of amino acid production, Progress in industrial microbiology,* vol. **24**, Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amsterdam.

.

Ajinomoto Co. Inc. Japan (1980) 'D-a-amino acids from 5-substituted hydantoin'. *Jpn.Kokai Tokkyo Koho JP* **55114292** 3 Sep 1980, Showa, 7 pp. (Japanese) (Japan) [C.A. 94: 45601].

Akai, H. (1986) 'Histidine' In: *Biotechnology of amino acid production, Progress in industrial microbiology.'* vol. **24**, [Aida, K.; Chibata, I.; Nakayama, K.; Takinami, K.; Yamada, H. (Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B. V., Amstedam, p. 247-256.

Albertsson, P.A. (1958) 'Partial fractionation in liquid two-phase system.' *Biochem. Biophys. Acta* **27**, 378-395.

Albertsson, P.A. (1960) in '*Partition of cell particles and macromolecules'*, John Wiley and Sons, New York.

Ames, G.F.; Prody, C.; Kustus, S.(1984) 'Simple, rapid and quantitative release of periplasmic proteins by chloroform.' *J. Bacteriol.* **16(3)**, 1181-1183.

Arcuri, M. B.; Antunes, O. A. C.; Sabino, S. J.; Pinto, G. F.; Oestreicher, E. G. (2000 'Resolution of D,L-hydantoins by D-hydantoinase from *Vigna angularis*: production of highly enantioenriched N-carbamoyl-D-phenylglycine at 100% conversion.' *Amino Acids,* **19(2)**, 477-482.

Arnold, W.N.(1972a) 'Location of acid phosphatase and beta-fructofuranosidasewithin yeast envelopes.' *J. Bacteriol.* **112(3),** 1346-1352.

Arnold, W.N. (1972b) 'The structure of the yeast cell wall.' *J. Biol. Chem*. **247(4)**, 1161-1169.

Asano, Y.; Kato, Y.; Yamazaki, K. (1994) 'Preparation of L-amino acids with microorganism'. *Jpn. Kokai Tokkoyo Koho JP* **06245781 A2** 6 Sep 1994, Heisei 20 pp. (Japan) [C.A.121: 299321].

Asenjo, J.A. (1990) 'Selection of operation in separation', in '*Separation processes in biotechnology',* Asenjo, J.A. ed., Marcel Dekker Inc. New York and Basel, pp. 3-16.

Astruc, Jean; Morelle, Jean; Lauzanne-Morelle, Eliane (1992) 'Acetylpolyaminoacids as insecticides against lice on humans.' *Fr. Demande FR* **2675015 A1** 16 Oct 1992, 7pp. (France) [C.A.118:141867].

Atkinson, B. and Mavituna, F. (1991) *'Biochemical Engineering and Biotechnology Handbook.'* 2nd edition, M Stockton Press, USA

Atkinson, T., Scawen, M.D.; Hammond, P.M. (1987) 'Large scale industrial techniques of enzyme recovery.' In: *Biotechnology,* Rehm, H.J. and Reed, G. eds., **vol. 7 a,** pp.279-323, VCH, Publishers, Germany

Baeyer, A. (1861) *Ann*. **117**:178.

Balk, R.; Eipel, H.; Pressler, U. (1998) 'Bioluminescence test for thedetection of microorganisms in aqueous dispersions of polymer and/ or pigments.' *Ger. Offen. DE* **19625137 Al** 8 Jan. 1998, 8 pp. (Germany) [C.A. 128: 125582].

Barthomeuf, Chantal; Regerat, Francoise; Pourrat, Henri (1994.) 'Improvement in tannase recovery using enzymic disruption of mycelium in combination with reverse micellar enzyme extraction.' *Biotechnol. Tech.* **8(2),** 137-42.

Basha, S.Y. and Palanivelu, P. (2000) 'A novel method for immobilization of invertase from the thermophilic fungus, *Thermomyces lanuginosus*.' *World J. Microbiol. Biotechnol.* **16(2)**, 151-154.

Battilotti, M.; Barberini, U. (1988) 'Preparation of D-valine from D,L-5-isopropylhydantoin by stereoselective active biocatalysis' *.J. Mol. Catal.*, **43(3)**, 343-52.

Berggern, K.; Veide, A.; Nygren, P.A. and Tjerneld, F. (1999) 'Genetic engineering of protein-peptide fusions for control of protein partitioning in thermoseparating aq. two-phase systems'. *Biotechnol. Bioeng*. **62(2)**, 135-144.

Bernath, P.R.; Venkatsubramanion, K. and Vieth, W.R.(1997)'Immobilized enzyme' in *'Annual reports on fermentation processes'*, **vol. 1**, edited by Perlman, D. and Tsao, G.T., p. 235-266, Academic Press, New York

Bernheim, F.; Bernheim, M.L.C. (1946) 'The hydrolysis of hydantoin by various tissues.' *J. Biol. Chem.* **163**, 683-685.

Bernheim, F. (1972) 'Effect of chloroform, phenol, alcohol and cyanogen iodide on swelling of *Pseudomonas aeruginosa* in various salts.' *Microbios,* **5 (18)**, 143. [CA 77: 83907q].

Bernheim, F. (1974) 'Differential effect of lipophilic and sulfhydryl agents on swelling and K-efflux of *Pseudomonas aeruginosa*.' *Cytobios,* **9 (33-34)**, 33 [CA 81: 115201f].

Bim, M.A. and Franco, T.T. (2000) 'Extraction of in aqueous two-phase systems of alkaline xylanase produced by *Bacillus pumilus* and its application in kraft pulp bleaching.' *Journal of chromatography B*. vol. **243 (1,2)**, 349-356.

Bio-Rad catalogue 1, 1983.

Biselli, Manfred Fridolin (I992) 'Enzyme-catalyzed racemate separation of amino acids with integrated reracemization using the conversion of D,L-alanine to L-alanine as an example.' *Ber. Forschungszent. Juelich, Juel* **2581**, 102 pp. (German) 1992 [C.A. 117:46617].

Blum, H.; Beier, H.; Gron, H.J. (1987) 'Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gel.' *Electrophoresis* **8**, 93-99.

Bommarius, Andreas; Schaefer, Matthias; Drauz, Karlheinz (1995) 'Production of cyclic N-carbamoyl-D-amino acids.' *Ger. Offen. DE* **4330678 A1** 16 Mar 1995, 4 pp. (Germany) [C.A.122: 237925].

Bommarius, Andreas; Drauz, Karlheinz; Kottenhahn, Matthias (1997) 'Production of (R)-tertiary leucine.' *Ger. Offen. DE* **19529211 A1** 13 Feb 1997, 3 pp. Germany) [C.A.126: 198637].

Bommarius, Andreas; Schwarm, Michael (2001) 'Preparation of D-(3-pyridyl)alanine.' *Eur. Pat. Appl. EP* **1083170 A1** 14 Mar 2001, 6 pp. [C.A. 134: 222631].

Brodelium, P. and Vandamme, E.J. (1987) 'Immobilized cell systems' in *'Biotechnology'*, **vol. 7a**, ed. by Rehm, H. J. and Reed, G.; p.405-464, VCH Germany

Brooks, Kathlean P.; Jones, Svan A.; KIm,Byung-DONg and Sander, Eugene G. (1983) 'Bovine liver dihydropyrimidine amidohydrolase :Purification, properties and characterization as a zinc metalloenzyme.' *Arch. Biochem. Biophys.* **226**,469-483.

Brown, M.R.W. and Richards, R.M.E. (1964) 'Effect of polysorbate (tween) 80 on the resistance of *Pseudomonas aeruginosa* to chemical inactivation.' *J. Pharm. Pharmacol. suppl.* **16**, 51-55 [C.A. 62: 5600h].

Brummer, W.; Gunzer, G. (1987) 'Laboratory techniques of enzyme recovery.' In '*Biotechnology'*. Rehm, H.J. and Reed, G.edited, **vol. 7a**, p.213-278, VCH Publishers, Germany

Buckland, B.C.; Lilly, M.D. and Dunnill, P. (1976) 'The kinetics of cholesterol oxidase synthesis by *Nocardia rhodocrous*.' *Biotechnol. Bioeng.* **18**, 601-621.

Builder, Stuart E. (1993) 'Hydrophobic Interaction Chromatography: Principle and Methods.' *Pharmacia catalogue*: 18-1020-90. ISSN 91- 970490-4-2.

Burton, S.G.; Dorington, R.A.;Hartley, C.;Kirchmann, S.; Matcher, G.and Phehane, V.(1998) 'Production of enantiometrically pure amino acids : characterization of South African hydantoinase and hydantoinase producing bacteria.' *J. Mol. Catal. B: Enzym.* **5(1-4),** 301-305 [CA 129: 259365].

Burtscher, Helmut; Lang, Gunter; Popp, Friedrich (1995) 'A thermostable D-hydantoinase from *Bacillus* for use in the manufacture of carbamoyl-a-amino acids and its manufacture by expression of the cloned gene.' *Ger. Offen. DE* **4328829 A1** 2 Mar 1995, 9 pp. (Germany). [C.A.122: 234314].

Caravaca, J. and Grisolia, S. (1958) 'Enzymatic decarbamylation of carbamyl beta-alanine and carbamyl beta-aminoisobutyric acid.' *J. Biol. Chem.* **231**, 357-365.

Carvalho, Walter; Silva, Silvio S.; Converti, Attilio; Vitolo, Michele; Felipe, Maria G. A.; Roberto, Ines C.; Silva, Messias B.; Mancilha, Ismael M. (2002).'Use of immobilized *Candida* yeast cells for xylitol production from sugarcane bagasse hydrolysate: Cell immobilization conditions.' *Applied Biochemistry and Biotechnology*, **98-100** (Biotechnology for Fuels and Chemicals), 489-496.

Cecere, F.; Galli, G.; Morisi, F. (1975) 'Substrate and steric specificity of hydropyrimidine hydrase.' *FEBS Lett.* **57**, 192-194.

Chao, Yun-Peng; Fu, Hongyong; Lo, Tsuey-Er; Chen, Po Ting; Wang, Jenn-Jye (1999) 'One-step production of D-p-hydroxyphenylglycine by recombinant *Escherichia coli* strains.' *Biotechnol. Prog.,* **15(6)**, 1039-1045.

Chen, Yi-Chuan; Yin, Bang-Ding; Lin, Sung-Chyr; Hsu, Wen-Hwei (1999) 'Production of N-carbamoyl-D-hydroxyphenyl-glycine by D-hydantoinase activity of a recombinant *Escherichia coli*.' *Process Biochem. (Oxford),* **35(3,4)**, 285-290.

Chevalier, Pierre; Roy, Denis; Morin, Andre (1989a) 'Hydantoinase activity of immobilized non-growing *Pseudomonas putida* cells.' *Appl. Microbiol. Biotechnol*., **30(5)**, 482-6.

Chevalier, P.; Roy, D.; Morin, A. (1989b) 'Study of a bacterial hydantoinase(dihydropyrimidinase) activity using alginate beads and a cell recycle system.' *Biotechnol. Tech*., **3(2)**, 135-8.

Chibata, Ichiro; Tosa, Tetsuya; Sato, Tadashi; Mori, Takao (1976) 'Production of L-amino acids by aminoacylase adsorbed on DEAE-Sephadex.' *Methods Enzymol*., **44**(Immobilized Enzymes), 746-59.[C.A.86:187636].

Chibata, I.; Tosa, T.; Kakimoto, T. (1986a) 'Alanine'. In: *Biotechnology of amino acid production, Progress in industrial microbiology,* vol.**24**, [Aida, K.; Chibata, I.; Nakayama,K.; Takinami, K.; Yamada, H. (Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p. 224-232.

Chibata, I.; Tosa, T.; Sato, T. (1986b) 'Aspartic acid', ibid. p. 144-151.

Chien, Hungchien Roger; Hsu, Wen-Hwei (1996) 'Rapid and sensitive detection of D-hydantoinase producing microorganisms by using microtiter plate assay.' *Biotechnol. Tech.,* **10(11),** 879-882

Choi, H.; Laleye, L.; Amantea, G.F.; Simard, R.E. (1997) 'Release of aminopeptidase from *Lactobacillus casei* sp. *casei* by cell disruption in a Microfluidizer.' *Biotechnol. Tech.* **11(7)**, 451-453.

Christidis, Yani; Schouteeten, Alain (1979) 'N-Acyl-a-aromaticglycine derivatives'. *Swiss CH* **614929** 28 Dec 1979, 4 pp. (French). (Switzerland) [C.A. 92:215778].

Coakley, W. T.; Bater, A. J.; Lloyd, D. (1977) 'Disruption of microorganisms.' *Adv. Microb*. *Physiol*., **16**, 279-341.

Cotoras, D. and Wagner, F. (1984) 'Stereospecific hydrolysis of 5-monosubstituted hydantoins', in *Third European Congress on Biotechnology,* **vol. 1,** Verlag Chemie, Weinheim. Pp. 351-356.

Cumming, R. H.; Tuffnell, J.; Street, G. (1985) 'The dependence of protein release from *Bacillus amyloliquefaciens* on the growth phase in batch culture.' *Biotechnol.Bioeng.* **27(6)**, 887-9.

Darbyshire, J. (1981) 'Large scale enzyme extraction and recovery'.' In *'Topics in enzyme fermentation biotechnology',* vol. **5,** 147-186, [Wiseman, A. Ed.], Ellis Horwood, Chichester, UK

Deulofen, V. (1936) *Ber Dtsch. Chem. Ges*. **69,** 2456.

Dinelli, D.; Marconi, W.; Cecere, F.; Galli, G.; Morisi, F. (1978) 'A new method for the production of optically active amino acids.' In *Enzyme Engineering,* **volume date 1975, 3**, [Pye E. K. and Weetall, H.H. Eds.], p. 477-481, Plenum, New York

Dinelli, Dino; Morisi, Franco; Zaccardelli, Delio (1980) 'Preparation of one only of the two stereoisomeric forms of a carbamyl amino acid.' *Swiss CH* **620943** 31 Dec 1980, 6 pp. (Italian) (Switzerland) [C.A. 94:172895].

Dotani, M.; Kondo, t.; Igarashi, H.; Uchiyama, T. (1985) 'Biochemical production of D-amino acids'. *Jpn. Kokai tokkyo Koho JP* **60184392 A2** 19 Sep 1985, Showa, 6 pp. (Japanese) (Japan) [C.A. 104: 166912].

