RESULTS AND DISCUSSIONS

3.1 Isolation and characterization of a bacteria capable of utilising acrylonitrile as a sole source of carbon and nitrogen

There have been many reports regarding the bacteria capable of utilising acetonitrile as a sole source of carbon and nitrogen (Digeronimo and Antonie, 1976; Firmin and Gray 1976 ; Linton and Knowles 1983; Arnaud *et al.*, 1977; Asano *et al.*, 1980). On the other hand reports regarding the organisms involved in the breakdown of acrylonitrile have been scarce. An organism *Arthrobacter sp I9* was isolated from aclamatised activated sludge and was shown to be capable of growing on acrylonitrile as a sole source of carbon and nitrogen (Yamada *et al.*, 1979). The organisms capable of utilising acrylonitrile as a sole source of carbon and nitrogen have enormous implication on treatment of waste waters containing acrylonitrile and other nitriles. As mentioned earlier acrylonitrile is a predominant chemical present in the acrylonitrile/acrylate (ACN\ACR) plant wastes of IPCL. Moreover, the nitrile utilising organisms are also important from the point of view of nitrile bioconversions leading to the manufacture of various amides and acids which are commercially important.

The results of earlier studies had shown that, four strains of bacteria namely *Pseudomonas, Aeromonas, Arthrobacter* and *Corynebacterium* were seen to treat ACN\ACR waste waters. Thus, the probability of isolating an organism capable of utilising

acrylonitrile from this mixture was high. In order to do so,this mixture of bacteria was inoculated into basal salt media (the composition of which is given under materials) containing 0.2% acrylonitrile and 1% glucose and allowed to grow aerobically on a rotary shaker. When turbidity (indicating growth) was observed, cells were subcultured. During subsequent subcultures glucose was slowly withdrawn from the medium, thus adapting the cells to grow on acrylonitrile as a sole carbon and nitrogen source. A loop full of this culture, after two or three subcultures, was then streaked on nutrient agar and acrylonitrile agar plates. Single isolated colonies obtained were then transferred individually into tubes containing 3ml of basal salt media or nutrient broth containing acrylonitrile (0.2%) with the help of a sterile tooth pick and allowed to grow overnight. Susequently 10% v/v of this culture was inoculated into 30 ml of basal salt medium containing 0.2% acrylonitrile as a sole souce of carbon and nitrogen and grown aerobically on a shaker. The culture which showed the best growth was taken for further studies. The bacterial identification was carried out as mentioned in the "Methods". The results of these experiments are shown in Table 10. In accordance with the results obtained, this organism was tentatively classified as belonging to the *Coryneform* group of bacteria and to the genus *Arthrobacter* and named as *Arthrobacter BC1*. It is interesting to note that there is a

Table 10. Identification of bacteria capable of utilizing acrylonitrile as a sole source of carbon and nitrogen

TEST	CHARACTERISTICS
Colony	circular, opaque, raised, cream colored, soft
Morphology	Gram +ve, rod, non-capsulating, and non-sporulating.
Broth culture	Slightly turbid, viscous sediment.
Growth on agar slants	opaque, soft, cream colored.
Gelatin liquification	negative
Fat hydrolysis	negative
Starch hydrolysis	negative
Urea hydrolysis	negative
Indole production	negative
Methyl red test	negative
Acetyl methyl carbinol test	negative
Citrate utilization	negative
H_2S production	negative
Catalase test	negative
Oxidase test	negative

(Contd.)

