



UNIVERSITI PUTRA MALAYSIA

**ISOLATION OF SERRATIA MARCESCENS (ISOLATE 30) AND
PARTIAL PURIFICATION OF ITS ACRYLAMIDE-DEGRADING
AMIDASE**

NINA SUHAITY BINTI AZMI.

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PURIFICATION OF ITS ACRYLAMIDE-DEGRADING AMIDASE**

By

NINA SUHAITY BINTI AZMI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
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Abstract of thesis presented to the Senate of University Putra Malaysia in
fulfilment of the requirements for the degree of Master of Science

**PARTIAL PURIFICATION AND CHARACTERIZATION OF AMIDASE
FROM ACRYLAMIDE-DEGRADING BACTERIA STRAIN 30**

BY

NINA SUHAITY BINTI AZMI

May 2005

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A total of 217 bacterial isolates were isolated by an enrichment procedure from Malaysian soils which had been treated and non-treated with acrylamide. From the preliminary screening using MTT (3-[4,5-dimethylthiazol-2-yl]-5-diphenyltetrazoliumbromide) assay, 10 bacterial isolates were found to be capable of utilizing acrylamide as a nitrogen source. Formation of the blue colour of formazan and high absorbance were the criteria in the selection of bacteria. These ten isolates were labeled as Isolate 5, 9, 10, 15, 30, 45, 60, 110, 115 and 145. The isolates were subjected to secondary screening using MTT assay, Direct Plate Count and High Performance Liquid Chromatography (HPLC). From the results obtained, five bacterial isolates, Isolate 9, 10, 15, 30 and 60 gave the highest colony count in CFU/ mL and absorbance reading from the MTT assay. These five bacterial isolates were then selected for further of examined for their ability to degrade acrylamide by HPLC. Isolate 30 showed the best degradation of acrylamide with 99.84% degradation after 48 hours incubation in

enrichment culture with acrylamide as a substrate at a final concentration of 100 mg/L. Isolate 60 showed the second highest degradation of acrylamide at 70.53%, followed by Isolate 10 at 50.8% degradation and isolates 15 and 9 at 16.24% and 14.58% respectively. Control without bacteria accounted only 0.16% acrylamide degradation.

Isolate 30 was selected for optimization of growth media with six different parameters. These parameters were temperature, pH, different types of carbon sources, different concentrations of carbon source, different amides as the substrate and effect of different concentrations of selected amide substrate. From the results obtained, Isolate 30 showed an optimum growth temperature at 27 ° C. Its highest colony count \log_{10} cfu/ml was directly proportional to the growth of bacteria. This isolate showed the highest growth at pH 7.5 where this type of bacteria grew well near neutrality and slightly alkaline media. For the carbon sources optimization, glucose was selected due to the high colony count. Isolate 30 was found to grow best at the glucose concentration of 1%. Among the amide substrates, acrylamide was found to be the best sole nitrogen source which can support bacterial growth in enrichment cultures and the bacteria showed the highest colony count when 400 mg/L acrylamide was provided.

From the macroscopic and microscopic observations of Isolate 30, the bacteria was gram-negative rod, might become differentiated into a convex, pigmented and relatively opaque centre and an effuse, colourless, almost transparent periphery with an irregular crenated edge. Biochemical tests using Microbact 24E

(12A + 12B) showed that the Isolate 30, which had been isolated from Tanjung Karang, Selangor gave 99.83% similarity to *Serratia marcescens*.

Amidase (EC 3.5.1.4) produced by *Serratia marcescens* strain 30 was partially purified and characterized. The purification procedure used, including ion-exchange chromatography, Mono-QTM strong-anion exchanger, ultrafiltration and gel filtration, yielded a partially purified amidase fraction having an overall purification factor of 13.83 fold and a yield of 48.33%. Indophenol blue method was used for the amidase assay and amidase characterization studies was done to determine the K_m and V_{max} value, the effect of temperature and pH on amidase activity and the effect of heavy metals on amidase activity. K_m values for acrylamide were determined by incubating amidase at 50° C with various concentrations of acrylamide (0.25 to 20mM) in phosphate buffer (50mM; pH 7.5). By using non-linear regression analysis, the K_m and V_{max} values were 0.9376mM acrylamide and 60.92 μ mol ammonia disappeared/min respectively. The amidase exhibited maximal activity at 50° C and pH value at 7.5. The result of effects of heavy metals on amidase activity showed that $AgNO_3$ and $CuCl_2$ inhibited amidase activity while HBO_3 enhanced amidase activity. The enzyme is a monomer with an apparent molecular weight of 111 kDa.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**PENULENAN SEPARA DAN PENCIRIAN AMIDASE DARIPADA
AKRILAMIDA YANG BOLEH DIURAI OLEH BAKTERIA STRAIN 30**

