MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

As mentioned earlier, the present studies were concerned with the isolation and characterization of an organism from petrochemical waste waters, which was capable of utilizing acrylonitrile. The capacity of this organism to utilize acrylonitrile as a carbon and nitrogen source was evaluated. Further the metabolic pathway of acrylonitrile was deduced from studies using resting cells and cell free extracts. The enzymes involved (nitrile hydratase and amidase were purified and characterized and their genetic basis was determined. The parameters determined during the studies were:-

a) Isolation and characterization of bacteria capable of utilizing acrylonitrile as a sole source of carbon and nitrogen, viz 1) Growth 2) Production of ammonia 3) Change of pH.

b) Metabolism in resting cells:-

1) Rate of utilization of nitriles, substrate specificity, optimum time, temperature, pH and the product formed.

c) Studies in cell free extracts

1) Characterization of enzyme with respect to optimum time, temperature, pH, substrate specificities, activators, inhibitors and localization of enzymes.

2) Enzymes involved in ammonia assimilation

3) Enzymes involved in utilization of acrylic acid.

4) Purification of the enzymes; nitrilase and amidase.

c) Isolation and characterization of plasmids, purification restriction analysis and curing studies.

d) Accumulation of acrylamide by intact cells and cells entrapped in poly acrylamide gel matrix.

2.1 MATERIALS

The common chemicals used in the experiments were of research grade purity and were obtained from British Drug House (India) Ltd.,. Sarabhai chemicals, Loba, Sisco Research Laboratories Ltd. Glaxo (Qualigens) The sources of other chemicals are given in Table 7.

The reagents and standards used in the experiments are given in the section 2.2 under materials.

2.1.1 Composition of modified basal salt medium (Willets and Child, 1978)

Composition	gms \ liter
K_2HPO_4	2
KH_2PO_4	1
$MgSO_4 7H_2O$	0.250
NaCl	1.0
Yeast extract (1%)	1 ml

Table: 7 SOURCE OF SPECIAL CHEMICALS, ENZYMES AND REAGENTS

Acetonitrile,		Merck (Ger.)
Acrylamide,		Merck (Ger.)
Acrylonitrile,		BDH (Eng.)
ADP		Sigma
Agar,		
Agarose,		BRL
Biogel,		Pharmacia
Bisacrylamide,		
Coomassie brilliant blue R		
Coomassie brilliant blue G 250		
ß- Cyano Alanine		
Xylene Cyanol		
DEAE Cellulose		
DEAE Sephadex		
Dithio-threitol		
DTT		Sigma
Anti foam "A" emulsion Emulsion		
Ethedium Bromide		
Glutamine		
Imidazole		
Lysozyme		Bethesda Reseach Laboratories (BRL)
NADP		Sigma
NADPH		Sigma
PancreaticRNAse		Betheda Research Lab (BRL)
Plasmids	pBR 322	
	pUC 19	Bhoreinger
	Blue script	Manneheim
	pKT 231	MTCC (Chandigarh)
Porapack Q		Hewlett Packard
Standard Protein molecular wt. markers		Sigma
Restriction Enzymes :	ECO RI	
	HIND III	Bethesda research
	NOT I	Laboratories (BRL)
SDS (Sod. Dodecy. Sulph.)		Sigma
Sephadex G-50; G-75; G-100; G-150; and G 200		Pharmacia
Standards DNA molecular wt markers		Bhoeringer Manneheim
TEMED		BDH (Eng.)
TSK gel		Toyo soda co. Japan.)
a-oxoglutarate		Sigma

Micronutrient solution 1 ml

1 ml of yeast extract of 1% is added asepticaly to the medium after sterilizing it.

1 ml of micronutrient medium contains 20 ug each of

$ZnSO_4 \cdot 7H_2O$

$MnSO_4 \cdot 5H_2O$

$CaCl_2$

$FeSO_4 \cdot 7H_2O$

$CuSO_4 \cdot 5H_2O$

$(NH_4)_2MoO_4 \cdot 4H_2O$

The pH was adjusted to 7.00

This media was dispensed in 100 ml vol. in 250 ml flasks plugged with cotton and sterilized at 15 psi for 10 min.