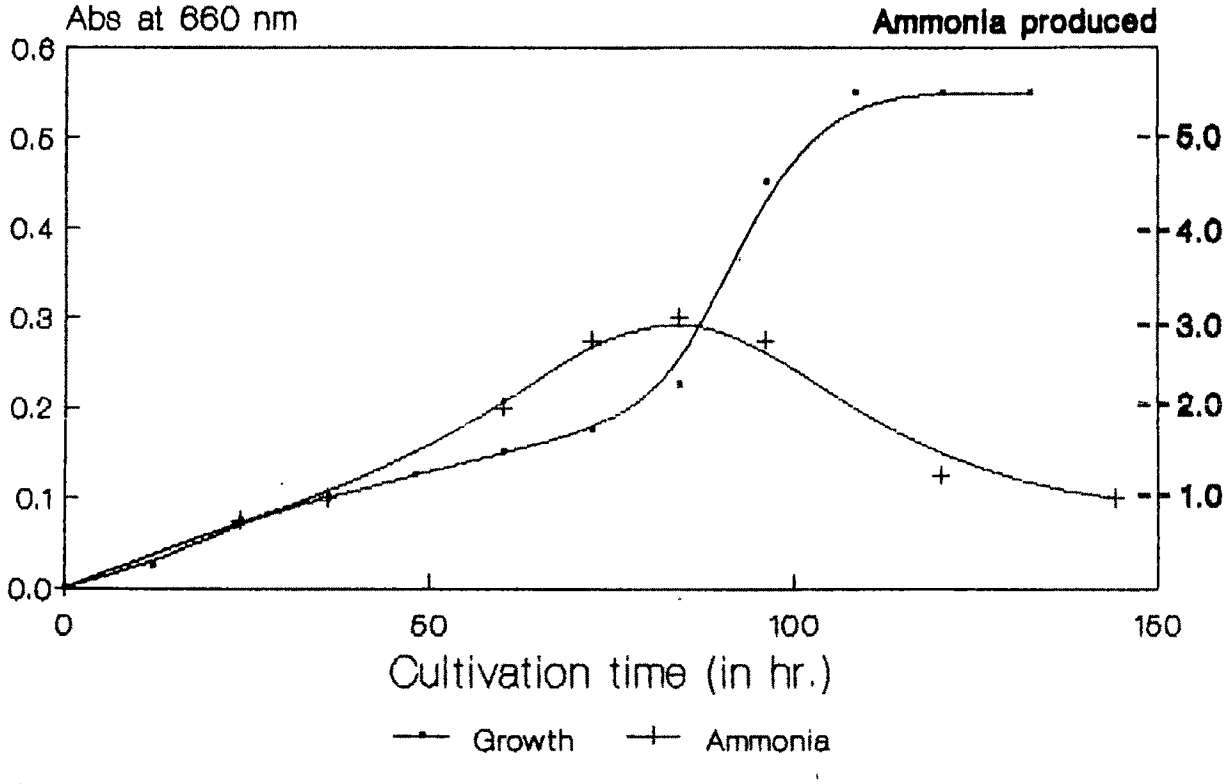
Growth in 7.5% NaCl	negative
Litmus milk test	unchanged
Carbohydrate fermentation	Only glucose
Acid from sugars tested	none
Acid and gas production from sugars	none
Suggested genus	*Arthrobacter*
Classified as	*Arthrobacter BC1*

dominance of the group of *coryneform* in nitrile metabolism. Many organisms reported so far, involved in the utilisation of various nitriles as a source of carbon and/or nitrogen belong to this group (Mimura *et al.*, 1969; Digeronimo and Antonie, 1976; Firmin and Gray, 1979; Asano *et al.*, 1980; Harper, 1977; Yamada *et al.*, 1979; Watanabe *et al.*, 1987). It has also been observed that the genus *Arthrobacter* occurs at a considerably good frequency in the waste waters of IPCL (Goud, 1987).