OLEH

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Sejumlah 217 isolat bacteria telah dipencilkan menggunakan prosedur pengayaan daripada tanah-tanah di Malaysia yang telah dirawat dan tidak dirawat dengan akrilamida. Daripada keputusan penyaringan permulaan menggunakan ujian MTT, 10 isolat bacteria berupaya menggunakan akrilamida sebagai sumber nitrogen. Pembentukan warna biru formazan dan bacaan absorban tertinggi adalah kriteria dalam pemilihan bacteria. Sepuluh isolat ini adalah Isolat 5, 9, 10, 15, 30, 45, 60, 110, 115 dan 145. Isolat-isolat ini telah dilakukan penyaringan kedua menggunakan asai MTT, kaedah pengiraan koloni bacteria dan "High Performance Liquid Chromatography (HPLC)". Daripada keputusan yang diperolehi, lima isolat bacteria, Isolat 9, 10, 15, 30 dan 60 telah memberi bacaan tertinggi dalam pengiraan koloni bacteria dalam CFU/mL dan bacaan absorban daripada asai MTT. Kelima-lima isolat bacteria dipilih untuk analisis lanjutan degradasi akrilamida menggunakan HPLC. Isolat 30 menunjukkan degradasi akrilamida tertinggi dengan 99.84% degradasi selepas 48 jam pengermanan di dalam larutan pengayaan dengan akrilamida sebagai substrat dan kepekatan akrilamida terakhir adalah 100 mg/L. Isolat 60 menunjukkan degradasi kedua tertinggi akrilamida dengan 70.53%, diikuti dengan 50.8% degradasi

oleh Isolat 10, 16.24% dan 14.58% oleh Isolat 15 dan 9. Pengawalan tanpa bakteria menunjukkan hanya 0.16% degradasi akrilamida.

Isolat 30 dipilih untuk lanjutan optimasi media pertumbuhan termasuk enam parameter berbeza. Parameter-parameter ini adalah suhu, pH, sumber karbon berbeza, substrat amida berbeza dan kesan kepekatan berbeza bagi substrat amida terpilih. Daripada keputusan yang diperoleh, Isolat 30 menunjukkan suhu optimum pada 27°C, koloni bakteria tertinggi \log_{10} CFU/mL adalah berkadar kepada pertumbuhan bakteria. Isolat ini menunjukkan pertumbuhan bakteria tertinggi pada pH 7.5 di mana bakteria tumbuh dengan baik berhampiran sifat neutral dan sedikit media beralkali. Untuk optimasi sumber karbon, glukosa telah dipilih berdasarkan bacaan optimum koloni bakteria \log_{10} CFU/mL. Bakteria strain 30 telah dijumpai tumbuh paling baik pada kepekatan glukosa 1%. Di kalangan substrat amida, akrilamida telah ditemui sebagai satu-satunya sumber nitrogen terbaik di mana pertumbuhan bakteria dapat disokong di dalam media pengayaan dan bakteria menunjukkan pengiraan koloni bakteria tertinggi apabila 400 mg/L akrilamida dibekalkan.

Daripada pemerhatian makroskopik dan mikroskopik ke atas Isolat 30, bakteria ini adalah gram negatif berbentuk rod, berupaya membeza kepada konveks, berpigmen dan agak legap di tengahnya dan efusi, tidak berwarna, kebanyakan jernih di pinggir dengan sisi berlekuk. Ujian Biokimia menggunakan Microbact 24E (12A+B) menunjukkan Isolat 30, yang dipencilkan dari Tanjung Karang, Selangor memberi 99.83% persamaan dengan bakteria daripada *Serratia marcescens*.