2.1.2 Composition of Luria Broth

Composition	gm./Lt.
Peptone	10
Yeast extract	5
NaCl	10

The pH of the solution was adjusted to 7.5 with NaOH and the volumes made up with distilled water. The broth was sterilized by autoclaving at 15 psi for 15 min.

For the preparation of plates and slants 2.5% of solid agar

agar was added to it. The agar dissolved on autoclaving and after cooling to 45°C appropriate antibiotics of acrylonitrile was added to it, and poured in plates or slants as required.

2.1.3 Composition of Acrylonitrile and Acrylamide agar :-

3% agar was added to the basal salt medium containing 100 mg % of yeast extract. The contents were autoclaved at 15 psi for 15 min. and on cooling to 45°C acrylonitrile or acrylamide was added to it and poured as plates or slants.

2.2 Reagents and solutions used for various assay procedures

Acrylonitrile (10 u moles) : 0.17 ml of acrylonitrile was dissolved in water and volume made upto 5.0 ml with GDW 0.1 ml of this gave 100 umoles.This was then diluted 10 times to obtain 10 umoles in 0.1 ml.

Acrylamide (10 umoles) :177 mg of acrylamide was dissolved in 5 ml GDW 0.1 ml of this solution gives 100 umoles,to obtain 10 umoles, This solution was diluted 10

times.

Acetonitrile (10 u mols) :0.53 ml acetonitrile was dissolved in 10 ml of water 0.1ml of this solution gives 100 umols 10 umols was obtained by diluting this solution 10 times and taking 0.1 ml

Acetamide (10 u mols) :590 mg Of solid actamide was dissolved in 10 ml of water 0.1 ml of this solution gives 100 u mols. To obtain 10 u mols this solution was diluted 10 times and 0.1 ml was taken.

Ammonium chloride (40 mM) :162.1 mg was dissolved in 5 ml of GDW.

Glutamine (0.03 M) :219 mg glutamine was dissolved in 5.0 ml.From this 0.2 ml was taken out for the assay.

Glutmine (0.5 mM) :5.48 mg was dissolved in 5.0 ml of GDW.

Arsenate (0.02 M) :312.5 mg was dissolved in

5.0 ml of GDW. 0.2 ml was added in assay.

ADP (4×10^{-4} M) : 6.8 mg of ADP was dissolved in 5.0 ml of GDW. 0.1 ml of this was taken in the assay system.

Hydroxylamine hydrochloride :209.0 mg was dissolved (0.06M) in 5.0 ml of GDW. 0.2 ml of this was taken in the assay.

Manganese chloride (0.03 M) :188 mg was dissolved in 5.0 ml GDW 0.2 ml of this was taken in the assay system.

NADP (0.25 mM) :9.56 mg was dissolved in 5.0 ml of GDW.

2% Acetonitrile in 0.07% H_3PO_4

(For HPLC analysis of nitriles, amides and acids) :To 100 ml milli Q™ reagent grade water, was added 7 ml of H_3PO_4 and 20 ml of acetonitrile shaken well and the volume was made upto 1000 ml in the same grade water, degassed, filterd

Sodium phenoxide reagent. :To 10 ml of 3.3 M NaOH was added 3.6 ml of redistilled liquefied phenol and the volume was made upto 100 ml with GDW, and stored in brown glass bottles at 4 °C for upto a week, in the refrigerator. The reagent turns yellow after about a week and cannot be used further.

Sodium Nitroprusside reagent :500 mg of solid sodium (0.1%) nitroprusside was dissolved in 500 ml in GDW and stored in brown bottles. This reagent is stable for months at room temperature.

Sodium hypochlorite reagent :This was made by diluting the commercially available grade of hypochlorite soln. (5% available chlorine) 25 times. This reagent is to be prepared fresh at the time of the assay.

DEAE cellulose, Sephadex :DEAE cellulose was swollen in water and activated as follows. The gel was washed with 1 N HCL and then with GDW. Then the gel was again washed with 1 N HCL and then with GDW. The gel was then packed as a slurry in a glass column and equilibrated with 2 column vol. of 100 mM KDE buffer.

4X gel buffer for Protein :16.95 gm of Electrophoresis Tris (1.5M) was dissolved in 90 ml of water and the pH was adjusted to 8.8 with conc. HCL. The volume was made upto 100 ml with GDW.