3.2 Optimization of growth conditions for *Arthrobacter BC1*

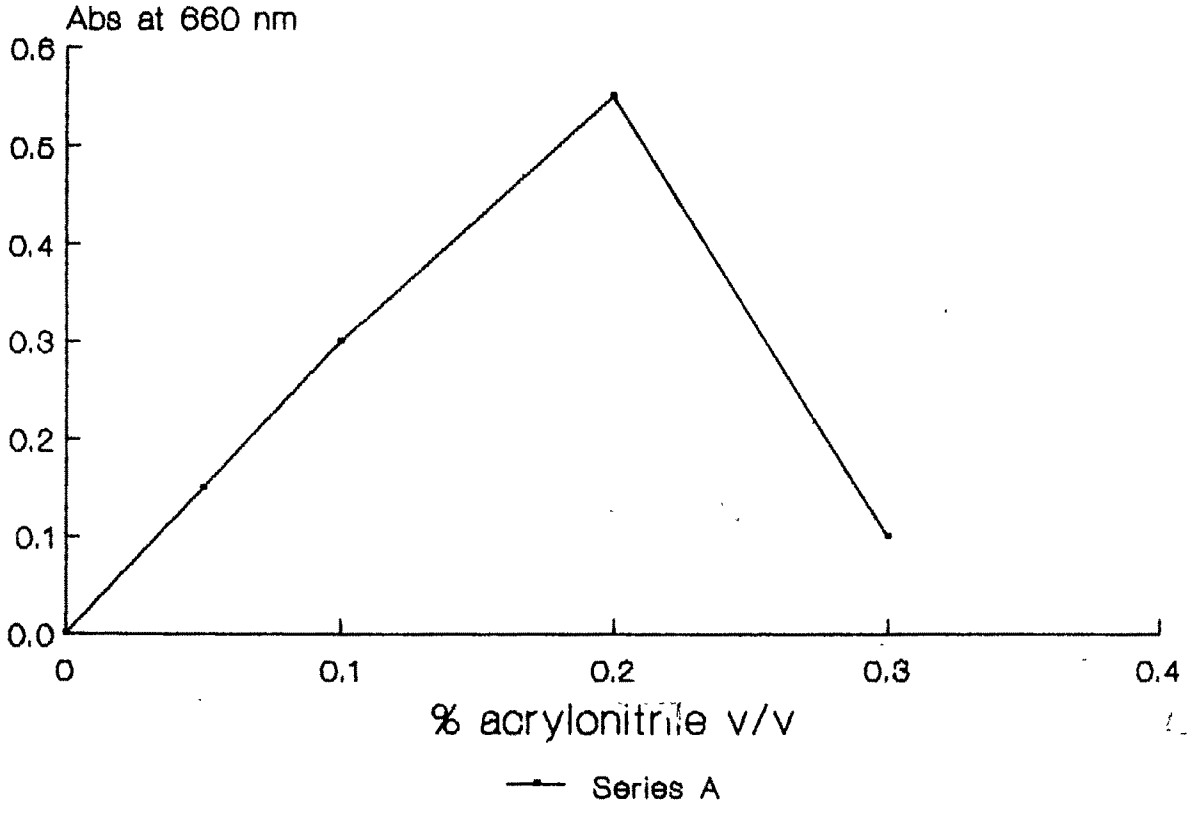
After obtaining an organism capable of growing in the presence of acrylonitrile, further studies were carried out to optimize the conditions for maximum growth and breakdown of acrylonitrile. Nitriles are known to be broken-down to the corresponding acid and ammonia with or without the formation of the intermediate amide. Thus ammonia was chosen as a parameter to evaluate the the extent to which nitrile is broken-down. Conditions for maximum growth and nitrile breakdown were studied. Fig. 7 shows the profile of growth and ammonia production, when *Arthrobacter BC1* was grown in basal salt medium containing acrylonitrile as a sole source of carbon and nitrogen. It is seen that ammonia production peaked at mid point of exponential growth phase indicating a rapid breakdown of acrylonitrile during this period. Initially time required for optimum growth was 96 hr., during the course of the study

Fig. 7 **Growth of Arthrobacter BC1 on Acrylonitrile (0.2% v/v) as a sole Carbon and Nitrogen source**