Dotani, M.; Kondo, T.; uragami, S.; Koga, H. (1989) 'Manufacture of L-a-amino acids with *Mycoplana* or *Protaminobacter* species from racemic amino acid amides.' *Jpn. Kokai Tokkyo Koho JP* **01277499 A2** 7 Nov 1989 Heisei, 8pp. (Japan) [C.A. 112: 196691]

Downs, John David (1983) 'Cell disruption.' *Eur. Pat. Appl. EP* **78556 A1** 11 May 1983, 22 pp. [C.A. 99: 103746].

D'Souza, Stanislaus; Deshpande, Ashwini (2001)'Simultaneous purification and reversible immobilization of D-amino acid oxidase from *Trigonopsis variabilis* on a hydrophobic support.' *Applied Biochemistry and Biotechnology,* **95(2)**, 83-92

Dudley, K.H.; Bius, D.L.; Butler, T.C. (1970) 'Metabolic fates of 3-ethyl-5-phenylhydantoin (ethotoin, peganone), 3-methyl-5-phenylhydantoin and 5-phenylhydantoin.' *J. Pharmacol. Exp. Ther.* **175(1)**, 27-37 [C.A.73: 107818g].

Dudley, K.H.; Bius, D.L. (1973) 'Synthesis of optically active 5-phenylhydantoins.' *J. Heterocycl. chem.* **10**,173-80 [C.A.79: 17907e].

Dunnill, P. (1975) 'The state of enzyme isolation technology.' In: *'Enzyme Engineering',* **vol. 3**; p. 207-215, [Pye, E.K. and Weethall, H.H. Eds.], Plenum Press, New York and London

Durham, Don R.; Weber, Jennifer E. (1995) 'Properties of D-hydantoinase from *Agrobacterium tumefaciens* and its use for the preparation of N-carbamyl D-amino acids.' *Biochem. Biophys. Res. Commun.*, **216(3)**, 1095-1100.

Durwald, H.; Hoffman-Berling, H. (1971) 'DNAsynthesis in nucleotide-permeable *Escherichia coli* cells. II. Synthesis of replicate form DNA of phage Φ X174.' *J. Mol. Biol.* **58**, 755-773.

Eadie, G.S.; Bernheim, F.; Bernheim, M.L.C. (1949) 'The partial purification and propertiesof animal and plant hydantoinases.' *J. Biol. Chem*. **181**, 449- 458.

Elks, J.; Heme, B.A.; Ryman, B.E. (1948) 'The synthesis of some alpha-amino-acids.' *J. Chem. Soc*. **1386-1389.**

Enei, H.; Yamada, H. (1986) '3.4-dihydroxyphenylalanine'. In: *Biotechnology of amino acid production, Progress in industrial microbiology,* vol. **24**, [Aida, K.; Chibata, I.; Nakayama, K.; Takinami, K.; Yamada, H. (Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p.280-285.

Eto, Morifusa; Tawata, Shinkichi; Sakamoto, Koichi; Oshima, Kohei (1978) 'Novel insecticidal five-membered cyclic phosphoramidothionates derived from L-amino acids.' *Nippon Noyaku Gakkaishi,* **3(2),** 161-3 (English) [C.A.89: 174974].

Falconer, R.J.; O'Neill, B.K.; Middelberg, P.J. (1997) 'Chemical treatment of *E. coli* 1. Extraction of intracellular protein from uninduced cells.' *Biotechnol. Bioeng.* **53(1)**, 453-458.

Fan, C.-H.; Lee, C.-K. (2001 'Purification of hydantoinase from adzuki bean and its immobilization for N-carbamoylphenylglycine production.' *Biochemical Engineering Journal* **8(2)** 157-164.

Felix, H. (1982) 'Permeabilized cells.' *Anal. Biochem.* **120**, 211-234.

Fenton, D. M. (1982) 'Solvent treatment for beta-D-galactosidase release from yeast cells.' *Enzyme Microb. Technol.* **4,** 229-232.

Feung, Chao-Shieung; Hamilton, Robert H.; Mumma, Ralph O. (1977) 'Metabolism of 2,4-dichlorophenoxyacetic acid. 11. Herbicidal properties of amino acid conjugates.' *J. Agric. Food Chem.,* **25(4)**, 898-900 [C.A.87: 34163].

Fotheringham, I. G.; Kidman, G. E.; McArthur, B. S.; Robinson, L. E.; Scollar, M. P. (1991) 'Aminotransferase catalysed conversion of D-amino acids to L-amino acids'. *Biotechnol. Prog.* **7(4)**, 380-1.

Fruton, J.S.; Simmond, S. [Eds.] (1961) 'Amino acid as structural units of proteins'. In: *General biochemistry,* Asia Publishing House, New York , p.45-84.

Gaebler, O.H.; Keltch, A.K. (1926) 'On the metabolism of hydantoins and hydantoic acid.' *J. Biol. Chem.* **70**, 763-777.

Garcia, Maria J.; Azerad, Robert (1997) 'Production of ring-substituted D-phenylglycines by microbial or enzymic hydrolysis/deracemization of the corresponding D,L-hydantoins.' *Tetrahedron: Asymmetry,* **8(1)**, 85-92.

Gavristov, A. V.; Karpova, M. A.; Mosichev, M. S.; Kuznetsov, V. D. (1981) 'Comparative study of procedures for isolation of glucose isomerase from *Streptomyces albogriseolus* mycelium. *Mikrobiologiya* **50(4)**, 626-30 (Russian) [C.A. 95:182879].

Gillonier, C.; Guivarch, M. (1980) 'D-a-amino acids'. *Ger. Offen. DE* **3018584,** 27 Nov 1980, 20 pp. (German) (Germany) [C.A. 94: 207143].

Godtfredsen, Sven Erik; Clausen, Kim; Ingvorsen, Kjeld; Hermes, Hubertus Fransiscus Maria; Van Balken, Johannes Arnoldus Maria; Meijer, Emmo Marinus (1989) 'Manufacture of optically active amino acids from amino acid amides with bacteria producing an amidase.' *PCT Int. Appl. WO* **8901525 A1** 23 Feb 1989, 25 pp. [C.A.112: 6077].

Gokhale, D.V.; Bastawde, K.B.; Patil, S.G.; Kalkote, U. R.; Joshi, R.R.; Joshi, R. A.; Ravindranathan, T.; Gaikwad, B. G.; Jogdand, V. V.; Nene, S. (1996) 'Chemoenzymic synthesis of D(-)phenylglycine using hydantoinase of *Pseudomonas desmolyticum* resting cells.' *Enzyme Microb. Technol.,* **18(5)**, 353-7.

Gokhale, D.V.; Bastawde, K.B.; Patil, S.G.; Kalkote, U. R.; Joshi, R.R.; Joshi, R. A.; Ravindranathan, T.; Gaikwad, B. G.; Jogdand, V. V.; Nene, S. (2000) 'Manuf. of D(-)-N-carbamoylphenylglycine from D,L-5-phenylhydantoin by fermentation with *Pseudomonas* NCIM 5070 ATCC 55940.*U.S. US* **6087136 A** 11 Jul 2000, 4 pp.

Gonzalez-Saiz, J. M.; Pizarro, C. (2001) 'Polyacrylamide gels as support for enzyme immobilization by entrapment. Effect of polyelectrolyte carrier, pH and temperature on enzyme action and kinetics parameters.' *Eur. Polym. J.,* **37(3),** 435-444.

Gornall, A.G.; Bardawill, C.S. and David, M.M (1949) 'Determination of serum protein by means of the biuret reaction.' *J. Biol. Chem.* **177**, 751-766.

Gray, G.W. and Wilkinson, S.G. (1965a)'The action of Ethylenediaminetetraacetic acid on *Pseudomonas aeruginosa*.' *J. Appl. Bacteriol.* **28(1)**, 153-164.

Gray, G.W.; Wilkinson, S.G. (1965b) 'The effect of ethylenediaminetetraacetic acid on cell wall of some gram negative bacteria.' *J. Gen. Microbiol.* **39(3**), 385-399.

Grifantini, Renata; Frascotti, Gianni; Galli, Giuliano; Grandi, Guido (1995) 'Production of D-a-amino acids from 5'-substituted hydrantoins using fermentation by microorganism transformed with plasmid encoding D-hydantoinase and D-N-carbamylase.' *Eur. Pat. Appl. EP* **677585 A1** 18 Oct 1995, 44 pp. [C.A. 124: 7179].

Grifantini, Renata;Carpani, Giovanna; Galli, Giuliano; Grandi,Guido (1997) 'Thermostable mutants of D-N-a-carbamoylase with normal catalytic activity for manufacture of D-amino acids from N-carbamoylamino acids.' *Eur. Pat. Appl. EP* **780473 A2** 25 Jun 1997,19 pp. [C.A.127: 92191].

Grifantini, Renata; Galli, Giuliano; Carpani, Giovanna; Pratesi, Claudio;Frascotti, Gianni; Grandi, Guido (1998) 'Efficient conversion of 5-substituted hydantoins to D-a-amino acids using recombinant *Escherichia coli* strains.' *Microbiology (Reading, U. K.),* **144(4)**, 947-954.

Groeger, U.; Sahm, H.; Leuchtenberger, W. (1986) 'Production of branched aliphatic or aromatic amino acids from alpha-ketocarboxylic acids by fermentation'. *Eur. Pat. Appl. EP* **166903 A2** 8 Jan 1986, 11 pp. [C.A. 104:147148].

Gross, C.; Syldatk, C.; Wagner, F.(1987) 'Screening method for microorganisms producing L-amino acids from D,L-5-monosubstituted hydantoins.' *Biotechnol. Tech.*, **1(2)**, 85-90.

Gueguen, Y.; Chemardin, P.; Janbon, G.; Arnaud,A.; Galzy, P. (1999) 'Use of b-glucosidase in the development of flavour in wines and fruit juices' *Methods Biotechnol.*, **10**(Carbohydrate biotechnology protocol), 323-331.

Guivarch, Marcel; Gillonnier, Claude; Brunie, Jean Claude (1980) 'Preparation of optically active amino acids with hydantoinases.' *Bull. Soc. Chim. Fr.,* **(1-2, Pt. 2),** 91-5 (French) [C.A.92: 176474]

Gutcho, S.Y. [Ed.] (1973) '*Chemicals by fermentation*'. Noyes Data Corporation, London, England.

Harada, Kaoru; Tamura, Minoru (1979) 'Sterically controlled synthesis of optically active organic compounds. XXVIII. Asymmetric synthesis of alanine by hydrogenolytic asymmetric transamination between (R)-2-amino-2-phenylethanol and ethyl pyruvate.' *Bull. Chem. Soc. Jpn.,* **52(4),** 1227-8 [C.A.91: 57458].

Harrison, Susan T. L.; Dennis, John S.; Chase, Howard A. (1991) 'Combined chemical and mechanical processes for the disruption of bacteria.' *Bioseparation* **2(2)**, 95-105.

Hartley, Carol J.; Kirchmann, Shaun; Burton, Stephanie G.; Dorrington, Rosemary A. (1998) 'Production of D-amino acids from D,L-5-substituted hydantoins by an *Agrobacterium tumefaciens* strain and isolation of a mutant with inducer-independent expression of hydantoin-hydrolyzing activity.' *Biotechnol. Lett*., **20(7)**,707-711.

Hartley, C.J.; Manford, F.; Burton, S.G.; Dorrington, R.A. (2001) 'Overproduction of hydantoinase and N-carbamoylamino acid amidohydrolase enzyme by regulatory mutants of *Agrobacterium tumefaciens*.' *Appl. Microbiol. Biotechnol.* **57**, 43-9.

Hashiyama, Tomiki; Harada, Naoyuki; Arakawa, Hiroaki; Kusama, Mari;Ozaki,Yasuhiko; Kuroda,Tooru; Seki, Masahiko (1998) 'Process for preparing optically active phenyloxirane compounds by epoxidation of styrene derivatives using chiral ketones or dioxiranes.' *PCT Int. Appl. WO* **9856762 A2** 17 Dec 1998, 178 pp. [C.A.130: 66383].

Hayashi, Katsuo; Kidouchi, Kiyoshi; Sumi, Satoshi; Mizokami, Masashi;Orito,Etsuro; Kumada,Kazunori; Ueda, Ryuzo; Wada, Yoshiro (1996). 'Possible prediction of adverse reactions to pyrimidine chemotherapy from urinary pyrimidine levels and a case of asymptomatic adult dihydropyrimidinuria.' *Clin. Cancer Res.*, **2(12)**, 1937-1941 [C.A.126: 84193].

Hearn, M.T.W. and Anspach, B. (1990) 'Chemical, physical and biochemical concept in isolation and purification of protein,' in *Handbook of enzyme biotechnology*, 2nd edition, Wiseman, A. ed., Ellis Horwood Limited, England, pp. 17-64.

Henrick, Clive A. (1980) 'Pyridyl esters of a-substituted amino acids' *U.S. US* **4226872** 7 Oct 1980, 8 pp. Cont.-in-part of U.S. Ser. No. 16, 775, abandoned.[C.A. 94:121339].

Hermes, Hubertus Franciscus Maria; Peeters, Wijnand Peter Helena; Peters, Peter Josephus Hubertus (1990) 'Amino acid amide racemase for preparation of optically active amino acids.' *Eur. Pat. Appl. EP* **383403 A1** 22 Aug 1990, 12 pp. [C.A.114: 99986].

Hoppe, B.; Martens, J. (1984) 'Amino acids - production and recovery.' *Chem Uunserer Zeit* **18,** 73- 86 [C.A. 101: 131045].

Hu, Yonghong; Yang, Yongge; Chen, Wuling; Cai, Li; Oyang, Pingkai (1998) 'Detn. of phenylglycine in dihydropyrimidinase conversion.' *Yaowu Fenxi Zazhi,* **18(4)**, 238-241 (Chinese) [C.A.130: 3106].

Huang, Hong-hui; Liu, Meng-liu; Hu, Zhuo-yi (2000) 'Isolation, purification and properties of dihydropyrimidinase from *Pseudomonas putida* 9801.' *Zhongguo Yaoke Daxue Xuebao,* **31(5),** 389-392 (Chinese) [C.A.134: 97052].