10X electrode buffer (For protein electrophoresis) : 30.25 gm of Tris base (0.25M) was dissolved alongwith 144 gms of glycine in 850 ml of water. The pH would be 8.3; if not, it was adjusted and

the volume made upto 1000 ml with GDW.

KDE buffer (Potassium phosphate,Dithio-threitol EDTA buffer (For enzyme preparation and storage)

:Stock solutions of 1 M Phosphate buffer and 0.5 M EDTA were suitably diluted DTT was added to give a concentration of 100:10:10 mM

Sephadex G-50 G-75 G-200

:Granules of and Biogel P-300 Sephadex G-50 were swollen in phosphate buffer 100 mM pH 7.0 overnight. The phosphate buffer volume was in excess of the water regain capacity of the gel. (1 gm of Sephadex G50 takes about 5 ml water). The excess water was decanted and the slurry was poured into a glass column with a glass wool plug at one end the column was equilibrated with two volumes of KDE buffer. The protein sample was applied to the column and eluted in KDE buffer. In the

	case of Bio-Gel P-300 the gel was swollen, equilibrated and eluted in the manner described earlier.
2X stacking gel buffer (protein electrophoresis)	:3 gms of tris base (0.25M) was dissolved in 90 ml GDW and the pH brought to 6.8 with HCL. The volume was then brought up to 100 ml with water.
Tris acetate EDTA (TAE) buffer for DNA electrophoresis (0.04M).	:A Stock of 50 X was prepared by dissolving 242 g of Tris base with 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0)in 1000 ml of GDW.
Tris Borate EDTA for (DNA electrophoresis) (0.089 M Tris Boric acid 0.002 M EDTA)	:5X Concentrated stock of was prepared by dissolving 54 gm Tris base, 27.5 gm 0.089 M Boric acid; to this was added 20 ml 0.5 M EDTA (pH 8.0) and the volume made upto 1000 ml with distilled water

Tris-Glycine buffer for (Protein electrophoresis in Native and SDS PAGE) (25mM Tris 250mM glycine) :Stock of 5X was prepared by dissolving 15.1 gm of Tris base, 94 gm of glycine in 1000 ml water.

Lysozyme (For DNA isolation) :Was prepared fresh to give a concentration of 5 mg/ml.

RNAse free from DNAse (For DNA isolation) :Pancreatic RNAse was dissolved in 10mM Tris, 15mM NaCl to give a final concentration of 10 mg/ml. The solution was heated at 100 ^{o}C for 15'and allowed to cool at room temperature. This was then made up to aliquots of 1 ml and stored at -20 ^{o}C.

NaCl (5M) (For DNA isolation) :29.2 gm of NaCl was dissolved in 100 ml of water and sterilized by autoclaving

SDS (Sodium dodecyl sulfate) :10 gms of SDS was added 10% to 90 ml of water, and heated to 70 ^{o}C till it dissolved. pH was adjusted to 7.2 with conc. HCl and the volume made upto 100 ml with water.

Tris (1 M) (For DNA work) :To 121 gm of tris base was added, 800 ml of water. The pH was adjusted to the desired value by adding the following amounts of conc HCl. pH 7.4 - 70 ml; 7.6 - 60 ml 8.0 - 42 ml

Tris/EDTA (TE) (For DNA)
10 mM Tris (pH 7.4) 1 mM
EDTA (pH 8.0) 0.1M NaCl
1 mM EDTA) :Prepared by diluting the stock 1 M Tris, 5 M NaCl and 0.5 M EDTA

Reagent "A" (for Plasmid lysis by method of Wheatcroft and williams) :To 5 ml of Tris HCl pH 8 was added 10ml of 0.5 M EDTA pH 8.0. To this was added 5ml of

antifoam "A" emulsion (Sigma) and 10 mg/Lt. Xylene cyanol. The volume was made upto 100 ml with distilled water. This regent was stable at room temperature for weeks.

Reagent "B" :10 ml of 10 M NaOH was mixed 10ml of 10% SDS. The volume was made upto 100ml with glass distilled water. This reagent was preferably made fresh at the time of use.