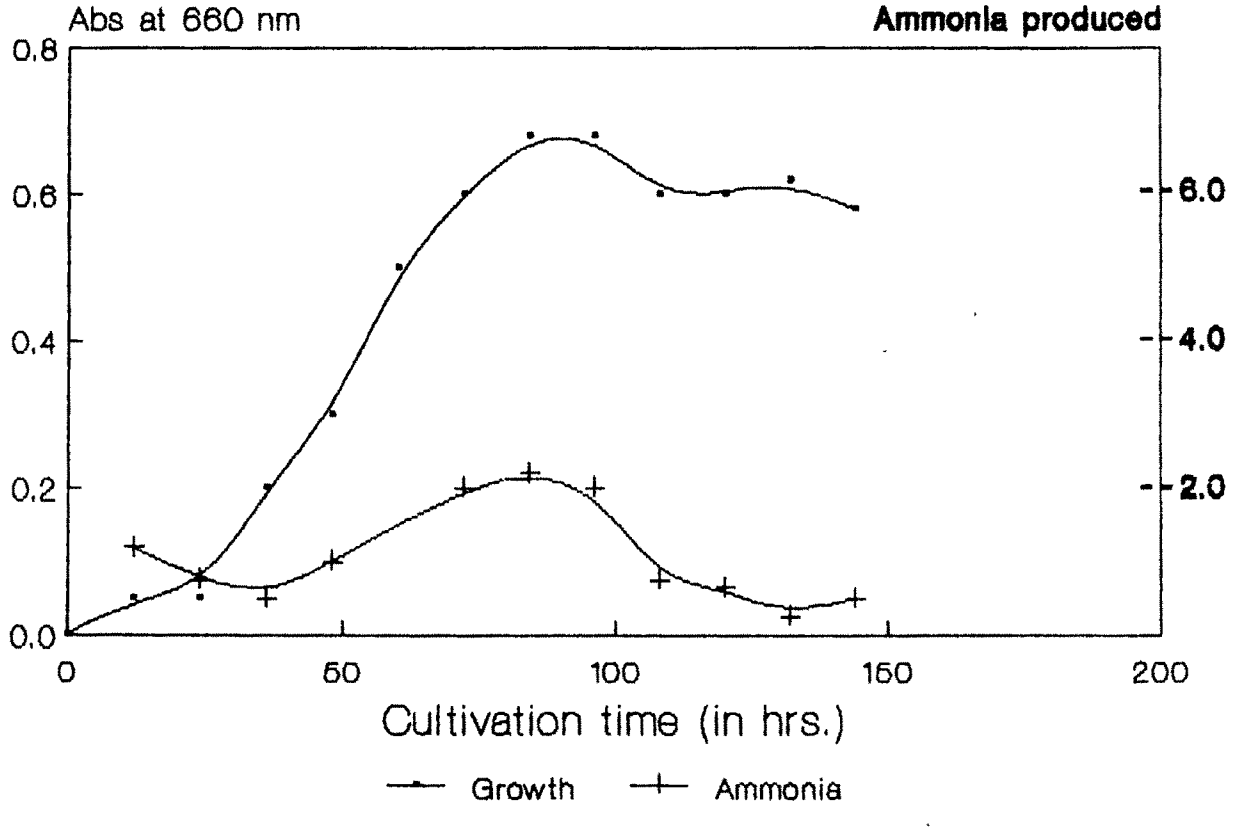
Ammonia production in μmoles/ml

Fig. 8 Effect of acrylonitrile concentrations on the growth of Arthrobacter BC1



All observations made after 96 hr.

Fig. 9 Growth of Arthrobacter on Glucose (0.2%) and acrylonitrile (0.2%)