Hudson, Harry R. (2000) 'Aminophosphonic and aminophosphinic acids and their derivatives as agrochemicals.' *Aminophosphonic Aminophosphinic Acids,* 443-482, [Edited by:Kukhar, Valery Pavlovich; Hudson, Harry R.], John Wiley & Sons Ltd.:Chichester, UK. [C.A.133: 218740].

Ichikawa, Shigeaki; Shibukawa, Mitsuru (1986) 'b-Lactam antibiotics-biosynthesis of amino acid precursors and ring systems.' *Prog. Ind. Microbiol*., **24(Biotechnol. Amino Acid Prod.)**, 309-28 [C.A.105: 224465].

Imaki, Sunao;Takuma, Isatatsu (1986a) '5-Arylidenehydantoins.' *Jpn.KokaiTokkyo Koho JP* **61037774 A2** 22 Feb 1986 Showa, 3 pp. (Japanese) (Japan) [C.A.105: 42807].

Imaki, Sunao; Takuma, Isatatsu (1986b) '5-Arylidenehydantions.' *Jpn. Kokai Tokkyo Koho JP* **61037775 A2** 22 Feb 1986 Showa, 3 pp. (Japanese) (Japan) [C.A.105: 42806]

Ingham, K.C. (1984) 'Protein precipitation with polyethyleneglycol,' in *Methods in Enzymology*, **vol. 104,** Jakoby, W.B.ed., Academic Press Inc, Orsando, Florida, pp.351-356.

Innocente, N. Palla, G. (1999) 'Occurance of D-amino acids in typical semi-hard cheese'. *J. Dairy Res.* **66(4),** 633-637.

.

Inoue, Masayasu; Morino, Yoshimasa; Kagamiyama, Hiroyuki (1976) 'Procedures for enzyme purification.' *Taisha,* **13(9),** 1521-34 (Japanese) [C.A. 86: 102297].

Ishii, K.; Takinami, K. (1978) 'L-Amino acids'. *Jpn. Kokai Tokkyo Koho JP* **53086093**, 29 Jul 1978, Showa, 6 pp. (Japanese) (Japan) [Chem. Abstr. 90: 4432].

Ishii, K.; Yokozeki, K.; Iwogami, S.; Takinami, K.(1978) 'L-Amino acids'. *Japan Kokai JP* **53034990**, 31 Mar 1978, Showa, 6 pp. (Japanese) (Japan) [C.A. 89: 105882].

Ishikawa, T.; Kumara, H. (1988) 'Manufacture of L-amino acid by *Pseudomonas* NS303'. *Jpn. Kokai Tokkyo Koho JP* **63063377 A2** 19 Mar 1988, Showa, 4 pp. [C.A. 109: 53244].

Ishikawa, Takahiro; Watabe, Ken; Mukohara, Yukuo; Nakamura, Hiroaki (1997) 'Mechanism of stereospecific conversion of D,L-5-substituted hydantoins to the corresponding L-amino acids by *Pseudomonas sp*.strain NS671.' *Biosci., Biotechnol., Biochem.*, **61(1)**,185-187.

Isobu, K.; Hirose, M. (1999) 'Enzymic manufacture of D-amino acids'. *Jpn. Kokai Tokkyo Koho JP* **11103887 A2** 20Apr 1999, Heisei, 9 pp. (Japanese) (Japan) [C.A. 130: 280931].

Issleib, Kurt; Moegelin, Werner; Balszuweit, Arno (1985) 'N-(Carboxyphosphinomethyl)-amino acids as herbicides and plant growth regulators.' *Ger.(East) DD* **228263 A1** 9 Oct 1985, 9 pp. [C.A.107: 78245]

Jiang, Ning; Ren, Yonge; Qiang, Yajing; Lu, Dajun; Sun, Wanru (1995) 'Breeding of hydantoinase-overproducing strains by resistance of substrate analog.' *Weishengwu Xuebao* **35(5)**, 342-5 (Chinese) [C.A.124: 140709]

Jung, Ju-Young; Yun, Hyun Shik; Kim, Eun-Ki (1997) 'Hydrolysis of olive oil by lipase, immobilized on hydrophobic support.' *J. Microbiol. Biotechnol*., **7(2),** 151-156.

Johansson, G. (1984) 'Affinity partitioning,' in *Methods in Enzymology*, **vol.104**, Jakoby, W.B. ed., Academic Press Inc, Orsando, Florida,, pp.356-364.

Kamphius, J.; Boesten, W.H.J.; Broxterman, Q.B.; Hermes, H.F.M.; van Balhen, J.A.M.; Meijer, E.M.; Schoemaker, H.E. (1990) 'New developments in the Chemo-enzymatic production of amino acids'. In*: Advances in biochemical engineeering/biotechnology,* vol. **42**, [Fitcher, A. (eds.)], Springer-Verlag Berlin Heidelberg, p.133-186.

Kanegfuchi Chem. Ind. Co. Ltd. Japan (1980) 'D-a-amino acids'. *Jpn. Kokai Tokkyo Koho JP* **55104890**, 11 Aug 1980, Showa, 17 pp. (Japanese) (Japan) [C.A. 93: 236942].

Karossi, A.T.; Pudjiraharti, S. and Budiwoti, T.A. (1998) 'Isolation of peroxidase from horseradish cell culture by precipitation with ammonium sulphate.' *Biotechnol. sustainable Util. Biol. Resour. Trop.* **12**, 211-214.

Kato, Masaaki; Morikawa, Tadashi; Miyoshi, Teruzo (1989) 'Manufacture of D-b-hydroxyamino acids with D-threonine aldolase.' *Jpn. Kokai Tokkyo Koho JP* **01317391 A2** 22 Dec 1989 Heisei, 8 pp. (Japan) [C.A.112: 234007].

Kawabe, Seiichiro; Kawabe, Yotaro (1981) 'Immobilization of papain by the use of polyacrylamide.' *Okayama Rika Daigaku Kiyo, A,* **17**, 45-56 (Japanese) [C.A. 97:68535]

Kawada, Mitsuru; Sugihara, Yoshihiro; Matsumura, Koichi; Kitamura, Teruo; Kondo, Katsumi; Kobi, Akio (1991) 'Preparation of chiral compounds as liquid crystal compositions.' *Jpn. Kokai Tokkyo Koho JP* **03109352 A2** 9 May 1991 Heisei, 29 pp. (Japan) [C.A.115: 195001].

Keil, Oliver; Schneider, Manfred P.; Rasor, J. Peter (1995) 'New hydantoinases from thermophilic microorganisms. Synthesis of enantiomerically pure D-amino acids.' *Tetrahedron: Asymmetry,* **6(6)**, 1257-60.

Kenney, Donald S.; Stein, Robert G. (1980) 'Herbicides derived from dihaloisonicotinoyl derivatives of amino acids.' *U.S.US* **4195984** 1 Apr 1980, 5 pp. [C.A. 93: 72294]

Kennedy, J.F. ; Cabral, J.M.S (1987) 'Enzyme immobilization' in '*Biotechnology',* vol. **7a**, ed. by Rehm, H.J. and Reed, G.; p.347-404, VCH, Germany

Key, B.A.; Gray, G.W. and Wilkinson, S.G. (1970) 'The effect of ethylenediaminetetraacetic acid on *Pseudomonas alcaligenes* and the composition of the bacterial cell wall.' *Biochem. J.* **117**, 721-732.

Kheirolomoom, A.; Ardjmand, M.; Fazelinia, H.; Zakeri, A. (2001) 'Isolation of penicillin G acylase from *Escherichia coli* ATCC 11105 by physical and chemical treatments.' *Biochemical Engineering Journal,* **8(3)**, 223-227

Kikuchi, M. and Nakao, Y.(1986) "Glutamic acid'. In: *Biotechnology of amino acid production, Progress in industrial microbiology,* vol. **24**, [Aida, K.; Chibata, I.; Nakayama, K.; Takinami, K.; Yamada, H. (Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p. 101-113.

Kikugawa, Mariko; Kaneko, Masae; Fujimoto-Sakata, Shigeko; Maeda, Mitsuko; Kawasaki, Koichi; Takagi, Tatsuya; Tamaki, Nanaya (1994) 'Purification, characterization and inhibition of dihydropyrimidinase from rat liver.' *Eur. J.Biochem.,* **219(1-2)**, 393-9.

Kim, D.M.; Kim, H.S. (1993) 'Enzymatic synthesis of D-p-hydroxyphenylglycine from D,L-p-hydroxyphenylhydantoin in the presence of organic cosolvent.' *Enzyme Microb. Technol.* **15**, 530-4

Kim, Geun-Joong; Kim, Hak-Sung (1995a) 'Optimal reaction conditions for the enzymic synthesis of optically active D-p-hydroxyphenylglycine from 5-substituted hydantoin using D-hydantoinase and N-carbamoylase.' *Ann. N. Y. Acad. Sci.,* **750(Enzyme Engineering XII)**, 185-9 [C.A. 124:56620]

Kim, G.-J.; Kim, H.-S. (1995b) 'Optimization of enzymic synthesis of D-p-hydroxyphenylglycine from D,L-5-substituted hydantoin using D-hydantoinase and N-carbamoylase.' *Enzyme Microb. Technol.* **17(1)**, 63-7.

Kim, Hak-Sung; Kim, Dong-Man (1997) 'Preparation of N-carbamoyl-D-amino acids with immobilized hydantoinase.' *Repub. Korea KR* **9702668 B1** 7Mar 1997, No pp. given (Korean)[C.A.133: 74328]

Kim, Dong-Man; Kim, Geun-Joong; Kim, Hak-Sung (1994) 'Enhancement of operational stability of immobilized whole cell D-hydantoinase.' *Biotechnol. Lett*., **16(1)**, 11-16.

Kim, Hak-sung; Lee, Seung-koo; Lee, Dong-chol (1997a) 'Process for the preparation of N-carbamoyl-D-amino acid using novel thermophilic microorganism having heat-resistant d-hydantoinase activity.' *Repub. Korea KR* **9705487 B1** 16 Apr 1997, No pp. given (Korean) [C.A. 133: 176266].

Kim, Hyung-soon; Hong, Sung-joo; Noh, Bum-sub; Kim, Mook; Choe, Hong-kyu; Park, Young-chol; Cho, Yong-joo (1997b) 'Process for preparing D-M-amino acids'.

Repub. Korea KR **9710133 B1** 21 Jun 1997, No pp. given (Korean) [C.A.133: 176261].

Kim, Hak-sung; Lee, Seung-koo; Lee, Dong-chol (1997c) 'Novel microorganism having heat-resistant d-hydantoinase activity.' *Repub. Korea KR* **122284 B1** 24 Nov 1997, No pp. given (Korean) [C.A.133: 192132].

Kim, Pan-Soo; Shin, Hyun-Dong; Park, Joong-Kon; Lee, Yong-Hyun (2000) 'Immobilization of cyclodextrin glucanotransferase on amberlite IRA900 for biosynthesis of transglycosylated xylitol.' *Biotechnol. Bioprocess Eng*. **5(3),** 174-180.

Kitagawa, Hiroyuki; Miyoshi, Teruzo; Kato, Masaaki; Ikemi, Masahisa; Omine, Hiroshi; Chiba, Susumu (1985) 'Novel hydantionase.' *Jpn. Kokai Tokkyo Koho JP* **60241888 A2** 30 Nov 1985 Showa, 8 pp. (Japanese) (Japan) [C.A.104: 205546].

Klages, Uwe; Weber, Alfred (1989) 'Microbial manufacture of L-amino acids and amino acid amides.' *PCT Int. Appl. WO* **8910969 A1** 16 Nov 1989, 18 pp. [C.A.113: 57454].

Komatsubara, S.; Kisumi, M. (1986) 'Isoleucine, valine and leucine'. In: *Biotechnology of amino acid production, Progress in industrial microbiology,* vol. **24**, [Aida, K.; Chibata, I.; Nakayama,K.; Takinami, K.; Yamada, H. (Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p. 233-246.

Kraatz, Udo; Andersch, Wolfram; Turberg, Andreas; Mencke, Norbert (1997) 'Preparation of amino acid fluorobutenyl esters as pesticides.' *Ger. Offen. DE* **19544674 A1** 5 Jun 1997, 17 pp. (Germany) [C.A.127: 81783].

Kroner, K.H.; Hustedt, H.; Granda, S.and Kula, M.R.((1978) 'Technicalaspect of separation using aqueous two-phase systems in enzyme isolation processes'. *Biotechnol. Bioeng.* **20,** 1967-1988.

Kroner, K.H.; Schutte, H.; Stach, W. and Kula, M.R. (1982) 'Scale-up of formate dehydrogenase by partition'. *J. Chem. Technol. Biotechnol*. **32**, 130-137.

Kula, M.R.; Kroner, K.H.; Hustedt, H. and Schutte, H. (1981) 'Technical aspect of extractive enzyme purification'. *Ann. N.Y. Acad. Sci*. **369,** 341-354.

Kula, M.R.; Kroner, K.H. and Hustedt, H. (1982) 'Purification of enzyme by liquid-liquid extraction,' in *Advances in Biochemical Engineering /Biotechnology*, **vol. 24,** Fiechter, A. ed., Springer-Verlag Berlin Heidelberg New York, pp.73-118.

Kumagai, H. (1986) 'Glutathione and gamma-glutamylpeptide'. In: *Biotechnology of amino acid production, Progress in industrial microbiology,*vol.**24**, [Aida, K.; Chibata, I.; Nakayama,K.; Takinami, K.; Yamada, H.

(Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p. 259-268.

Kung, Wei Jen Hu; Cesa, Mark Clark; Burrington, James David (1986) 'Entioselective hydrolysis of N-acylamino acid esters using a combined enzyme system. *Eur. Pat. Appl. EP* **182517 A2** 28 May 1986, 12 pp. [C.A.106: 17022].

Kutsuki, Hidetoshi; Mori, Natsuki; Hasegawa, Junzo; Watanabe, Kyoshi (1988) 'Optically active sulfonyloxy compounds, their preparation, and manufacture of optically active 1-methyl-3-phenylpropylamine using them.' *Jpn. Kokai Tokkyo Koho JP* **63190868 A2** 8 Aug 1988 Showa, 8 pp. (Japan) [C.A.112: 178064].

Kuwahara, Masao; Tagawa, Michito; Furusato, Takashi; Narushima, Hiroyuki; Shinke, Shuzo (1990) 'Process for producing optically active compounds.' *Eur.Pat. Appl. EP* **358428 A2** 14 Mar 1990, 17 pp. [C.A. 113: 76613].