Amidase (EC 3.5.1.4) yang dihasilkan oleh *Serratia marcescens* strain 30 telah dilakukan penulenan separa dan pencirian. Prosedur penulenan yang digunakan termasuk kromatografi penukar ion, penukar anion- kuat Mono-QTM, penurasan ultra dan penurasan gel menghasilkan fraksi amidase separa tulen yang mempunyai keseluruhan factor penulenan melebihi 13.83 ikatan dan kadar hasil 48.33%. Kaedah “Indophenol blue” digunakan untuk kajian asai dan pencirian amidase telah dijalankan untuk kajian pencirian amidase bagi menentukan K_m dan V_{max} , kesan suhu dan pH ke atas aktiviti amidase dan kesan logam berat ke atas aktiviti amidase. Nilai K_m untuk akrilamida dapat ditentukan dengan eraman amidase pada suhu 50°C dengan kepekatan akrilamida pelbagai (0.25 hingga 20 mM) di dalam penimbal fosfat (50mM; pH 7.5). Dengan menggunakan analisis regresi tidak linear, nilai K_m dan V_{mak} adalah 0.9376 mM akrilamida dan 60.92 μ mol ammonia dihasilkan/ min. Kemunculan aktiviti amidase yang maksimum adalah pada 50° C dan nilai pH 7.5. Daripada keputusan kesan logam berat pada aktiviti amidase menunjukkan $AgNO_3$ dan $CuCl_2$ merencat aktiviti amidase manakala HBO_3 meningkatkan aktiviti amidase. Enzim ini adalah monomer yang muncul dengan berat molekul 111 kDa.

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I certify that an Examination Committee met on 24th May 2005 to conduct the final examination of Nina Suhaity Azmi on her Master of Science thesis entitled "Isolation of *Serratia marcescens* (Isolate 30) and Partial Purification of its Acrylamide-Degrading Amidase" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



NINA SUHAITY BINTI AZMI

Date: 19th July 2005

TABLE OF CONTENTS

	Page
ABSTRACT	ii
ABSTRAK	v
ACKNOWLEDGEMENT	viii
APPROVALS	ix
DECLARATION	xi
LIST OF TABLES	xvi
LIST OF FIGURES	xvii
LIST OF ABBREVIATIONS	xx
 CHAPTER	
1 INTRODUCTION	1
 2 LITERATURE REVIEW	4
2.1 Acrylamide	4
2.2 Physical and chemical properties of acrylamide	4
2.3 The uses of acrylamide and polyacrylamide	6
2.4 Production of acrylamide	12
2.5 Acrylamide toxicity	15
2.6 Acrylamide as a pollutant	20
2.7 Biodegradation	23
2.8 Amidase	29
2.9 Amide hydrolysis	32
2.10 Amide transfer activity	34
2.11 Ping Pong Bi Bi Reactions	35
2.12 Enzyme purification	38
 3 METHODOLOGY	45
3.1 Equipments	45
3.1.1 Chemicals	45
3.1.2 Phosphate Buffered Medium (PBM) or Enrichment culture	45
3.1.3 Nutrient Agar (NA)	46
3.1.4 Nutrient Broth (NB)	46
3.2 Soil Sampling	46
3.3 Isolation and Culture technique	47
3.4 Measurement of Growth	47
3.4.1 Plate Count	48
3.4.2 Spectrophotometry	49
3.5 MTT Assay	50
3.6 Screening of acrylamide degraders	51
3.6.1 Preliminary screening of acrylamide degraders	52
3.6.2 Secondary screening of acrylamide degraders	53