Reagent "C" :To 25 ml of melted redistilled Phenol was added 24 ml of chloroform and 1ml of isoamyl alcohol to give the following composition. Phenol:Chloroform :Isoamylalcohol 25:24:1.

Potassium acetate

3M w.r.t potassium

5M w.r.t acetate :To 60 ml of 5M potassium acetate was added 11.5 ml glacial acetic acid. The

resultant solution was 3M w.r.t potassium and 5M w.r.t acetate.

Sodium acetate 3 M pH 7.0 :41 gms of Sodium acetate was dissolved in 80 ml of water the pH adjusted to 7.0 or 5.2 with glacial acetic acid.

Ammonium acetate (10M) :77 gm of Ammonium acetate was dissolved in 100ml of glass distilled water and filter sterilized.

EDTA (0.5 M) :186.1 gm of disodium EDTA was dissolved in 800ml water the pH was adjusted to 8.0 with NaOH and sterilized by autoclaving.

2.3 Methods

2.3.1 Isolation of acrylonitrile utilizing organism:-

The isolation was done using enrichment culture technique. A mixture of four bacteria which were earlier used to treat acrylonitrile/acrylate plants wastes were used. The mixture contained organisms of the following genera, *Aeromonas,*

Pseudomonas, Arthrobacter, and Cornyebacterium. This mixture was inoculated in basal salt media, the composition of which is given under Materials. It contains 1% glucose and 0.2% acrylonitrile. When growth was obtained the organisms were progressively subcultured in medium containing lesser amounts of glucose i.e. 0.5%, 0.2%, 0.1%. Finally glucose was totally withdrawn from the media and the cultured organisms were adapted to grow on acrylonitrile as the sole source of carbon and nitrogen. Repeated subculturing was done until a good growth was obtained. During the course of the subcultures, a loopful of the growth was streaked on nutrient agar plates, acrylonitrile and acrylamide plates whose composition has been mentioned in Materials. The isolated colonies were obtained and transferred to tubes containing 3 ml of basal salt medium and 0.2% v/v acrylonitrile and grown overnight. These were then transferred to 100 ml of basal salt media containing acrylonitrile. The purity of the culture was routinely monitored by streaking it on nutrient agar plates, acrylonitrile plates and acrylamide plates and observing the colonies obtained.

2.3.2 Identification of bacteria :-

The organism which showed maximum growth in medium contained acrylonitrile as a sole source of carbon and nitrogen, was isolated and identified in accordance of tests described in the

manual of microbiological methods (Society of American Bacteriologists, 1957), Cruikshank (1972), Norris and Ribbons (1971) and Skerman (1967). With the results thus obtained the bacteria was classified into its genera according to Bergy's Manual of Determinative Bacteriology 8th Ed. (Buchanan *et al.* 1979).

2.3.3 Growth of bacteria

Growth of bacteria was routinely determined by measuring the absorbance of the bacterial culture at 660 nm in an Erma photoelectric colorimeter model AE 80 pH was measured using a pH meter.

2.3.4 Estimation of ammonia

Ammonia was estimated by indophenol method (Fawcett and Scott, 1960). A suitable aliquot (0.1 to 0.5 ml) was taken and the volume was made up to 0.5 ml with glass distilled water to this was added 0.5 ml of sodium phenoxide reagent; 0.5 ml of nitroprusside reagent and 0.5 ml of sodium hypochlorite reagent. A blue color was developed by placing the tubes in boiling waterbath for 5 min. the intensity of the color was measured at 660 nm in an Erma photoelectric colorimeter. Ammonium sulfate was used as a standard. The range of this method was found to be between 50 and 400 nmols of ammonia.