Kyowa Hakko Kogyo Co., Ltd., Japan (1982) 'Benzothiazolyl amino acid derivatives.' *Jpn. Kokai Tokkyo Koho JP* **57149280 A2** 14 Sep 1982 Showa, 21 pp. (Japanese) (Japan) [C.A. 98: 72084].

Laemmli, U.K. (1970)'Cleavage of structural protein during the assembly of the head of bacteriophase T4.' *Nature* **227,** 680-685.

Lautenschlaeger, Hans Heiner; Dereu, Norbert; Niemann, Reinhard; Hameister, Walter (1986) 'N-(2-Hydroxyalkyl) amino acids, their derivatives and their pharmaceutical use.' *Ger. Offen. DE* **3544045 A1** 26 Jun 1986, 48 pp. (German) (Germany) [C.A.105:171847].

Lazarus, N.R., Ramel, A.H., Rustum, Y.M. and Barnard, E.A.(1966) 'Yeast hexokinase. I. Preparation of the pure enzyme.'. *Biochemistry* **5,** 4003-4016.

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

Lee, S.-G.; Lee, D.-C.; Hong, S.-P.; Sung, M.-H.; Kim, H.-S. (1995) 'Thermostable D-hydantoinase from thermophilic *Bacillus stearothermophilus* SD-1: characteristics of purified enzyme.' *Appl. Microbiol. Biotechnol.* **43(2),** 270-6.

Lee, Dong-Cheol; Lee, Seung-Goo; Kim, Hak-Sung (1996a) 'Production of D-p-hydroxyphenylglycine from D,L-5-(4-hydroxyphenyl)hydantoin using immobilized thermostable D-hydantoinase from *Bacillus stearothermophilus* SD-1.' *Enzyme Microb. Technol*., **18(1)**, 35-40.

Lee, Dong-Cheol; Lee, Seung-Goo; Hong, Seung-Pyo; Sung, Moon-Hee; Kim, Hak-Sung(1996b) 'Cloning and overexpression of thermostable D-hydantoinase from

thermophile in *E. coli* and its application to the synthesis of optically active D-amino acids'. *Ann. N. Y. Acad. Sci.*, **799(Enzyme Engineering XIII),** 401-405.

Lee, Cheng-Kang; Lin, Kung-Chern (1996) 'N-Carbamoyl-D-p-hydroxyphenyglycine production using immobilized D-hydantoinase from recombinant *E. coli*.' *Enzyme Microb. Technol*., **19(8)**, 623-627.

Lee, Dong-Cheol; Kim, Geun-Joong; Cha, Yoo-Kyong; Lee, Cheo-Young; Kim, Hak-Sung (1997a) 'Mass production of thermostable D-hydantoinase by batch culture of recombinant *Escherichia coli* with a constitutive expression system.' *Biotechnol. Bioeng.,* **56(4)**, 449-455.

Lee, Seung-Goo; Lee, Dong-Cheol; Kim, Hak-Sung (1997b) 'Purification and characterization of thermostable D-hydantoinase from thermophilic *Bacillus stearothermophilus* SD-1.' *Appl. Biochem. Biotechnol*., **62(2/3),** 251-266

Lee, Dong-Cheol; Kim, Hak-Sung (1998) 'Optimization of a heterogeneous reaction system for the production of optically active D-amino acids using thermostable D-hydantoinase.' *Biotechnol. Bioeng.*, **60(6)**, 729-738.

Lee, Dong-Cheol; Kim, Geun-Joong; Cha, Yoo-Kyong; Lee, Cheo-Young; Kim, Hak-Sung (1998) 'A cultivation strategy of recombinant *Escherichia coli* for mass production of thermostable D-hydantoinase.' *Ann. N. Y. Acad. Sci.*, **864(Enzyme Engineering XIV)**, 371-374.

Lee, C.-K.; Fan, C.-H. (1999) 'Pressure swing reactor for converting suspension of solid substrate p-hydroxy-phenylhydantoin by immobilized D-hydantoinase.' *Bioprocess Eng.,* **21(4)**, 341-347.

Lee, Sang Yup; Lee, Young; Wang, Fulai (1999) 'Chiral compounds from bacterial polyesters: sugars to plastics to fine chemicals.' *Biotechnol. Bioeng.,* **65(3),** 363-368 [C.A.132: 63185].

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

Lee, C.-K.; Fan, C.-H. and Yang, P.-F. (2001b) 'Modelling and simulation of a pressure-swing reactor for the conversion of poorly soluble substrate by immobilized enzyme: a case of hydantoinase reaction.' *Biochemical Engineering Journal* **7(3)**, 233-239.

Leive, L. (1968) 'Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate.' *J. Biol. Chem.* **243(9**), 2373-2380.

Lewis, David John; Farrand, Stephen Giles (1985) 'Amino acids via bioconversion.' *Eur. Pat. Appl. EP* **152275 A2** 21 Aug 1985, 26 pp. [C.A.103: 176978].

Li, Zhichang (1985) 'Recent advances in amino acid agrochemicals.' *Xinan Shifan Daxue Xuebao, Ziran Kexueban,* **(4)**, 77-80 (Chinese) [C.A.104: 124828]

Li, Jun; Hu, Zhuoyi (1999) 'The effect of inducer on enzyme activity of dihydropyrimidinase.' *Zhongguo Yaoke Daxue Xuebao* **30(3)**, 220-223 (Chinese) 1999 [C.A.132: 20388].

Li, Jun; Hu, Zhuoyi; Cai, Ping (1999) 'Screening of strains producing dihydropyrimidinase and fermentation conditions.' *Yaowu Shengwu Jishu* **6(2)**, 90-94 (Chinese) [C.A.132: 221391].

Linz, R. (1948) 'Lysis by chloroform and somatic antigen.' *Ann. Inst. Pasteur.,* **75**, 544 [C.A. 45: 2056c].

Liu, Yang; Kong, Degang; Gao, Peiji (1996) 'Regulation of nitrogen sources on *Corynebacterium crenatum* glutamine synthetase and effect of lysozyme treatment on cell disruption.' *Weishengwuxue Tongbao*, **23(3)**, 150-154 (Chinese) [C.A. 126: 115518].

Liu, Chuanbin; Bai, Fengwu; Feng, Pusun; Miao, Weirong; Su, Zhiguo (2000) 'Trehalose extraction from *Saccharomyces cerevisiae* after microwave treatment'. *Huagong Xuebao* (Chin. Ed.), **51(6),** 810-813 (Chinese) [C.A. 134: 236303].

Louwrier, Ariel; Knowles, Christopher J. (1997) 'The aim of industrial enzymic amoxycillin production: characterization of a novel carbamoylase enzyme in the form of a crude, cell-free extract.' *Biotechnol. Appl. Biochem*., **25(2)**, 143-149.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J. (1951) 'Protein measurement with folin phenol reagent.' *J. Biol. Chem.* **193**, 265-275.

Lunkenheimer, Winfried; Scheinpflug, Hans; Brandes, Wilhelm (1986) 'Preparation of (E)-isomers of N-a-[2-cyano-2-(alkoxyimino)acetyl]amino acid derivatives as pesticides and fungicides.' *Eur. Pat. Appl. EP* **206004 A1** 30 Dec 1986, 41 pp. [C.A.107: 218071].

Luo, Chengxiang; Chen, Tietao (1999) 'Extraction of permeable lactase from *Kluyveromyces lactis*.' *Faming Zhuanli Shenqing Gongkai Shuomingshu CN* **1225389 A** 11 Aug 1999, 7 pp. (Chinese) [C.A. 133:86102].

Luksa, V.; Starkuviene, V.; Starkuviene, B.; Dagys, R. (1997) 'Purification and characterization of the D-hydantoinase from *Bacillus circulans.* '*Appl. Biochem. Biotechnol.*, **62(2/3),** 219-231.

Maguire, James H.; Dudley, Kenneth H. (1978b) 'Partial purification and characterization of dihydropyrimidinases from calf and rat liver.' *Drug Metab. Dispos*., **6(5)**, 601-5 [C.A. 90:18150].

Makryleas, K. and Drauz, K. (1990) 'Manufacture of D-amino acids with *Agrobacterium*'. *Ger. DE* **3918057C1** 3May 1990, 4 pp.(Germany). [C.A. 114: 4882].

Manjon, A.; Iborra, J.L. and Arocas, A.(1991) 'Shortchain flavour ester synthesis by immobilized lipase in organic media' *Biotechnol. Lett.* **13(5)**, 339-344.

Mappes, C.J.; Pommer, E.H.; Rentzea, C.; Zeeh, B. (1980) '1,3 Bis-(trihalomethylsulfenyl)-imidazoline-2,4-diones.' *U.S. Pat.* **4198423** 15 Apr 1980, 6 pp. [C.A. 93: 71784]

Martin, A. B.; Picciolato, M.; Heras, A. (1998) 'Coimmobilization of enzymes and cells on chitosan and derivatives.' *Prog. Biotechnol.*, **15**(Stability and Stabilization of Biocatalysis), 679-684

Matos, Jose Ricardo (1988) (Texas A and M Univ., College Station, TX, USA) 'Chemical and enzymic synthesis of amino acids and penicillins.' 241 pp. Avail. *Univ. Microfilms Int.*, Order No. **DA8822205.** From:Diss. Abstr. Int. B 1989, 49(8), 3198 (English) [C.A. 112:77894].

May, O.; Siemann, M.; Syldatk, C.; Niefind, K.; Schomburg, D. (1996) 'Crystallization and preliminary X-ray analysis of a hydantoinase from *Arthrobacter aurescens* DSM 3745.' *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, **D52(6)**, 1209-1210

May, Oliver; Siemann, Martin; Pietzsch, Markus; Kiess, Michael; Mttes, Ralf; Syldatk, Christoph (1998) 'Substrate-dependent enantioselectivity of a novel hydantoinase from *Arthrobacter aurescens* DSM 3745: Purification and characterization as new member of cyclic amidases.' *J. Biotechnol.*, **61(1)**, 1-13

McCabe, B.K.; Kuek, C.; Gordon, G.L.R.; Phillips, M.W. (2001) 'Immobilization of monocentric and polycentric types of anaerobic chytrid fungi in Ca-alginate.' *Enzyme Microb. Technol.* **29(2-3**), 144-149

Mcguire, Sean M.; Olsen, Gilbert G.,II.; Wohnlich, Lisa; Wilson, Jenifer (1996) 'Preliminary purification of thermostable urease 408.' *Proc. ERDEC Sci. Conf. Chem. Biol. Def. Res*. Meeting Date **1995**, 827-833 [C.A. 127: 94132].

McLean, E. A.; Schaschke, C. J. (1996) 'Disruption of yeast cells using high-pressure carbon dioxide.' *IChemE Res. Event, Eur. Conf. Young Res. Chem. Eng*., 2nd, Volume **1,** 85-87.

Mehta, N.B.; Diuguid, C.A.R.; Soroko, F.F. (1981) 'Potential anticonvulsants. 1. Benzylhydantoins.' *J. Med. Chem.* **24,** 465-8.

Meiller, Francois; Mirabel, Bernard (1979) 'Support-aminoacylase complexes.' *U.S. US* **4132596** 2 Jan 1979, 4 pp. (English). (United States of America) [C.A. 90:99265]

Melling, J. and Scott, G.K. (1972) 'Preparation of gram quantities of purified R-factor-mediated penicillinase from *Escherichia coli* strain W 3310'. *Biochem. J.* **130**, 55-62.

Meyer, Patrick; Runser, Serge(1993).'Efficient production of the industrial biocatalysts hydantoinase and N-carbamyl amino acid aminodhydrolase: novel nonmetabolizable inducers.' *FEMS Microbiol. Lett.* **109(1),** 67-73

Mikhaleva, N. I. (1983) 'Comparative study of the effect of different cell disruption methods on the membrane apparatus of E. coli.' *Nek. Aspekty Fiziol. Mikroorg.*, 41-50. Edited by: Gaziev, A. I. Akad.Nauk SSSR, Nauchn. Tsentr Biol. Issled.: Pushchino, USSR. (Russian) [C.A.100: 3129].

Mirelman, D.; Nuchamowitz, Y. (1979) 'Biosynthesis of peptidoglycan in *Psedomonas aeruginosa*. 1.The incorporation of peptidoglycan into the cell wall.' *Eur. J. Biochem.* **94**, 541-548.

Mirelman, D.; Yashouv-Gan, Y.; Schwarz, U. (1977) 'Regulation of murein biosynthesis and septum formation of in filamentous cells of *Escherichia coli* PAT 84.' *J. Bacteriol*. 1**29(3)**, 1593-1600.

Mitsuhashi, Kazuya; Yamamoto, Hiroaki; Matsuyama, Akinobu; Tokuyama, Shinji (2000) 'Fungal D-aminoacylases and method for producing D-amino acids.' *Eur. Pat.Appl. EP* **976828 A1** 2 Feb 2000, 32 pp. [C.A.132:104690].

Mitsuhashi, Kazuya; Yamamoto, Hiroaki; Matsuyama, Akinobu; Tokuyama, Shinji (2001) 'Protein and cDNA sequences of *Hypomyces mycophilus* D-aminoacylase and their uses for producing D-amino acids.' *Eur. Pat. Appl. EP* **1120465 A1** 1 Aug 2001, 33 pp. [C.A.135: 149149].

Mitsui Toatsu Chemicals, Inc., Japan (1983) 'a-Amino fatty acid anilide derivatives.' *Jpn. Kokai Tokkyo Koho JP* **58150548 A2** 7 Sep 1983 Showa, 9 pp. (Japanese). (Japan).[C.A. 100:139629]

Miyoshi, T. Kitagawa, H. Kato, M.; Chiba, S. (1985) 'L-Amino acids. *Eur. Pat. Appl. EP* **159866 A2,** 30 Oct 1985,17 pp. [C.A. 104: 67504].

Moeller, A.; Syldatk, C.; Schulze, M.; Wagner, F. (1988) 'Stereo- and substrate-specificity of a D-hydantoinase and a D-N-carbamyl-amino acid amidohydrolase of *Arthrobacter crystallopoietes* AM 2.' *Enzyme Microb. Technol.*, **10(10)**, 618-25.