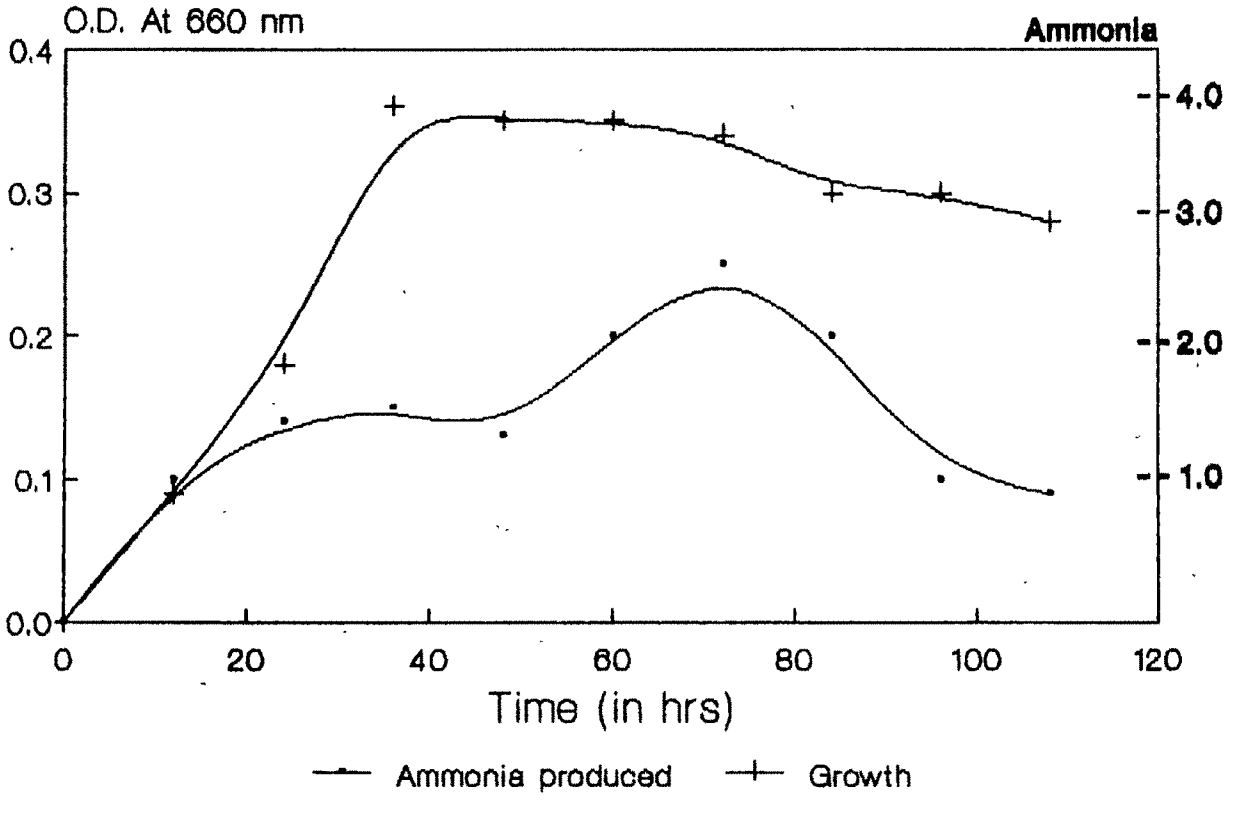


Ammonia in μmoles/ml

it was seen that, as the organism adapted to acrylonitrile the lag phase was reduced and the organism showed optimum growth within 48 hr. When the concentration of acrylonitrile was varied and growth was observed at the end of 96 hr. it was seen that a concentration of 0.2% v/v of acrylonitrile showed maximum growth Fig 8. It was also seen that at concentrations beyond 0.2% v/v tended to inhibit growth.