3.6.3	Screening of the best acrylamide degrader using High Performance Liquid Chromatography (HPLC)	54
3.7	Bacteria Identification	55
3.7.1	Gram staining method	56
3.7.2	Microbact kit and the principle of each reaction	58
3.7.2.1	Lysine test	59
3.7.2.2	Ornithine test	60
3.7.2.3	H ₂ S test	60
3.7.2.4	Glucose, mannitol and xylose test	60
3.7.2.5	ONPG test	60
3.7.2.6	Indole test	61
3.7.2.7	Urease test	61
3.7.2.8	VP test	61
3.7.2.9	Citrate test	61
3.7.2.10	TDA test	62
3.7.2.11	Gelatin test	62
3.7.2.12	Malonate test	62
3.7.2.13	Inositol, sorbitol, rhamnose, sucrose, lactose, 60 arabinose, adonitol, raffinose and salicin test	63
3.7.2.14	Arginine test	63
3.8	Growth and maintenance of bacteria culture	63
3.8.1	Microbank	64
3.9	Growth optimization of Isolate 30	64
3.9.1	Optimization of pH	65
3.9.2	Temperature optimization	65
3.9.3	Carbon source optimization	65
3.9.4	The effect of concentration of carbon sources on growth of Isolate 30	66
3.9.5	The effects of different amides on growth of Isolate 30	66
3.9.6	The effects of different concentrations of amide on bacterial growth	66
3.10	Biodegradation of acrylamide by Isolate 30 after growth Optimization	67
3.11	Protein determination	68
3.12	Enzyme assay	69
3.13	Standard curve for ammonia	69
3.14	Partial purification of acrylamide-degrading enzyme	71
3.14.1	Preparation of crude enzyme	72
3.14.2	Ion-exchange chromatography using DEAE cellulose	73
3.14.3	Partial purification using Mono-Q TM strong anion Exchanger	75
3.14.4	Ultrafiltration	76
3.14.5	Partial purification using Superose TM gel filtration	77
3.14.6	Kinetic study of partially purified amidase after ion-exchange chromatography	78
3.14.6.1	Effect of pH on enzyme activity	78
3.14.6.2	Effect of temperature on enzyme activity	79
3.14.6.3	Effect of heavy metals on enzyme activity	79
3.15	Molecular weight determination	79

3.15.1	Gel filtration	79
3.15.2	SDS polyacrylamide gel electrophoresis	81
4.0	RESULTS	85
4.1	Soil sampling, isolation and culture techniques	85
4.2	Screening of acrylamide degraders	87
4.2.1	Preliminary screening of acrylamide degraders	87
4.2.2	Secondary screening of acrylamide degraders	90
4.3	Bacteria Identification	96
4.3.1	Colony examination of Isolate 30	96
4.3.2	Gram staining method	98
4.3.3	Microbact kit	99
4.4	Optimization of growth media	101
4.4.1	Temperature optimization	101
4.4.2	pH optimization	102
4.4.3	The optimization of carbon sources	103
4.4.4	The effect of glucose at different concentrations	104
4.4.5	The effects of different amides on growth of Isolate 30	105
4.4.6	The effects of different concentrations of acrylamide on bacterial growth	106
4.5	Partial purification of acrylamide-degrading amidase	108
4.6	Amidase characterization studies	113
4.6.1	Determination of Km and Vmax for amidase	113
4.6.2	Effects of different temperatures on amidase activities	115
4.6.3	Effects of different pH on amidase activity	117
4.6.4	Effects of heavy metals on amidase activity	118
4.6.5	Molecular weight determination	119
4.6.5.1	Molecular weight from gel filtration on Superose™	119
4.6.5.2	SDS polyacrylamide gel electrophoresis	120
5.0	DISCUSSIONS	125
5.1	Soil sampling, isolation and culture techniques	125
5.2	Screening of acrylamide degraders	129
5.2.1	Preliminary screening of acrylamide degraders	129
5.2.2	Secondary screening of acrylamide degraders	130
5.3	Bacteria identification	133
5.3.1	Colony examination of Isolate 30	133
5.3.2	Gram staining method	133
5.3.3	Microbact kit	135
5.4	Optimization of growth media	138
5.4.1	Temperature optimization	138
5.4.2	pH optimization	142
5.4.3	The optimization of carbon sources	144
5.4.4	The effect of glucose at different concentrations	146
5.4.5	The effects of different amides on growth	149
5.4.6	The effects of different concentrations of acrylamide on bacterial growth	150
5.5	Partial purification of acrylamide-degrading amidase	151