2.3.5 Preparation of whole (resting) cells.

The organism was grown for 48 hr. in basal salt medium containing acrylonitrile as a sole source of carbon and nitrogen. The cells were harvested by centrifugation at 5000 g for 10 min. in a Sorvall model RC5B refrigerated centrifuge maintained at 4 °C. The cells were the washed thrice with basal salt medium (containing no acrylonitrile) after which they were resuspended in basal salt medium (containing no carbon or nitrogen source) and allowed to incubate for 12 hr. on a rotary shaker. The cells were then collected by centrifugation as mentioned earlier and resuspended in, 1/10th volume of original culture volume, of 50 mM phosphate buffer pH 7.0. This was termed as whole or resting cells. Suitable aliquots were taken out and used for the measurement of nitrile or amide breakdown by either, measuring the nitrile or amide by gaschromatography (GC) or High performance liquid chromatography (HPLC), or by measuring the ammonia released by indophenol method. The assay system was as given below:-

Phosphate buffer pH 7.0, :100 u mols

substrate (Nitrile or amide) : 20 u mols

Resting cell suspension : 0.1 to 0.5 ml; The volume was made upto 1 ml with glass distilled water. this was incubated at 37 °C for 30 to 45 min. If the activities were low the time of incubation was extended to 1hr.; if high the cell suspension

was suitably diluted. At the end of incubation time the reaction was terminated by tranferring a suitable aliquot (0.1 to 0.5 ml) into tube containing 0.5 ml of sodium phenoxide reagent the volume was made upto 0.5 ml with water the ammonia was estimated as mentioned earlier.

Part of the assay system left was transferred into microcentrifuge tubes (Eppendorf type) and this was centrifuged at 15000 rpm for 10 min. the supernatent was collected and a suitable aliquot was injected into GC or HPLC for the estimation of nitriles amides and acids.

2.3.6 Gas chromatography of nitriles and amides

Nitrile and amides were estimated in a Hewlett Packard model 5840A gas chromarograph equipped with a flame ionization detector. The chromatographic conditions were:

Column	:	Stainless steel 2mm i.d 2mt long packed with porapack Q 80-100 mesh(Hewlett Packard)
Oven temperature	:	200 °C
Detector temperature	:	250 °C
Injection temperature	:	225 °C
Flow rate of carrier gas (N_2)	:	25ml/min

Authentic standards of acetonitrile, acrylonitrile, actamide,

acrylamide, acetic acid and acrylic acid were used to determine the retention time and to normalize for the detector responses.

2.3.7 Determination of nitriles and amides by High Performance Liquid Chromatography (HPLC) (Amerant *et al.*, 1989).

The HPLC assembly consisted of Beckman model 110A and 110B pumps, Beckman model 160A fixed wave length UV-VIS monitor. The column used was Ultrasphere ODS (Altex). The solvent composition was 2% acetonitrile in 0.07% ortho phosphoric acid. The detection was at 210 nm. Authentic standards of acrylonitrile, acrylamide, acetamide, acetic acid and acrylic acid were used to determine the retention time and to calibrate the system.

2.3.8 Preparation of cell free extracts :-

Cells at mid point of exponential growth phase were harvested by centrifugation at 5,000 g for 10' in a Sorvall model RC5B refrigated centrifuge maintained at 4 ^{o}C. The pelleted cells were washed thrice with 50 mM phosphate buffer pH 7.0. The cells were then resuspended in 1/10 the original culture volume of KDE buffer (Phosphate, DTT & EDTA) pH 7.0. The suspension was chilled on ice salt bath and sonicated after addition of glass powder in a Branson model ultrasonic cell disrupter at 20 KHz. The cells were exposed for 5 min. to ultrasonic waves, with intervals of 2 min. The total exposure was for 30 min. The glass powder, whole cells, and cell debris were removed by

centrifugation for 30 min. at 25,000g. The clear supernatant thus obtained was termed as cell free extract.

2.3.9 Enzyme assays:-

2.3.9.1 Nitrilase and Amidase -

The activity of nitrilase and amidase were routinely estimated by measuring the amount of ammonia produced from nitrile or amide respectively. The assay system is given in Table 8. The reaction was terminated by the addition of 0.5 ml of sodium phenoxide reagent. To this, was added 0.5 ml of nitroprusside reagent, and 0.5 ml of sodium hypochlorite reagent. the blue color developed was read at 660 nm in a Erma photoelectric colorimeter. To estimate the nitrile or amide formed by GC or HPLC the reaction was terminated with 6% perchloric acid, centrifuged at 10,000g and the supernatant injected into HPLC or GC.

2.3.9.2 Glutamine Synthetase (GS)

GS was measured by the g-glutamyl transfer reaction catalyzed by the enzyme. The assay system and the procedure is shown in Table 9.