Moriguchi, Mitsuaki; Sakai, Kenji; Miyamoto, Yoshiro; Wakayama, Mamoru (1993) 'Production, purification, and characterization of D-aminoacylase from *Alcaligenes xylosoxydans subsp*. *xylosoxydans* A-6.' *Biosci*., *Biotechnol., Biochem.* **57(7)**, 1149-52. [C.A.119: 265080]

Morin, Andre; Hummel, Werner; Schuette, Horst; Kula, Maria Regina (1986a) 'Characterization of hydantoinase from *Pseudomonas fluorescens* strain DSM 84.' *Biotechnol. Appl. Biochem.*, **8(6)**, 564-74

Morin, Andre; Hummel, Werner; Kula, Maria Regina (1986b) 'Production of hydantoinase from *Pseudomonas fluorescens* strain DSM 84.' *Appl. Microbiol. Biotechnol.,* **25(2)**, 91-6.

Morin, A.; Hummel, W.; Kula, M.R.(1986c) 'Rapid detection of microbial hydantoinase on solid medium.' *Biotechnol. Lett*. **8**, 571-576.

Morin, Andre; Hummel, Werner; Kula, Maria Regina (1987) 'Enrichment and selection of hydantoinase-producing microorganisms.' *J. Gen.Microbiol.*, **133(5)**, 1201-7..

Morin, Andre; Leblanc, Danielle; Paleczek, Albert; Hummel, Werner; Kula, Maria Regina (1990) 'Comparison of seven microbial D-hydantoinases.' *J. Biotechnol.*, **16(1-2)**, 37-47 [C.A.114: 19910]

Morin, Andre; Touzel, Jean Pierre; Lafond, Alain; Leblanc, Danielle (1991) 'D-Hydantoinase from anaerobic microorganisms.' *Appl. Microbiol. Biotechnol.*, **35(4)**, 536-40.

Morin, Andre (1993) 'Use of D-hydantoinase extracted from legumes to produce N-carbamyl D-amino acids.' *Enzyme Microb. Technol*., **15(3)**, 208-14.

Morin, Andre; Poitras, Elyse; Moresoli, Christine; Brion, Francois (1995a) 'Extraction of cyclic amide amidohydrolase from green hulls of *Pisum sativum* and its use as biocatalyst for N-carbamyl amino acids.' *Bioresour. Technol*., **53(1)**, 31-7

Morin, A.; Tran Trung, N. H.; LaPointe, G.; Dubeau, H. (1995b) 'Conditions used with a continuous cultivation system to screen for D-hydantoinase-producing microorganisms.' *Appl. Microbiol. Biotechnol.* **43(2)**, 259-66 [C.A.123: 28982].

Morin, Andre; Leblanc, Danielle; Lingnau, Andreas; Lapointe,Gisele(1997)'Cyclic amide amidohydrolase activities in *Pisum sativum* hulls.' *Bioresour. Technol.,* **61(1)**, 91-97.

Morinaga, Y.; Yamada, H. (1986) 'Serine'. In: *Biotechnology of amino acid production, Progress in industrial microbiology,* vol. **24**, [Aida, K.; Chibata, I.; Nakayama,K.; Takinami, K.; Yamada, H. (Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p. 207-216.

Mukaiyama, Teruaki; Iwasawa, Nobuharu; Stevens, Rodney W.; Haga, Toru (1984) 'Synthetic control leading to chiral compounds.' *Tetrahedron,* **40(8)**, 1381-90 [C.A. 102: 23719].

Murakami, Hisamichi (1989) 'Utilization of optically active compounds.' *Kikan Kagaku Sosetsu,* **(6)**, 212-25 (Japanese) [C.A.112: 20520].

Muramatsu, Hiroshige; Ueda, Teruo (1982) 'Halogenated a-amino acids.' *U.S. US* **4351954 A** 28 Sep 1982, 5 pp. [C.A.97: 216728].

Nagata, Yoko (1999) 'D-amino acids in nature'. *In:Adv. biochirality,[Symp. Biol. homochirality]* Meeting Date **1998,** [Palyi, G.; Zucchi, C.; Caglioti, L.(Eds.)], Elesevier Science, Oxford, U.K.[C.A.. 132: 148087].

Nagata, Y.; Tanaka, K.; Iida, T.; Kera,Y.; Yamada, R.-H.; Nakajima, Y.; Fujiwara, T.; Fukumori,Y.; Yamanaka, T.; Koga, Y.; Tsuji, S.; Kawaguchi-Nagata, K. (1999) 'Occurance of D-amino acids in a few Archaea and dehydrogenase activities in hyperthermophile *Pyrobaculum islandicum'. Biochim. Biophys. Acta* **1435** (1-2), 160-166.

Naglak, T.J.; Wang, H.Y. (1990) 'Recovery of foreign protein from the periplasm of *E.coli* by chemical permeabilization.' *Enzyme Microb. Technol*. **12**, 603-611.

Naglak, T.J., Hettwer, D.J.; Wang, H.Y. (1990) 'Chemical Permeabilization of cells for intracellular product release.' In: *Separation processes in biotchnology*, Asenjo J.A. ed., p. 177-205, Marcel Dekker, Inc., New York.

Nagubandi, Sreeramulu (1985a) 'Phosphonomethylated amino acids.' *U.S. US* **4548758 A** 22 Oct 1985, 4 pp. [C.A.104: 207450]
Nagubandi, Sreeramulu (1985b) 'Phosphonomethylated amino acids.' *U.S. US* **4491548 A** 1 Jan 1985, 6 pp. [C.A.102: 204293]

Nakamori, S.; Yokozeki, K.; Mitsugi, K.; Eguchi, C.; Iwagami, H.((1978) 'D-a-amino acids'. *Ger. Offen. DE* **2825245,** 14 Dec 1978, 38 pp. (German) (Germany) [C.A. 90: 136262].

Nakamori, S. (1986) 'Threonine and homoserine'. In: *Biotechnology of amino acid production, Progress in industrial microbiology,* vol. **24**, [Aida, K.; Chibata, I.; Nakayama, K.; Takinami, K.; Yamada, H. (Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p. 173-182.

Nakayama, Kiyoshi; Ozawa, Tatsuya (1990) 'Enzymic or microbial manufacture of L-homophenylalanine from 5-benzylmethylhydantoin.' *Jpn.Kokai Tokkyo Koho JP* **02119796 A2** 7 May 1990 Heisei, 4 pp.(Japan) [C.A. 113: 150893].

Nanba, Hirokazu; Yajima, Kazuyoshi; Takano, Masayuki; Yamada,Yukio; Ikenaka, Yasuhiro; Takahashi, Satomi (1996a) 'Cloning of gene for hydantoinase of *Bacillus, Agrobacterium and Pseudomonas* and use of the gene for producing D-N-carbamoyl-a-amino acid.' *PCT Int. Appl. WO* **9620275 A1** 4 Jul 1996, 53 pp. [C.A. 125: 162106].

Nanba, Hirokazu; Tamada, Yukio; Yajima, Kazuyoshi; Takano,Masayuki; Ikenaka, Yasuhiro; Takahashi, Satomi; Ohashi, Takehisa (1996b) 'Process for producing D-amino acid by using composite immobilized enzyme preparation.' *PCT Int. Appl. WO* **9600296 A1** 4 Jan 1996, 45 pp. [C.A.124: 115602]

Nanba, Hirokazu; Ikenaka, Yasuhiro; Yamada, Yukio;Yajima, Kazuyoshi;Takano, Masayuki; Takahashi, Satomi (1998) 'Isolation of *Agrobacterium sp*. strain KNK712 that produces N-carbamyl-D-amino acid amidohydrolase, cloning of the gene for this enzyme, and properties of the enzyme.' *Biosci., Biotechnol., Biochem.*, **62(5),** 875-881

Nandakumar, R.; Gounot, A. M.; Mattiasson, B. (2000) 'Gentle lysis of mucous producing cold-adapted bacteria by surfactant treatment combined with mechanical disruption.' *J. Biotechnol*., **83(3)**, 211-217

Nath, Mala; Yadav, Rakesh; Gielen, Marcel; Dalil, Hassan; De Vos, Dick; Eng, G. (1997) 'Synthesis, characteristic spectral studies and in vitro antimicrobial and antitumor activities of organotin (IV) complexes of Schiff bases derived from amino-acids.' *Appl. Organomet. Chem.,* **11(9)**, 727-736. [C.A.127: 278248].

Nelson, J.M. and Griffin, E.G.(1916) 'Adsorption of invertase' *J. Am.Chem. Soc*. **38,** 1109

Nishida, Yutaka; Nakamichi, Katsuhiko; Nabe, Koichi; Tosa, Tetsuya (1987) 'Enzymic production of L-tryptophan from DL-5-indolylmethylhydantoin by *Flavobacterium sp*.' *Enzyme Microb. Technol*., **9(12)**, 721-5.

Novella, I.S.; Fargues,C.; Gresillot, G. ((1994) 'Improvement of the extraction of penicillin acylase from *E. coli* by combined use of chemical methods.' *Biotechnol. Bioeng.* **44**, 379-382.

Ofuna, Yasushi; Kurokawa, Natsuko; Hori, Keiko (1987) 'A process for the preparation of imino acids-containing peptides useful in preparing drugs and

agrochemicals.' *Jpn. Kokai Tokkyo Koho JP* **62148458 A2** 2 Jul 1987 Showa, 7 pp. (Japan) [C.A.108: 56624].

Ogawa, T.; Kimoto, M.; Sasaoka, K. (1977) 'Identification of D-aspartic acid and D-glutamic acid in pea seedlings.' *Agric. Biol. Chem.* ***4*1(9)**, 1811-12.

Ogawa, Jun; Honda, Michinari; Soong, Chee Leong; Shimizu, Sakayu (1995) 'Diversity of cyclic ureide compound-, dihydropyrimidine-, and hydantoin-hydrolyzing enzymes in *Blastobacter sp.* A17p-4.' *Biosci. Biotechnol. Biochem.* **59(10)**, 1960-2.

Ogawa, Jun; Shimizu, Sakayu (2000) 'Stereoselective synthesis using hydantoinases and carbamoylases.' *Stereoselective Biocatalysis*, **1-21.** Edited by: Patel, Ramesh N. Marcel Dekker, Inc.: New York, N. Y. (English) [C.A.134: 29662]

Ohfune, Yasufumi; Sakaitani, Masahiro (1987) '[(Tert-Butyldimethylsilyl)oxy]carbonyl amino acids as intermediates for agrochemicals, cenral nervous system agents, and antibacterials.' *Eur. Pat. Appl. EP* **217243 A2** 8 Apr 1987, 32 pp. [C.A. 107: 176471].

Ohleyer, Eric (1989) 'Stereospecific hydrolysis of hydroxyphenylhydantoin to hydroxyphenylglycine by *Agrobacterium*.' *Eur. Pat. Appl. EP* **309310 A1** 29 Mar 1989, 7 pp. [C.A112: 117372].

Ohta, H.; Jikihara, T.; Wakabayashi, K.; Fujita, T. (1980) *Pestic Biochem. Physiol*.**14**, 153

Okii, Mitsuyoshi; Matsukuma, Ikuo; Teranishi, Masayuki; Konnai, Makoto; Takematsu, Tetsuo (1980) 'Herbicidal activity of amino acid related compounds. I. Herbicidal activity of thiocarbonyl amino acid derivatives.' *Zasso Kenkyu*, **25(2)**, 98-103 (Japanese) [C.A.94: 115852]

Olama, Z. A. (1999) 'Production, characterization and immobilization of L-aminoacylase of *Bacillus cereus*.' *Egypt. J. Microbiol*., **Volume Date 1998**, 33(4), 575-593 [C.A.133:134221].

Olivieri, R.; Fascetti, E.; Angelini, L.; Degen, L. (1979a) 'Enzymic conversion of N-carbamoyl-D-amino acids to D-amino acids.' *Enzyme Microb. Technol*.**1**, 201-4

Olivieri, R.; Viglia, A.; Degen, L.; Angelini, L.; Fascetti, E. (1979b) 'D-amino acids'. *Ger. Offen. DE* **2920963** 29 Nov 1979, 20 pp. (German) (Germany). [C.A. 92: 92733].

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

Omori, Yoshiyuki; Ichida, Taizo; Ukita, Rie; Osumi, Masako; Ueda, Mitsuyoshi; Tanaka, Atsuo (1989) 'A novel method-a "freeze-blast" method-to disrupt microbial cells.' *J. Ferment. Bioeng.*, **67(1)**, 52-6.

Oner, M. D. and Akar, B. (1993) 'Separation of theproteolytic enzymes from fig tree latex and its utilization in Gaziantep cheese production.' *Food Sci. Technol.* (London) **26(4)**, 318-321. [C.A. 120: 75904]

Park, Joo-Ho; Kim, Geun-Joong; Lee, Seung-Goo; Kim, Hak-Sung (1998) 'Biochemical properties of thermostable D-hydantoinase from *Bacillus thermocatenulatus* GH-2.' *Ann. N. Y. Acad. Sci.*, **864** (Enzyme Engineering XIV), 337-340.

Park, Joo-Ho; Kim, Geun-Joong; Lee, Seung-Goo; Lee, Dong-Cheol; Kim, Hak-Sung (1999) 'Purification and characterization of thermostable D-hydantoinase from *Bacillus thermocatenulatus* GH-2.' Appl. *Biochem. Biotechnol*., **81(1)**, 53-65.

Park, Joo-Ho; Kim, Geun-Joong; Kim, Hak-Sung (2000) 'Production of D-amino Acid using whole cells of recombinant *Escherichia coli* with separately and coexpressed D-hydantoinase and N-carbamoylase.' *Biotechnol. Prog.,* **16(4)**, 564-570.

Patil, Anita R.; D'souza, Stanislaus F. (1997) 'Measurement of in situ halophilic glyceraldehyde-3-phosphate dehydrogenase activity from the permeabilized cells of archaebacterium *Haloarcula vallismortis.' J. Gen. Appl. Microbiol*., **43(3)**, 163-167 (English) 1997

Peppler, H. J. (1979) 'Production of yeast and yeast products.' In: *Microbial Technology: Microbial Processes*, Peppler, H. J. and Perlman, D. eds., **vol. 1,** pp. 157-185, Academic press, London.

Petersson, E.A. and Torre, A.R. (1984) 'Displacement chromatography of protein,' in *Methods in Enzymology*, vol.**104,** Jakoby, W.B. ed., Academic Press Inc, Orsando, Florida., pp.113-133.