5.6	Amidase characterization studies	154
5.6.1	Determination of amidase K_m and V_{max}	154
5.6.2	Effects of different temperatures on amidase activity	155
5.6.3	Effects of different pH on amidase activity	156
5.6.4	Effects of heavy metals on amidase activity	157
5.6.5	Molecular weight determination	158
	5.6.5.1 Molecular weight from gel filtration on Superose™	158
	5.6.5.2 SDS polyacrylamide gel electrophoresis	159
6.0	CONCLUSION	160
	REFERENCES	162
	APPENDICES	179
	BIODATA OF THE AUTHOR	191

LIST OF TABLES

Table		Page
1	Industrial application of anionic polyacrylamide	10
2	Amidase purification	44
3	Locations of sample collection, pH, GPS location and temperatures of the soil and number of bacteria isolated from each location	86
4	The absorbance readings of seven isolates from the MTT assay during the preliminary screening	88
5	The absorbance reading of five isolates from the MTT assay during the secondary screening	91
6	Microscopic and macroscopic observations of Isolate 30	97
7	Biochemical test result using Microbact 24E(12A+12B) kit	99
8	Amidase purification table	112

LIST OF FIGURES

Figures		Page
1	Chemical structures of polyacrylamide (A) and acrylamide (B)	5
2	Comparative flowsheet for the microbial and conventional processes	14
3	Proposed pathway for mineralization of acrylamide by soil bacteria	28
4	Fold of amidase	30
5	Chemical and enzymatic pathways for nitrile hydrolysis	33
6	Ping Pong bisubstrate reaction	36
7	Studies of <i>Mycobacterium avium</i> catalysed hydroxamate formation from butyric or valeric acid and hydroxylamine	37
8	General reaction pathway of the combined hydrolysis and acyl-transfer activity of amide-hydrolysing enzymes	37
9	Large scale growth of Isolate 30	72
10	Schematic representation of gel filtration	80
11	MTT assay using 96 well microtiter plates	89
12	Acrylamide degradation by five isolates in acrylamide enrichment culture	93

13	Bacterial growth curve using bacterial count method (CFU/mL)	94
14	Biodegradation of acrylamide by bacteria Isolate 30	95
15	Colonies formed on agar-containing medium on a Petri plate after 24 hours incubation period at room temperature	97
16	Photomicrograph of bacteria Isolate 30	98
17	Result of biochemical test using Microbact Identification kit 24E (12A+12B) for bacteria strain 30	100
18	The effect of temperatures for the growth of <i>Serratia marcescens</i> strain 30	101
19	The effect of pH on growth of Isolate 30	102
20	A study of different carbon sources to the growth of <i>Serratia marcescens</i> strain 30	103
21	The effect of glucose concentration to the growth <i>Serratia marcescens</i> strain 30	104
22	The effect of amides (substrate) to the growth <i>Serratia marcescens</i>	105
23	The effect of different concentrations of acrylamide (substrate) on the growth of <i>Serratia marcescens</i> strain 30	107
24	Amidase elution profile using ion-exchange chromatography (DEAE cellulose)	109
25	Amidase elution profile using Mono-Q™ strong anion exchanger column	110

26	Amidase elution profile using Superose™ gel filtration column	111
27	Michaelis-Menten curve for amidase with acrylamide as the substrate	114
28	Effect of assay temperatures on amidase activity with acrylamide as the substrate	116
29	Effect of different pH on amidase activity with acrylamide as the substrate	117
30	Effect of heavy metals on amidase activity	118
31	The molecular weight of standard proteins versus their retention time using Superose™ column bed	119
32	Molecular weight determination on Superose™ column bed	121
33	Relationship between the molecular weight of proteins and retention time during gel filtration in Superose™	122
34	Polyacrylamide gel electrophoresis of amidase after crude extraction	123
35	Polyacrylamide gel electrophoresis of amidase after gel filtration	124

LIST OF ABBREVIATIONS

CFU	colony forming unit
DDT	dichlorodiphenyltrichloroethane
GPS	Global Positioning System
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
K_m	Michaelis Menten Constant
MARDI	Malaysian Agricultural Research and Development Institute
mAu*s	milliabsorbance unit
MTT	3-[4,5-dimethylthiazol-2-yl]-,5- diphenyltetrazoliumbromide
PAM	Polyacrylamide
PBM	Phosphate buffered medium
V_{max}	maximum initial velocity
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Manufactured chemicals present in our environment present possible health hazards. The United States Environmental Protection Agency (USEPA) categorized chemicals by their known or suspected effects as carcinogens, mutagens or as developmental, reproductive, acute, chronic or neurological toxins, and many chemicals fall into more than one of these categories. Over 10,000 pounds of carcinogens, agents known to increase cancer risks, have been released into the soil of more than 50% of the United States. Obviously, exposure to these and other chemicals released into the environment can have both acute and cumulative effects on health (Skipper and Turco, 1995).