2.3.9.3 Glutamate dehydrogenase (GDH) and Glutamate synthase (GOGAT).

The activities of GDH and GOGAT enzymes were estimated spectrophotometrically by measuring the rate of oxidation of

Table: 8 Assay system for Nitrile Hydratase and Amidase

	Nitrile hydratase	Amidase
1) Basis of Method	-----	------
2) E.C No.	E.C.3.5.5.1	E.C.3.5.1.4
3) Substrate	Acrylonitrile Acetonitrile	Acrylamide Acetamide
4) Cofactor	None	None
6) Buffer	Phosphate 100 u mols	Phosphate 100 u mols
7) pH	7.0	7.0
8) Total Volume	0.5 ml	0.5 ml
9) Time of incubation	30 to 40'	30 to 40'
10) Start of reaction	Addition of enzyme	Addition of enzyme
11) Temperature of incubation	60 C	60 C
12) Mode of termination	Addition of 0.5 ml of Sod.Phenox. reagent	Addition of 0.5 ml of Sod.Phenox. reagent
13) Treatment of blank	1.Enzyme added after termination 2. Enzyme incubated without substrate till the end of reaction	1.Enzyme added after 2.Enzyme incubated with out the substrate till the end rection time
14) Parameter measured	Ammonia liberated (Fawcett & Scott 195) Nitrile left over or amide formed by GC or HPLC	Ammonia liberated & (Fawcett& Scott
15) definition of Unit	Enzyme required to catalyze the formation 1 u mole of product (Ammonia or amide)	Enzyme required to catalyse the formation of 1 umol of ammonia

Table: 9 Assay System for Glutamine Synthtase, Glutamate dehydrgenase and Glutamate synthase (GOGAT)

	GS	GDH	GOGAT
1) Substrate	Glutamine	a Keto glutarate Ammonia	a-Ketoglutarate Glutamine
2) Cofactor	$MnCl_2$ (0.03M) Sod. Arsinite	NADPH	NADPH
3) Buffer	Imidazole (0.02M)	Tris-Glycine	Tris-Glycine
4) pH	7.5	7.6	7.6
5) Total volume	2.0	2 ml	2.0 ml
6) Time of incubation	30'		
7) Start of reaction	addition of enzym	Addition of NADPH	Addition of NADPH
8) Temperature	37 °C	37 °C	37 °C
9) Mode of terminatio	additon of 1 ml solution contn. 24% TCA, 6N HCl 10% $FeCl_3$	-----	-----
10) Treatment of blank	1.Enzyme incubated without substrate 2.Reaction terminated prior to addition enzyme	1.Enzyme incubated without substrate 2.Enzyme incubated without NADPH	1.Enzyme incubated Without substrate 2.Enzyme incubated with out NADPH
11) Parameter measured	Glutamyl ferric hydroximate spectrophoto-metrically	Oxidation of NADPH measured at 340 nm spectrophoto-metrically	Oxidation of NADPH measured at 340 nm Spectrophoto-metrically
12) Defenition of 1"unit"	Enzyme required for the production of 1 u mol of Glutamyl Ferric hydroximate	Enzyme required for the oxidation of 1 umole of NADPH	Enzyme required for the oxidation of 1 umole of NADPH

NADPH, which was indicated by the decrease in absorbance at 340 nm. A suitable aliquot of the cell free extract was added to the assay system as shown in Table 9.

2.3.9.4 Protein estimations

Protein was estimated by method of Lowry et al. (1956), by method of Bradford (1976) or by measuring the UV absorbance at 260 nm and 280 nm. The amount of protein was calculated as follows

Amount of protein (mg/ml) = 0.75 X abs. at 260 nm - 1.4 X abs. at 280 nm

2.3.10 High speed size exclusion chromatography for protein separation

The system consisted of Beckman model 110 A&B pumps and organizers, beckman model 160A fixed wave length UV-VIS detector. The columns used were TSK 2000, 3000, 4000 (Toyo soda company, Japan). Elution was done using 100 mM KDE buffer containing 100 mM NaCl. The eluted fractions were continuously monitored at 280 nm. Fractions were collected in a Gilson model fraction collector. Standard protein markers were used to calibrate the system.