Pharmacia Catalogue: Gel filtration: theory and practice.

Pietzsch, M.; Syldatk, C.; Wagner, F. (1990) 'Isolation and characterization of a new, non pyridoxal-5'-phosphate dependent hydantoin racemase.' *DECHEMA Biotechnol. Conf*., **4**(Pt. A, Lect. DECHEMA Annu. Meet. Biotechnol.,8th, 1990), 259-62 [C.A.115: 226676].

Poduska, Karel; Zaoral, Milan; Slama, Karel; Sorm, Frantisek (1976) 'Insecticidal amino acid derivatives with juvenile hormone activity.' *Czech. CS* **164370** 15 Aug 1976,8 pp. [C.A. 86:155989].

Powell, Lawson William; Power, John Anthony (1988) 'Immobilized enzyme preparation and its use'. *Eur. Pat. Appl. EP* **261836 A1** 30 Mar 1988, 10 pp. [C.A.110: 91273].

Poznanski, S.; Bednarski, W.; Kowalewska, Jadwiga; Mieczkowski, M. (1979) 'Method of disintegration of microbe cells and enzymic activity of b-galactosidase preparations.' *Bull. Acad. Pol. Sci., Ser. Sci. Biol*., **27(12),** 999-1003.

Price, N. C. (1992) 'Techniques for enzyme extraction.' *Enzyme Assays*, 255-75, Edited by: Eisenthal, Robert; Danson, Michael J. IRL: Oxford, UK. (English) 1992.

Pu, Wang; Yang, Li-Rong; Wu, Jian-Ping (2001) 'Immobilization of lipase by salts and the transesterification activity in hexane.' *Biotechnology Letters,* **23(17),** 1429-1433.

Putman, C. W. M. M.; Rotteveel, J. J.; Wevers, R. A.; Van Gennip, A.H.;Bakkeren, J. A. J. M.; De Abreu, R. A. (1997) 'Dihydropyrimidinase deficiency, a progressive neurological disorder?'. *Neuropediatrics (Stuttgart)*, **28(2),** 106-110 [C.A.127: 203972].

Qin, Yeya (2001) 'Process for preparing L-amino acid by resolution with immobilized amino acylase.' *Faming Zhuanli Shenqing Gongkai Shuomingshu CN* **1306092 A** 1 Aug 2001, 7 pp. (Chinese) [C.A. 136: 133688].

Ragnitz, K.; Syldatk, C.; Pietzsch, M. (2001) 'Optimization of the immobilization parameters and operational stability of immobilized hydantoinase and L-N-carbamoylase from *Arthrobacter aurescens* for the production of optically pure L-amino acids.' *Enzyme Microb. Technology*, **28(7-8),** 713-720.

Rai, Rekha; Taneja, Veena (1998a)'Papain catalyzed hydantoin hydrolysis in the synthesis of amino acids.' *Biochem. Biophys. Res. Commun.*, **244(3)**, 889-892.

Rai, R.; Taneja, V. (1998b) 'Production of D-amino acids using immobilized D-hydantoinase from lentil, *Lens esculenta*, seeds'. *Appl. Microbiol. Biotechnol*., **50(6)**, 658-662.

Rammler, D.H.; Okabayashi, T.; Delk, A (1965) 'Isolation and characterization of soluble ribonucleic acid obtained by solvent extraction bacteria.' *Biochemistry* **4(10)**, 1994-2001.

Ranjan, P.; Dave, S. R. (2000) 'A hydantoin hydrolysing bacteria: isolation, characterization and bioconversion.' *Curr. Sci.*, **78(6)**, 679-680.

Rehnberg, Bradley G.; Schultz, Donald A.; Raschke, Ronald L. (1981) 'Algal cell disruption using a Pyrex tissue grinder and the B. Braun homogenizer.' *J. Phycol.*, **17(3)**, 281-2.

Rhone-Poulenc Industries S. A., (Fr.) (1977) 'Aminoacylase-support comlpexes.' *Belg. BE* **855051** 25 Nov 1977,14 pp. (French) (Belgium) [C.A.88: 185354].

Robert, N.A.; Gray, G.W.; Wilkinson, S.G. (1970) 'Bactericidal action of ethylenediaminetetraacetic acid on *Pseudomonas aeruginosa*.' *Microbios.* **2(7-8)**, 189-208 [CA 74: 84387v].

Robinson, Donald; Stirling, John L.; Hutchinson, Winston; Koshy, Achamma (1980) 'Pitfalls and perspectives, in enzyme purification.' *Birth Defects, Orig. Artic. Ser*., **16** (1, Enzyme Ther. Genet.Dis. 2), 1-16

Roeda, D.; Westera, G. (1981) 'The synthesis of some carbon-11-labeled antiepileptic drugs with potential utility as radiopharmaceuticals: hydantoins and barbiturates.' *Int. J. Appl. Radiat. Isot.* **32,** 843-5.

Roi, A. A.; Nikonenko, V. U.; Gvozdyak, P. I. (1982) 'Effect of the method used for cell disruption of *Bacillus subtilis* 21/3 on the enzymic activity of cell-free extracts.' *Mikrobiol. Zh. (Kiev),* **44(1)**, 15-17 (Russian) [C.A. 96: 177557].

Rozzel, D.J.and Wagner, F. [Eds.] (1992) In: *Biocatalytic production of amino acids and derivatives,* Hanser Publishers Munich, Vienna, New York, Barcelona.

Runser, Serge; Ohleyer, Eric (1990) 'Properties of the hydantoinase from *Agrobacterium sp.* IP I-671.' *Biotechnol. Lett.*, **12(4)**, 259-64.

Runser, S.; Chinski, N. and Ohleyer, E. (1990) 'D-p-hydroxyphenylglycine production from D,L-5-p-hydroxyphenylhydantoin by *Agrobacterium sp*.' *Appl. Microb. Biotechnol.* **33,** 382-388.

Runser, Serge M.; Meyer, Patrick C.(1993)'Purification and biochemical characterization of the hydantoin hydrolyzing enzyme from *Agrobacterium species.* A hydantoinase with no 5,6-dihydropyrimidine amidohydrolase activity.' *Eur. J. Biochem*., **213(3),** 1315-24'

Ryu, Sangryeol; Lee, Keho (1997) 'Comparison of immobilization matrix for ethanol fermentation by *Zymomonas mobilis* and *Sachharomyces cerevisiae*.' *J. Microbiol. Biotechnol.* **7(6),** 438-440.

Saikawa, Isamu; Takano, Shuntaro; Yoshida, Chosaku; Takashima, Okuta; Momonoi, Kaishu; Kuroda, Seietsu; Komatsu, Miwako; Yasuda, Takashi; Kodama, Yutaka (1981) 'Amino acid derivatives of 2,3 dioxopiperazine.' *Pat. Specif. (Aust.) AU* **518792 B2** 22 Oct 1981, 35 pp. [C.A.97: 92762].

Sambale, Clemens; Kula, Maria Regina; Hummel, Werner; Drauz, Karlheinz (1987) 'Manufacture of optically active amino acids by microbial or enzymic

resolution of racemic amino acid carbamates.' *Ger. Offen. DE* **3606401 A1** 3 Sep 1987, 9 pp. (Germany) [C.A.108: 185294].

Sakai, H. (1986) 'Microbial amino acid antimetabolites'. In: *Biotechnology of amino acid production,Progress in industrial microbiology,* vol.**24**, [Aida, K.; Chibata, I.; Nakayama,K.; Takinami, K.; Yamada, H. (Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p. 286-308.

Sakai, Kenji; Obata, Taketeru; Ideta, Kohtaro; Moriguchi, Mitsuaki (1991) 'Purification and properties of D-aminoacylase from *Alcaligenes denitrificans subsp. xylosoxydans* MI-4.J.' *Ferment. Bioeng.,* **71(2)**, 79-82 (English) 1991.[C.A. 114:138602]

Sano, K.; Yokozeki, K.; Yamada, K.; Yasuda, H.; Eguchi, C.; Noda, I.; Mitsugi, K.(1977) 'L-amino acids' *Japan Kokai JP* **52055891** 5 Feb 1977 Showa, 6 pp. [C.A.87: 2312].

Sato, Yasuhiro; Takeuchi, Koichi; Ogata, Hideo; Suzuki, Kenji (1988)'Hydantoin intermediates for amino acids, agrochemicals, pharmaceuticals and food additives.' *Jpn. Kokai Tokkyo Koho JP* **63017868 A2** 25 Jan 1988 Showa, 4 pp. (Japan) [C.A. 109: 39741]

Save, S. S.; Pandit, A. B.; Joshi, J. B. (1994) 'Microbial cell disruption: role of cavitation.' *Chem. Eng. J. (Lausanne)*, **55(3)**, 67B-72B.

Scawen, M. D.; Atkinson, A.; Darbyshire, J. (1980) 'Large-scale enzyme purification.' *Appl. Protein Chem.,* 281-324, Edited by Grant, R. A., Appl. Sci.: Barking, Engl. (English) 1980.

Scawen, M.D. and Melling, J. (1985) 'Large-scale extraction and purification of enzyme and other proteins,' in *Handbook of enzyme biotechnology,* 2nd edition, Wiseman, A. ed., Ellis Horwood Limited, England, pp.15-53.

Schmierer, Roland; Maier, Thomas; Handte, Reinhard (1986) 'Preparation of N-(2,6-dialkylphenyl)-N-formylglycines.' *Ger. Offen. DE* **3515094 A1** 30 Oct 1986, 11 pp. (Germany)[C.A.106: 120228].

Schmitt-Rozieres, Murielle; Vanot, Gullaume; Deyric,Valerie; Comeau, Louis-Claude (1999) 'Borago Officinalis oil : Fatty acid fractionation by immobilized *Candida rugosa* lipase.' *J. Am. Oil Chem. Soc.* **76(5)**, 557-562

Schroder, Ludwig; Stransky, Werner; Mengel, Rudolf; Lust, Sigmund; Linden, Gerbert; Raddatz, Erich; Schneider, Gerhard (1983) 'Hydantoins and their application.' *Eur. Pat. Appl. EP* **91596 A2** 19 Oct 1983, 47 pp. [C.A.100: 85695].

Schuette, H.; Kula, M.R.(1990) 'Pilot and processes scale techniques for cell disruption'. *Biotechnol. Appl. Biochem.* **12,** 599- 620.

Schutte, Horst; Kula, Maria-Regina (1993) 'Cell disruption and isolation of non-secreted products' *Biotechnology (2nd Ed.)*, **Volume 3,** 505-26. Edited by: Stephanopoulos, Gregory. VCH: Weinheim, Germany.

Schuette, H.; Kroner, K. H.; Kula, M. R. (1984) 'Recent studies of mechanical disintegrators for the large scale disruption of microorganism.' *Eur. Congr. Biotechnol., 3rd,* **Volume 1**, 621-8. Verlag Chemie: Weinheim, Fed. Rep. Ger. (English) 1984.

Sedmak, J.J. and Grossberg, S.E. (1977) 'A rapid sensitive and versatile assay for protein using Coomassie Brilliant Blue G-250.' *Anal. Biochem.* **79,** 544-552.

Senuma, Masaru; Ishihara, Nobuhiko; Nishimoto, Shigeru; Yamato, Eisaku; Shigematsu, Noboru (1980) 'Process for preparing a hydroxyphenylglycine compound.' *Brit.GB* **1576678** 15 Oct 1980, 4 pp.[C.A. 95:42459]

Serrano, R., Gancedo, J.M.; Gancedo, C. (1973) "Assay of yeast enzymes in situ". *Eur. J. Biochem.* **34**, 479-483.

Sharma, Rakesh; Vohra, Rakesh M. (1997a) 'A thermostable D-hydantoinase isolated from a mesophilic *Bacillus sp.* AR9.' *Biochem. Biophys. Res. Commun.,* **234(2)**, 485-488.

Sharma, Rakesh; Vohra, Rakesh M. (1997b) 'A thermostable D-hydantoinase isolated from a mesophilic *Bacillus sp.* AR9.' [Erratum to document cited in CA127: 30739] *Biochem. Biophys. Res. Commun.,* **237(3)**, 759.

Shi, Ya-wei; Li, Han-qing; Yuan, Jing-ming; Ding, Yu-hua; Lian, Hui-yong; Qi, Yang-hong (2001) 'Purification of D-hydantoinase from *Pseudomonas* 2262'. Shanxi Daxue Xuebao, Ziran Kexueban, 24(3), 255-258 (Chinese) [C.A. 135: 354516].

Shimizu, Sakayu; Shimada, Hiroaki; Takahashi, Satomi; Ohashi, Takehisa; Tani, Yoshiki; Yamada, Hideaki (1980) 'Microbial transformation of hydantoins to amino acids. Part V. Synthesis of N-carbamyl-D-2-thienylglycine and D-2-thienylglycine by microbial hydantoinase.' *Agric. Biol. Chem.*, **44(9)**, 2233-4.

Shimizu, Masami; Komatsu, Toshiteru; Shimizu, Masao; Kase, Minoru (2000) 'Immobilization of a lipolytic enzyme by adsorption onto a porous, anion-exchange resin and its use for lipolysis and esterification of fats and oils.' *Eur. Pat. Appl. EP* **1008647 A2** 14 Jun 2000, 8 pp. [C.A.133: 28052].

Shintani, Yasushi; Fukuda, Hirosuke; Okamoto, Noriko; Murata, Kousaku; Kimura, Akira (1984) 'Isolation and characterization of N-long chain acyl aminoacylase from *Pseudomonas diminuta*.' *J. Biochem. (Tokyo),* **96(3)**, 637-43. [C.A. 102: 20107].

Shio, I. (1986) 'Tryptophane, phenylalanine and tyrosine'. In: *Biotechnology of amino acid production, Progress in industrial microbiology,* vol. **24**, [Aida, K.; Chibata, I.; Nakayama,K.; Takinami, K.; Yamada, H.(Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p. 188-206.

Shiotani, Takenaga; Kitamura, Shiro; Fukuda, Hideki (1991) 'Preparation of immobilized microorganism, especially hydantoinase-producing microorganism.' *Jpn. Kokai Tokkyo Koho JP* **3133386 A2** 6 Jun 1991 Heisei, 5 pp. (Japan) [C.A. 115:178204].

Shuey, C.D. (1990) 'Ion exchange processes,' in *Handbook of Enzyme Biotechnology*, 2nd edition, Wiseman, A. ed., Ellis Horwood Limited, England, pp. 263-286.