Acrylamide is an important industrial chemical, used primarily in the production of polymers and copolymers. The widespread use and indiscriminate discharge of acrylamide and polyacrylamide has led to the contamination of soil (Lande *et al.*, 1979; Nawaz *et al.*, 1992) and plants (Nishikawa *et al.*, 1978; Nawaz *et al.*, 1992).

There is abundant evidence, however, that most chemicals are degraded or dissipated in our not-so-fragile environment, despite efforts by environmental ethicists and the media to convince us otherwise (Skipper and Turco, 1995). But for most scientists



involved in reduction of environmental contaminants, there is indeed room for improvement in virtually all spheres.

The desire to minimize health effects has led to large expenditures to combat pollution. Bioremediation is one technology which shows great promise in terms of cost-effective pollution abatement. Bioremediation is the use of microorganisms or plants to detoxify an environment, mostly by transforming or degrading pollutants. The utility of bioremediation in the degradation of pollutants in the environment has been successfully demonstrated for several chemical compounds. Biodegradation is an important removal mechanism for chemicals released in large quantities to aquatic and terrestrial environments. It results in a decrease in the mass or load of chemicals present in the environment and is important in preventing the accumulation and persistence of chemicals (Shimp *et al.*, 1990; Babu *et al.*, 1996).

Biodegradation is the major route of removal acrylamide from soils. In aerobic soils, the chemical is 74- 94 % degraded in 14 days. Depending on the soil type, estimated half- lives range from 21- 36 hours. This study attempts to isolate and identify the bacteria, which utilize acrylamide as a carbon source. Microbial amidases (amidohydrolase, EC 3.5.1.4) catalyze the hydrolysis of aliphatic amides to their corresponding carboxylic acids and ammonia. Although numerous microorganisms catabolize aliphatic amides, acrylamide, because of its inhibitory effect on sulfhydryl proteins, inhibits their growth. Therefore, few microorganisms capable of degrading

acrylamide have been isolated (Nawaz *et al.*, 1994).

The objectives of this study are firstly, to isolate and screen acrylamide-degrading bacteria from previously treated and untreated Malaysian soil. Secondly, to identify and characterized the best acrylamide degrading bacterium by measuring acrylamide degradation by the bacterium in the shortest period and lowest residue left. Thirdly, is to partially purify the enzyme(s) involved in acrylamide degradation from the chosen bacterium.

CHAPTER 11

LITERATURE REVIEW

2.1 Acrylamide

Acrylamide (C.A.S. 79-06-1) is an odorless, free flowing white crystalline powder used as a chemical intermediate in the production and synthesis of polyacrylamides. These high-molecular weight polymers can be modified to develop nonionic, anionic, or cationic properties for specific uses. Acrylamide, an aliphatic amide, is extensively used in numerous industrial processes.

Global production of acrylamide has been estimated to be over 200,000 tons (ca. 180, 000 metric tons) (Nawaz *et al.*, 1994). Synonyms for acrylamide are acrylamide monomer, acrylic amide, propenamide, 2-propenamide, acrilamida (DOT Spanish), acrylamide (DOT French), acrylamide solution , acrylic acid amide (50%), acrylic amide (50%), ethylene carboxamide, ethylenecarboxamide, propenamide (50%), propenoic acid, amide, RCRA Waste Number U007, UN 2074, and vinyl amide (WHO, 1985).

2.2 Physical and chemical properties of acrylamide

Acrylamide occurs in crystalline form and in aqueous solution. The solid monomer is a colorless to white, free flowing crystal that is soluble in water, methanol, ethanol, dimethyl ether, and acetone, are insoluble in benzene and heptane. The chemical formula of acrylamide is C_3H_5NO and its molecular