2.3.11 SDS polyacrylamide gel electrophoresis

Analytical slab gel electrophoresis were performed by method of Lammelli (1970) The composition of buffers and acrylamide bis

acrylamide solutions are given in table under "materials". 1 Lt. of acrylamide:bis acrylamide was deionised using mixed bed resin (Biorad) and filtered through whatman filter paper no.1 and stored at 4 °C in brown bottles. The glass plates were washed in acid and rinsed thoroughly in glass distilled water, ethanol and dried. The glass plates were attached to each other separated by lightly greased spacers, this becomes the cast for polymerizing the gel. 25 ml of deionised 30% acrylamide:bis acrylamide was mixed with 15 ml of 4X gel buffer. To this was added 100 ul of (100 mg/ml)ammonium per sulfate solution. The volume was then adjusted to 60ml with water. This gives 12.5% gel. The polymerization was initiated by the addition of 50 ul TEMED. The resultant solution is immediately poured into the plates prepared as described earlier. Polymerization is usually complete within 1 hr. Stacking gel is prepared by mixing 1 ml of acrylamide:bisacrylamide to 4 ml of H_2O to this is added 5ml of 2X stacking gel buffer and mixed well and 10 ul of ammonium per sulfate solution and 5 ul of TEMED is added to start polymerization. This solution was then layered on top of the the running gel already polymerized and a comb was placed to form the wells. This set up was then attached to a vertical gel electrophoresis system model V16-2 (Bethedsa Research Laboratories Inc.. The buffer tank was filled with Tris-Glycine buffer pH 8.9. The sample wells were cleaned well by pipetting the electrode buffer in and out. The protein samples were mixed

with sample loading buffer and loaded into the wells. The electrophoresis was performed at 100 mv till the blue tracking dye reached the edge of the gel. The protein bands were visualized by staining with Coomassie brilliant blue or with silver nitrate.

2.3.12 Antibiotic Sensitivity:-

Antibiotic sensitivity of the isolate was determined tentatively by examining the zone of diffusion. Further, the minimum inhibitory concentrations of these antibiotics was determined as described by Sambrook *et al.* (1989). Single colonies of this culture grown overnight were picked up using a sterile toothpick and transferred to Luria broth, containing different amounts of (i.e. 100 to 1000 ug/ml) of appropriate antibiotic and allowed to grow aerobically overnight. Growth after 12 hr. was measured by using colorimeter and the minimum inhibitory concentration was calculated.

2.3.13 Screening and Detection of Plasmids:-

This was done using various methods.

a) Alkaline lysis method : Sambrook et al. (1989)

In this method the cell wall is removed using lysozyme, and the cells are lysed using a detergent - sodium dodecyl sulfate, at alkaline pH. Under these conditions DNA and nuclease denature. Upon neutralization of the solution, the topologically linked

strand of plasmid DNA aggregates precipitates as potassium dodecyl sulfate in the cold after neutralization. Both the aggregated chromosomal DNA and the insoluble detergent are removed by low speed centrifugation leaving the plasmid DNA in solution. A brief deproteinization with phenol chloroform ensures the removal of a majority of the proteins. The plasmid DNA is then precipitated with ethanol. The details of the protocol are -

1) The bacterial colonies were inoculated with appropriate antibiotic or grown overnight in media containing nitriles.

2) 1.5 ml of these cultures were taken into different eppendorf tubes and centrifuged to pellet the cells. (If found less, 1.5 ml of the cells were pelleted in the same tube.

3) The medium was either decanted or removed by aspiration leaving the bacterial pellet as dry as possible.

4) The pellet was resuspended by vortexing in 100 ul containing: 50 mM glucose
10 mM EDTA
25 mM Tris HCL pH 8.0

5) This was allowed to incubate at room temperature for 5 min.

6) To this was added 200 ul of a freshly prepared solution of 0.2N NaOH containing 1% SDS.

The cap of te tube was closed and the contents were mixed by inverting the tube rapidly two or three times. After the solution becomes clear and extremely viscous, the tube was incubated on ice for 5 min.

7) To this was added 150 ul of ice cold solution on potassium acetate pH 5.3 the cap was closed and vortexed gently in an inverted position.

8) The contents were centrifuged for 10' at 15,000 rpm.