Siemann, Martin; Alvarado-Marin, Adrian; Pietzsch, Markus; Syldatk, Christoph (1999) 'A D-specific hydantoin amidohydrolase: properties of the metalloenzyme purified from *Arthrobacter crystallopoietes*.' *J. Mol. Catal. B: Enzym.*, **6(3)**, 387-397.

Smalla, Kornelia; Hermann, Peter; Willhardt, Ingo; Turkova, Jaroslava; Coupek, Jiri (1985) 'Recovery of optical active a-amino acids from racemic mixtures.' *Ger. (East) DD* **216918 A1** 2 Jan 1985, 8 pp. (German) (German Democratic Republic).[C.A. 103: 86513].

SNAM Progetti S.p.A., Italy (1978) 'L-Carbamyl amino acids and the corresponding L-amino acids.' *Span. ES* **459856** 1 Apr 1978, 20 pp. (Spanish) (Spain) [C.A. 90: 4446]

Sobotka, H. (1932) 'd-5-Phenyl-5'-ethylhydantoin.' *U.S. Pat*. **1861458,** June 7,1932 [C.A.26: P4133].

Song, Joe Jun; Zhang, Shiming; Lenz, Robert W.; Goodwin, Steve (2000) 'In vitro polymerization and copolymerization of 3-hydroxypropionyl-CoA with the PHB sythase from *Ralstonia eutropha.*' *Biomacromolecules* **1(3),** 433-439 [C.A. 133: 292789]

Soong, C.L.; Ogawa, J.; Shimizu, S. (2001) 'Cyclic ureide and imide metabolism in microorganism producing a hydantoinase useful for amino acid production.' *J. Molecular Catalysis B: Enzymatic* **12(1-6)**, 61-70.

Soubeyrand, Sebastein; Manjunath, Puttaswamy (1998) 'Single step isolation of bovine seminal platelet- activating factor acetyhydrolase.' *J. Androl.* **19(2)**, 226-231[CA 129:78294].

Spector, T. (1978) 'Refinement of the Coomassie Blue method of protein quantitation.' *Anal. Biochem.* **86,** 142-146.

Spurlock, J.J. (1945) '5-substituted 5-(2-thienyl)hydantoins.' *U.S. Pat.***2366221,** January 2, 1945 [C.A. 39: 1736].

Srinivas, M.D.; Rashmi, K.R.; Raghavarao, K.S.M.S. (1999) 'Extraction and purification of the plant peroxidases by aqueous two-phase extraction coupled with gel filtration'. *Process Biochem.* (Oxford) **35 (1,2),** 43-48.

Su, Zhi-Guo;Feng,Xiao-Li (1999) 'Process integration of cell disruption and aqueous two-phase extraction.' *J. Chem. Technol. Biotechnol.*, **74(3)**, 284-288

Sudge, S. S.; Bastawde, K. B.; Gokhale, D. V.; Kalkote, U. R.; Ravindranathan, T. (1998) 'Production of D-hydantoinase by halophilic *Pseudomonas sp*. NCIM 5109.' *Appl. Microbiol. Biotechnol.,* **49(5)**, 594-599.

Suesse, Manfred; Schaks, Angela; Johne, Siegfried (1987) 'Preparation of (1,2,3,4-tetrahydro-4-oxo-2-thioxoquinazolin-3-yl)-alkanoates as agrochemicals.' *Ger. (East) DD* **252603 A1** 23 Dec 1987, 5 pp. (German Democratic Republic) [C.A.109: 73469].

Sugie, Makiko; Suzuki, Hideo (1980) 'Optical resolution of D,L-amino acids with D-aminoacylase of *Streptomyces*.' *Agric. Biol. Chem.,* **44(5)**, 1089-95 [C.A. 93: 40961].

Sugimoto, H. (1974) 'Synergistic effect of ethanol and sodium chloride on autolysis of baker's yeast for preparing food-grade yeast extract .' *J. Food Sci.* **39,** 939-942 [CA 82: 84437 b].

Sun, Wanru (1983a) 'Properties of dihydropyrimidinase in intact cells of *Pseudomonas putida*.' *Weishengwu Xuebao,* **23(3)**, 257-64 (Chinese) [C.A.100: 31970].

Sun, Wanru (1983b) 'Screening of strains producing dihydropyrimidinase and fermentation conditions.' *Weishengwu Xuebao* **23(2),** 133-42 (Chinese) [C.A. 99:103655].

Sun, Sufang; Li, Xuyuan; Nu, Shaoli; You, Xin (1999) 'Immobilization and characterization of b-galactosidase from the plant gram chicken bean (*Cicer arietinum*). Evolution of its enzymic actions in the hydrolysis of lactose.' *J. Agric. Food Chem*., **47(3)**, 819-823.

Sutherland, A.; Willis C.L.(1998) 'Chemoenzymic synthesis of 4-amino-2- hydroxy acid: A comparison of mutant and wild type oxidoreductase'. *J. Org. Chem.* **63(22),** 7764-7769 [C.A.129: 342728].

Syldatk, Christoph; Cotoras, Davor; Moeller, Angela; Wagner, F. (1986) 'Microbial, enantioselective hydrolysis of D,L-5-monosubstituted hydantoins for the production of D- and L-amino acids.' *Biotech-Forum*, **3(1)**, 9-19 (German) [C.A.107:196408].

Syldatk, C.; Cotoras, D.; Dombach, G.; Gross, C.; Kallwass, H.; Wagner, F. (1987) 'Substrate- and stereospecificity, induction and metallodependence of a microbial hydantoinase.' *Biotechnol. Lett.*, **9(1)**, 25-30 [C.A.107: 3102].

Syldatk, C.; Laufer, A.; Muller, R.; Hoke, H. (1990) 'Production of optically pure D and L-alpha amino acid by bioconversion of D,L-5-mono-substituted hydantoin derivatives'. In: *Advances in biochemical engineering /biotechnology*, vol. **41**, [Fiechter, A. (Ed.)], Springer-Verlag, Berlin, Heidelberg, p.31-75.

Syldatk, C.; Muller, R.; Siemann, M.; Krohn, K.; Wagner, F.(1992a) 'Microbial and enzymatic production of D-amino acids From D,L-monosustituted hydantoins'. In: *Biocatalytic production of amino acids and derivatives*, Rozzell, J.D. and Wagner, F. (EDS.); Hanser publishers, Munich Vienna New York Barcelona, pp. 75-128.

Syldatk, C.; Lehmensiek, V.; Ulrichs, G.; Bilitewski, U.; Krohn, K.; Hoeke, H.; Wagner, F. (1992b) 'Biotechnological production of unnatural L-amino acids from D,L-5-monosubstituted hydantoins. I. Derivatives of L-phenylalanine'. *Biotechnol. Lett.* **14(2)**, 99-104 [C.A. 116: 212897].

Syldatk, C.; Voelkel, D.; Bilitewski, U.; Krohn, K.; Hoeke, H.; Wagner, F. (1992c) 'Biotechnological production of unnatural L-amino acids from D,L-5-monosubstituted hydantoin. II L-a-and L-b-naphthyl-alanine'. *Biotechnol. Lett.* **14(2)**, 105-10 [C.A. 116: 192552].

Syldatk, C.; May, O.; Altnbuchner, J.; Mattes, R.; Siemann, M.(1999) 'Microbial hydantoinases-industrial enzymes from the origin of life?' *Appl. Microbiol. Biotechnol.*, **51(3)**, 293-309.

Tagawa, M.; Okamoto, E.; Akutsu, S.; Kuwahara, M. (1994) 'Process for producing optically active D-amino acids'. *PCT Int. Appl. WO* **9401577 A1** 20 Jan 1994 25 pp. [C.A. 120: 215483].

Tahara, S.; Miura, Y.; Mizutani, J. (1979) 'Hydantoin derivatives of S-alk(en)yl-L-cysteine-S-oxide. III. Antimicrobial L-5-alk(en)ylthiomethylhydantoin-(±)-S-oxides: mode of action.' *Agri. Biol. Chem.* **43,** 919-24.

Takahashi, Satomi; Kii, Yoshiaki; Kumagai, Hidehiko; Yamada,Hideaki (1978) 'Microbial transformation of hydantoins to amino acids. II. Purification, crystallization and properties of hydantoinase from *Pseudomonas striata*.' *J. Ferment. Technol.*, **56(5)**, 492-8.

Takahashi, Satomi; Ohashi, Takehisa; Kii, Yoshiaki; Kumagai, Hidehiko; Yamada, Hideaki (1979) 'Microbial transformation of hydantoins to amino acids. III. Microbial transformation of hydantoins to N-carbamyl-D-amino acids.' *J. Ferment. Technol.*, **57(4)**, 328-32.

Takahashi, Satomi (1983) 'Microbial synthesis of D-amino acids from D,L-5-substituted hydantoins.' *Hakko Kogaku Kaishi*, **61(3)**, 139-51(Japanese) 1983 [C.A. 99:51815].

Tamura, Minoru; Harada, Kaoru (1980) 'Sterically controlled synthesis of optically active organic compounds. XXX. Asymmetric synthesis of aspartic acid by hydrogenation of an optically active 1,4-oxazine derivative'. *Bull. Chem. Soc. Jpn.,* **53(2)**, 561-2 [C.A. 93:47136].

Tan, Tianwei; Yang, Yuanzhong; Ma, Runyu (1996) 'Selective release of superoxide dismutase from *Saccharomyces cerevisiae* by alcoholic solvent.' *Shengwu Gongcheng Xuebao*, **12(2)**, 148-151 (Chinese) [C.A. 126: 46333].

Tanaka, H.; Soda, K. (1986) 'Methionine'. In: *Biotechnology of amino acid production, Progress in industrial microbiology,* vol. **24**, [Aida, K.; Chibata, I.; Nakayama,K.; Takinami, K.; Yamada, H. (Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p.183-187.

Tanaka, H.; Kuboi, R.; Kumassawa, I.; Tsuchido T. (1993) 'Control of autolysis of *Bacillus subtilis* for selective production of intracellular enzyme glucose-6-phosphate dehydrogenase in aqueous two-phase systems.' *J. Ferment. Bioeng.* **75(6)**, 424-429 [CA 119: 137447].

Tang, Jia-fang; Zhou, Jiu-yuan; Liu, Zhi-lan; Xiao, Yu-xiu; Wu, Zheng-hui; Wu, Kai-nang (1999) 'Synthesis of N-(O,S-dimethylthiophosphoryl) a-L-amino acid esters and their insecticidal activity.' *Wuhan Daxue Xuebao, Ziran Kexueban,* **45(6)**, 861-864 (Chinese) [C.A.132: 265248].

Tani, Yoshiki; Yamada, Hideaki; Kumatani, Hidehiko (1978)'Synthesis using hydrolases. II. Phosphatase, hydantoinase, and tryptophanase.' *Kagaku To Seibutsu,* **16(7)**, 449-59 (Japanese) [C.A. 90: 2095].

Tao, Zhongkan; Li, Zhichang (1984) 'Rapiddevelopment of amino acid agrochemicals.' *Huaxue Tongbao,* **(3)**, 43-5 (Chinese) [C.A. 101: 49987].

Tarui, Naoki; Nagano, Yoichi; Izawa, Mikio; Kawata, Mitsuru; Yamano,Toru; Nakahama, Kazuo (2000) 'Enzymic or microbial manufacture of N-carbamoylamino acid and hydroxycarboxylic acid.' *Jpn. Kokai Tokkyo Koho JP* **2000308498 A2** 7 Nov 2000, 26 pp. (Japanese) (Japan) [C.A.133: 334175].

Tawaki, S.; Takeichi, M.; Hagiwara, T.; Tarukawa, H. (1986) *Jpn. Kokai Tokkyo Koho JP* **61181391 A2** 14 Aug 1986, Showa 4 pp. (Japan) [C.A. 106: 17042].

Tawaki, Shinichiro; Tarukawa, Hitoshi; Aikawa, Toshikazu; Takeichi, Mamoru (1988) 'Fermentative manufacture of D-N-carbamyl-a-amino acids as intermediates for drugs and agrochemicals.' *Jpn. Kokai Tokkyo Koho JP* **63071196 A2** 31 Mar 1988 Showa, 4 pp. (Japan) [C.A.109: 91276].

Terada, Atsusuke; Naruto, Shunji; Matsueda, Rei; Higashida, Susumu; Kitano, Kunitoshi (1985) 'Amino acid esters of milbemycins.' *Jpn. Kokai Tokkyo Koho JP* **60184085 A2** 19 Sep 1985 Showa, 11 pp. (Japanese) (Japan) [C.A.104: 207055].

Terasawa, Masato; Fukushima, Makiko; Shimazu, Mitsunobu; Yamagata, Hisashi; Yugawa, Hideaki (1990) 'Racemization of amino acids with bacteria.' *Jpn.Kokai Tokkyo Koho JP* **02124097 A2** 11 May 1990 Heisei, 4 pp. [C.A. 113:76625].

Thibault, T.D.; St.Clair, R.L. (1982) 'Imidazoles, compositions and herbicidal method.' *U.S. Pat.* **4345936** A 24 Aug 1982, 13 pp. [C.A.98: 16687p].

Tielemann, H. (1971) *Sci. Pharm.* **39**, 8.

Tokuyama Co., Japan (1984) 'Method for extraction of enzymes from microbial cells.' *Jpn. Kokai Tokkyo Koho JP* **59166080 A2** 19 Sep 1984 Showa, 3 pp. (Japanese) (Japan) [C.A.102: 42171].

Torrey, S. (ed.) (1983) 'Enzyme immobilization' in '*Enzyme technology*', p.38-106, Noyes Data Corporation, U.S.A.

Tosaka, O.; Takinami, K, (1986) 'Lysine'. In: *Biotechnology of amino acid production, Progress in industrial microbiology,* vol. **24**, [Aida, K.; Chibata, I.; Nakayama,K.; Takinami, K.; Yamada, H. (Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p. 152-172.

Tramper, J.; Luyben, K. C. A. M. (1984) 'Classical chemistry versus modern biotechnology. Biotechnological production of D(-)-4-hydroxy-phenyl-glycine.' *PT-Procestech.,* **39(4)**, 61-7 (Dutch) [C.A.101:149699].

Triadan, H. (1968) *Ober die parodontate Nebenwirkung der chronischen Hydantoinbe handlung Huthig*, Heidelberg.