9) The supernatant was transferred to a fresh tube and to it was added equal volume of chloroform, isoamyl alcohol, phenol and the contents mixed by vortexing. The tubes were briefly centrifuged and the supernatant collected in a fresh tube.

10) To the supernatant was added, half the volume of 7.5M ammonium acetate, 2 vol. of ethanol mixed well and allowed to stand at room temperature for 10 min..

11) Centrifuged at room temperature for 10'

12) The supernatant was decanted and the tubes were placed in inverted position to ensure all the liquid is drained away.

13) The pellet was resuspended in 10 ul of T_{10} E_{1} and 10 to 50ul were taken to analyze DNA by electrophoresis.

b) Plasmid preparation by Method of Wheatcroft and Williams (1981):

This method relies mainly on the fact that plasmids, which are closed circular DNA are more resistant to shearing when

vortexed rapidly in the presence of an antifoam agent. Chromosomal debris formed during this procedure can be separated during electrophoresis.

The protocol was -

1) The culture was grown in 30 ml medium containing appropriate antibiotic or acrylonitrile overnight.

2) 1.5 ml of this culture was transferred into sterile eppendorf tubes and centrifuged at 10,000 rpm for 30' in a biofuge.

3) The supernatant was decanted, 100 ul of reagent A, (see Materials), was added and the cells were resuspended by pippeting up and down with an autopipette.

4) To this was added 30 ul of reagent B (see Materials).

5) The contents were inverted gently, 20 times over a period of 2 min. during which the solution turns viscous and the color changes from blue to green.

6) The contents were then rapidly vortexed for 1'at full speed.

7) To this was added double the vol. of reagent C.

8) The contents were vortexed for 30 sec. and the emulsion formed was broken by centrifugation at 15,000 g for 30 min.

9) The upper phase was transferred into a clean eppendorf tube and to this was added 0.5 vol. of amm. acetate (7.5 M) and 2.1 vol. of chilled absolute ethanol and allowed to stand at -20 C for 30'.

10) The solution was centrifuged at 15,000 rpm at room

temperature for 10' and the supernatant decanted.

11) The pellet was rinsed with 70% alcohol and centrifuged and the supernatant decanted.

12) The pellet was vacuum desiccated in a Hetovac system for 10'.

13) The dried pellet was then dissolved in suitable volume of T E buffer and loaded on agarose gel for DNA analysis by
10 1
electrophoresis.

2.3.14 Other methods:

Restriction digestion, RNAse treatment, agarose gel electrophoresis, Ethedium bromide staining etc. were routinely performed in accordance to the protocols suggested in the "Manual of molecular cloning" by Sambrook *et al.* (1989).

Sephadex G-50; G-75; G-200; Biogel P-300; DEAE cellulose; DEAE sephadex etc. were washed activated swollen and equilibrated according to the protocols suggested in the manufacturers user manuals and in accordance to the protocols suggested in the "Methods in enzymology" vol. 1.

2.3.15 Immobilization of cells in poly acrylamide gel matrix; Watanabe *et al* (1987).

Cells harvested from 2 lt. of medium were resuspended in 100 ml of 50 mM phosphate buffer pH 7.0, and the cell suspension was cooled to 4 °C. To this cell suspension equal volume of

acrylamide-bis-acrylamide (29:1) was added. The polymerization was initiated by the addition of 5ml of 10% ammonium per sulfate and 5 ml of TEMED. The polymerization reaction starts within 5 min. The temperature of the reaction mixture increases to about 30 °C, and the polymerization reaction is complete within 1 hr. the gel formed was separated from the reaction vessel, cut into cubes and granulated in a polytron. The granules were washed several times with phosphate buffer pH 7.0, and small particles were decanted. These granules were then used to study the accumulation of acrylamide.

2.3.15.1 Accumulation of acrylamide by intact cells and immobilized cells.

Intact cells or immobilized cells were incubated in conical flasks either at room temperature or at 10 °C along with acrylonitrile 100 to 400 mM (approx.0.65% to 2.65%) and phosphate buffer 100 mM pH 7.0. At the end of reaction time the intact cells or immobilized cells were removed by centrifugation and the supernatent was assayed for nitrile amide and acid by HPLC.