Tripathi, C. K. M.; Bihari, Vinod; Tyagi, R. D. (2000) 'Microbial production of D-amino acids.' *Process Biochem. (Oxford)*, **35(10)**, 1247-1251 [C.A. 133: 206812].

Tsushima, Tadahiko; Ishihara, Shoichi; Fujita, Yusuke (1990) 'Fluorine-containing amino acids and their derivatives. 9. Synthesis and biological activities of difluoromethylhomocysteine.' *Tetrahedron Lett.*, **31(21)**, 3017-18 [C.A.113: 212593].

Vadehra, D.V., Wallace, D.L.; Harman, L.C. (1965) "Comparison of methods of extracting intracellular protease from bacteria". *Appl. Microbiol.* **13(6)**, 1010-1013.

Van Gennip, A. H.; Abeling, N. G. G. M.; Stroomer, A. E. M.; Van Lenthe, H.; Bakker, H. D. (1994) 'Clinical and biochemical findings in six patients with pyrimidine degradation defects.' *J. Inherited Metab. Dis.,* **17(1)**, 130-2 [C.A.121: 80294].

Van Gennip, Albert H.; De Abreu, Ronney A.; Vreken, Peter; Van Kuilenburg, Andre B. P. (1998) "Clinical and biochemical aspects of dihydropyrimidinase deficiency.' *Adv. Exp. Med. Biol.*, **431**(Purine and Pyrimidine Metabolism in Man IX, 1998), 125-128 [C.A.129: 288375].

Van Kuilenburg, A. B. P. Van; Van Lenthe, H.; Van Gennip, A. H. (1999) 'Radiochemical assay for determination of dihydropyrimidinase activity using reversed-phase high-performance liquid chromatography.' *J. Chromatogr., B: Biomed. Sci. Appl.* **729(1 + 2)**, 307-314.

Vercauteren, Ronny Leontina Marcel; Nguyen, Van Sau; Heylen, An Amanda Jules (2001) 'Method for enzymatically hydrolysing mixtures of isomaltulose and trehalulose.' *Eur. Pat. Appl. EP* **1153930 A1** 14 Nov 2001, 12 pp. [C.A.135: 356843].

Vosberg, H.; Hoffman-Berling, H. (1971) 'DNA synthesis in nucleotide-permeable *E. coli* cells. I. preparation and properties of the ether treated cells.' *J. Mol. Biol.* **58**, 739-753.

Wada, M. (1934) *Proc. Imp. Acad. Japan* **10**, 17.

Wagner, Fritz; Voelkel, Dirk; Bommarius, Andreas; Drauz, Karlheinz (1994) 'Microorganisms, their use and method of production of L-a-amino acids.' *Eur. Pat. Appl*. *EP* **625571 A2** 23 Nov 1994, 18 pp. [C.A.122: 104046].

Wakamoto, A.; Takahashi, O.; Furuhashi, K. (1989) 'Manufacture of optically pure amino acids by enzymic hydrolysis of 2-amino nitrile compounds'. *Eur. Pat. Appl. EP* **332379 A2** 13 Sep 1989, 18 pp. [C.A.112: 75347].

Waki, Hiroshi; Higuchi, Seiichiro; Tanaka, Shigeyuki (1980) 'Raman circular intensity differential spectra of some chiral compounds with related structures.' *Spectrochim. Acta, Part A*, **36A(7)**, 659-64 [C.A.93: 238063]
Wallach, D.P.; Grisolia, S. (1957) 'The purification and properties of hydropyrimidine hydrase.' *J. Biol. Chem.* **226**, 277-288.

Wallenfels, K.; Bender, H.; Roched, J.R. (1966) 'Pullulanase from *Aerobacter aerogenes*; production in a cell-bound state. Purification and properties of the enzyme.' *Biochem. Biophys. Res. commun.* **22(3)**, 254-261

Wang, Hasting; Potter, Charles R. (1976) 'Resolution of optically active compounds'. *Brit. GB* **1455710** 17 Nov. 1976, 6 pp. [C.A.86: 155969].

Wang, D.I.C.; Cooney, C.L.; Domain, A.L.; Dunnill,P.; Humphrey, A.E.; Lilly, M.D.[ed.](1979) 'Enzyme kinetics and immobilization' in ' *Fermentation and enzyme technology',* p.311-338, John Wiley & Sons, New York

Ware, E. (1950) *Chem. Rev*. **46**, 403.

Wasserman, Harry H.; Lipshutz, Bruce H. (1978) 'b-Lactams and intermediates for their manufacture.' *Ger.Offen. DE* **2747494** 3 May 1978, 44 pp. (German) (Germany) [C.A.89: 129384].

Weber, Jennifer Elizabeth; Durham, Donald Richard (1996) 'Hydantoinase from *Agrobacterium tumefaciens* and its use for the preparation of optically pure N-carbamyl-D-amino acids from racemic hydantoins.' *Eur. Pat. Appl. EP* **739978 A2** 30 Oct. 1996, 17 pp.

Weitzman, P.D.J. (1973) 'Behavior of enzymes at high concentration. Use of permeabilised cells in the study of enzyme activity and its regulation.' *FEBS-Lett.* **32**, 247-250.

West, Thomas P. (1997) 'Reductive catabolism of uracil and thymine by *Burkholderia cepacia*.' *Arch. Microbiol*., **168(3)**, 237-239.

Westhoff, Doris; Kamp, Gunter (1997) 'Glyceraldehyde 3-phosphate dehydrogenase is bound to the fibrous sheath of mammalian spermatozoa.' *J. Cell Sci.*, **110(15)**, 1821-1829 [CA 127:232502].

Whitaker, John R. (1994) 'Enzyme purification.' *Food Sci. Technol.* (N. Y.), **61**, 63-117

Wilchek, M.; Miron, T. and Kohen, J. (1984) 'Affinity chromatography,' in *Methods in Enzymology*, vol.**104,** Jakoby, W.B. ed., Academic Press Inc, Orsando, Florida, pp.3-55.

Wiseman, A.; Jones, P.R. (1971) 'Assay-increment and solubilization of alpha-glucosidase and invertase in disruption of Brewers' yeast" *J. Appl. Chem. Biotechnol.* **21**, 26-28.

Woldringh, C.L. (1970) 'Lysis of cell membrane of *Escherichia coli* K12 by ionic detergents.' *Biochim. Biophys. Acta* **224**, 288-290.

Xiu, Zhilong; Su, Zhiguo (1993) 'Simultaneous cell disruption and aqueous two-phase extraction for separation of alcohol dehydrogenase from yeast.' *Shengwu Gongcheng Xuebao*, **9(4)**, 342-7 (Chinese) [C.A.122: 49706].

Xiu, Zhilong; Su, Zhiguo (1994)'Cell disruption kinetics in horizontal bead mill.' *Huagong Xuebao (Chin. Ed.)*, **45(1)**, 51-7 (Chinese) [C.A. 120: 215450]

Xiu, Zhilong; Jiang, Wei; Su, Zhiguo (1994) 'Achievements and future trends on disruption of microbial cells.' *Huagong Jinzhan,* **(1)**, 15-21 (Chinese) [C.A.121: 132236].

Xu, Jien (1984) 'Applications of amino acids.' *Yiyao Gongye,* **(3),** 31-5 (Chinese) [C.A.101:34555].

Xu, Guorong; West, Thomas P. (1994) 'Characterization of dihydropyrimidinase from *Pseudomonas stutzeri'. Arch. Microbiol.*, **161(1)**, 70-4.

Yamada, Hideaki; Takahashi, Satomi; Sumino, Kazushige; Yoneda, Koji (1978a) 'Production of dihydropyrimidinase'. *Jpn. Kokai Tokkyo Koho JP* **53136583** 29 Nov 1978 Showa, 10 pp. (Japanese) (Japan) [C.A.90: 136254].

Yamada, H.; Takahashi, S.; Yoneda, K. (1978b) 'D-N-carbamoyl-a-amino acids'. *Ger. Offen. DE* **2757980** 6 Jul 1978, 34 pp. (German) (Germany) [C.A. 90: 4442].

Yamada, H.; Takahashi, S.; Kii, Y.; Kumagai, H.(1978c) 'Distribution of hydantoin hydrolysing activity in microorganisms.' *J. Ferment. Technol.* **56**, 484-491.

Yamada, Hideaki; Takahashi, Satomi; Sumino, Kazunari; Yoneta, Koji (1979) 'Microbial production of hydantoinase preparation.' *Jpn. Kokai Tokkyo Koho JP* **54084086** 4 Jul 1979 Showa, 10 pp. (Japanese) (Japan) [C.A.91: 138875].

Yamada, Hideaki; Shimizu, Sakayu; Yoneda, Koji (1980a)'Synthesis of D-amino acids using hydantoinase of microorganism. Production of p-D-hydroxyphenylglycine.' *Hakko to Kogyo,* **38(10)**, 937-46 (Japanese) [C.A.94: 63680].

Yamada, Hideaki; Shimizu, Sakayu; Shimada, Hiroaki; Tani, Yoshiki; Takahashi, Satomi; Ohashi, Takehisa (1980b) 'Production of D-phenylglycine-related amino acids by immobilized microbial cells.' *Biochimie*, **62(5-6)**, 395-9.

Yamada, Masahiko; Kamyama, Noboru; Yasohara, Yoshihiko; Takahashi, Satomi; Hasegawa, Junzo (1994) 'Manufacture of optically active b-amino acids with bacterial dihydropyrimidinase.' *Jpn. Kokai Tokkyo Koho JP* **06261787 A2** 20 Sep 1994 Heisei, 9 pp. (Japan) [C.A.122: 54174].

Yamada, Hideaki (1998) 'Screening of novel enzymes for the production of useful compounds.' *Stud. Org. Chem. (Amsterdam)*, **53**(New Frontiers in Screening for Microbial Biocatalysts), 13-17 [C.A.129: 105736].

Yang, Liu; Sun, Wanru (2001) 'Recent progress in study of hydantoinase.' *Shengwu Gongcheng Jinzhan* **21(3)**, 13-16, 7 (Chinese) [C.A.135: 328583].

Yin, Bang-Ding; Chen, Yi-Chuan; Lin, Sung-Chyr; Hsu, Wen-Hwei (2000) 'Production of D-amino acid precursors with permeabilized recombinant *Escherichia coli* with D-hydantoinase activity.' *Process Biochem. (Oxford)*, **35(9)**, 915-921.

Yokoyama, Shinichi; Inoue, Kuniyo (2000) 'Pre-treatment method using an alkaline solution for measuring bacteria.' *Jpn. Kokai Tokkyo Koho JP* **2000014398 A2** 18 Jan 2000, 5 pp. (Japanese) (Japan) [C.A. 132: 90370].

Yokozeki, K.; Nakamori, S.; Eguchi, C.; Yamada, K.; Mitsugi, K. (1987) 'Screnning of microorganisms producing D-p-hydroxyphenylglycine from D,L-5-(p-hydroxyphenyl)hydantoin.' *Agri. biol. Chem*. **51**, 355.

Yokozeki K., Nakamori S.,Yamanaka S., Eguchi C., Mitsugi K. and Yoshinaga F. (1987a) 'Optimal condition for the enzymatic production of D-amino acids from the corresponding 5-substituted hydantoins.' *Agric. Biol. Chem.* **51,** 715-719.

Yoshida, H. (1986) 'Arginine, citrulline and ornithine'. In: *Biotechnology of amino acid production, Progress in industrial microbiology,* vol. **24**, [Aida, K.; Chibata, I.; Nakayama,K.; Takinami, K.; Yamada, H. (Eds.)], Kodansha Ltd., Tokyo and Elsevier Science Publishers B.V., Amstedam, p. 131-143.

Young-Jin Pharmaceutical Industries, Ltd., S. Korea (1991) 'Novel Streptomyces and its use for manufacturing hydantoinase. *Jpn. Kokai Tokkyo Koho JP* **03067580 A2** 22 Mar 1991 Heisei, 8 pp. (Japan) [C.A. 115:69944].

Yungjin Pharmaceutical Industry Co., Ltd., S. Korea (1992) 'Hydantoinase production by a new *Streptomyces* isolate.' *Neth. Appl. NL* **9001680 A** 17 Feb 1992, 19 pp. (Netherlands) [C.A.116: 212991].

Zhang, Mingjun; Li, Jinghua; Zhang, Yankun; Li, Shuguang; Du, Jiangying; Li, Bo; Yu, Xingju (1999) 'Membrane enzymic reactor for producing D-p-hydroxyphenylglycine.' *Faming Zhuanli Shenqing Gongkai Shuomingshu CN*

1210890 A 17 Mar 1999, 6 pp. (Chinese) (People's Republic of China) [C.A.132: 331343].

Zhang, Yankun; Zhang, Mingjun; Li, Jinghua; Li, Shuguang; Yu, Xingju (2000) 'Kinetics of enzymic production of D-p-hydroxyphenylglycine by *Pseudomonas putida* sp. 2262 cell.' *Huagong Xuebao (Chin. Ed.)*, **51(1)**, 57-62 (Chinese) [C.A. 132: 307288].

List of publications

1. **Gaikwad, B.G**.; Nene, S. And Kulkarni, B.D. (2002) 'Studies on the production of hydantoinase using *Pseudomonas* strain'. (Communicated to Indian Journal of Biotechnology).

2. **Gaikwad, B.G**.; Nene, S. And Kulkarni, B.D. (2002) 'Extraction of hydantoinase from cells. 1. Use of solvents for rupturing or permeabilizing cells'. (Paper is accepted for publication in Asian Journal of Biotechnology, Microbiology and Environmental Sciences).

3. **Gaikwad, B.G**.; Nene, S. and Kulkarni, B.D. (2003) 'Extraction of hydantoinase from cells. 2. Use of solvents and detergents for rupturing or permeabilizing cells'. (Under preparation for Biotechnology and Applied Biochemistry).

4. **Gaikwad, B.G.**; Nene, S. and Kulkarni, B.D.(2003) 'Purification of hydantoinase from *Pseudomonas* strain'. (Under preparation for Applied Biochemistry and Biotechnology).

5. **Gaikwad, B.G**.; Nene, S. and Kukarni, B.D. (2003) 'Chiral compounds and use of hydantoinase in preparation of chiral compounds'. (Review under preparation for FEMS Microbiol. Lett.)