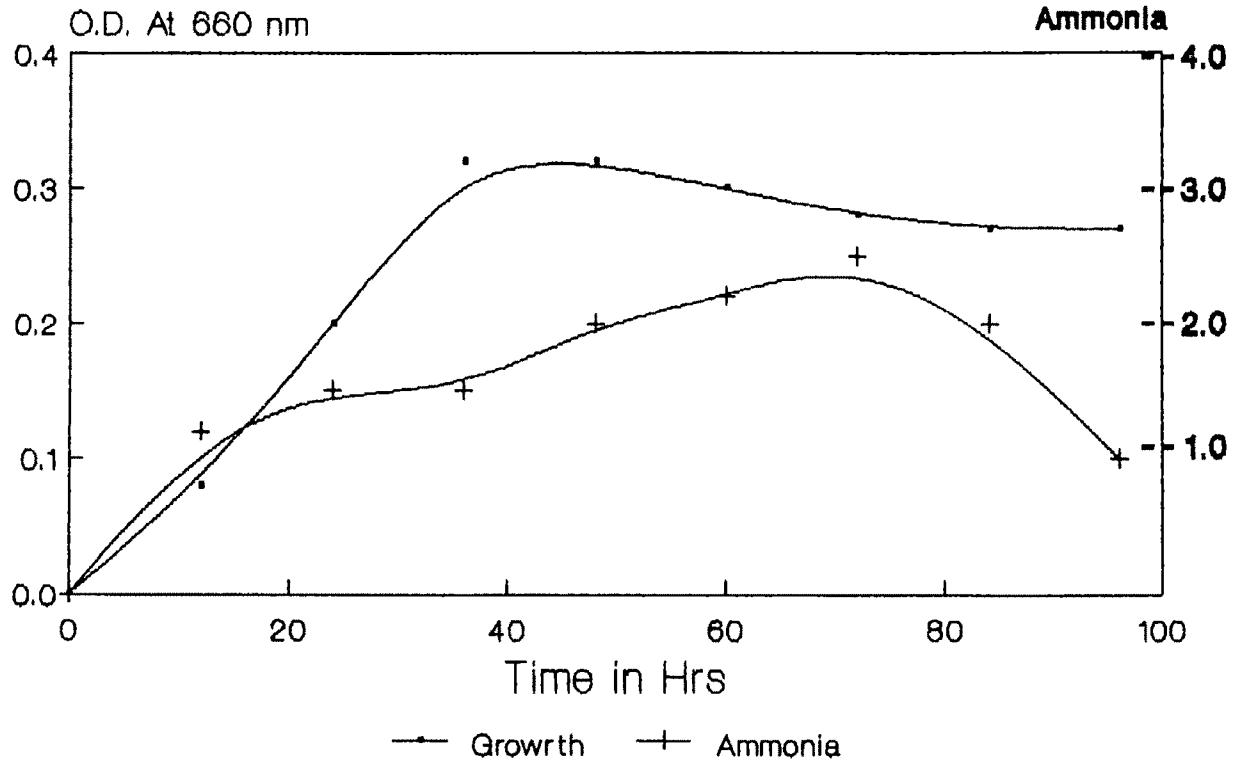
Acrylonitrile can be utilized either as a sole source of carbon and nitrogen or as a source of nitrogen in the presence of an added carbon source. In order to determine the influence of added carbon source on nitrile breakdown, 0.2% glucose w/v was added along with acrylonitrile (0.2%v/v) in a basal salt medium. Under these conditions the growth obtained was higher, the lag period seemed to be reduced as compared to its growth when acrylonitrile is used as a sole source of carbon and nitrogen. However in this case a lower ammonia production was observed Fig 9. This could probably indicate a lower breakdown of nitrile as under these conditions nitrile has to be broken down only for the production of nitrogen, as the requirement of nitrogen is 20 fold less than the requirement of carbon. Alternatively, this lower level of ammonia could also result from enhanced ammonia utilization as, in this case, carbon source is available in plenty. Repression of the enzymes involved in nitrile utilization by glucose and a few other

Fig. 10 **Growth of Arthrobacter BC 1 On acetonitrile as a sole carbon and nitrogen source (0.2% v/v)**



ammonia produced as μmoles/ml

Fig. 11 Growth of Arthrobacter BC1 on acetamide (0.2%) as a sole carbon and nitrogen source



Ammonia expressed as μ moles /ml

carbon sources viz. succinate, acetate has been reported by many workers (Asano *et al.*, 1980; Bandhopadyay *et al.*, 1986; Miller and Gray, 1982). Similar repressive action of glucose in the enzymes of nitrile utilization mechanism, in this organism, could result in reduced nitrile breakdown. This can be confirmed only by studying the induction/repression of the enzymes involved *in vitro*.

As acrylonitrile is not the only nitrile present in ACN/ACR waste waters, the ability of this microorganism to grow on other nitriles and amides were determined. Amides were chosen because it is a known intermediate in nitrile metabolism, moreover, amides are also found in appreciable quantities in ACN/ACR plant wastes (Table 3). Fig. 10 and Fig. 11 indicate that *Arthrobacter BC1* was capable of growing on other nitrile and amides when nitriles and amides were provided as a sole source of carbon and nitrogen. The organism also showed appreciable growth when nitriles were provided as a nitrogen source along with an easily metabolisable carbon source such as succinate, acetate etc. The ability to grow on variety of nitriles makes this organism a potent candidate to be evaluated for its ability to treat ACN/ACR plant waste waters in pure cultures. Interestingly, it was observed that acrylic acid the putative end product of acrylonitrile breakdown could not support growth as a carbon source when it was provided in a

basal salt media containing ammonium sulfate as a source of nitrogen. Two reasons can be attributed for this anomalous behavior:

a. It is known that acrylic acid spontaneously polymerizes in solution in which case the organism will not have access to the carbon source, hence will not be able to utilize it.

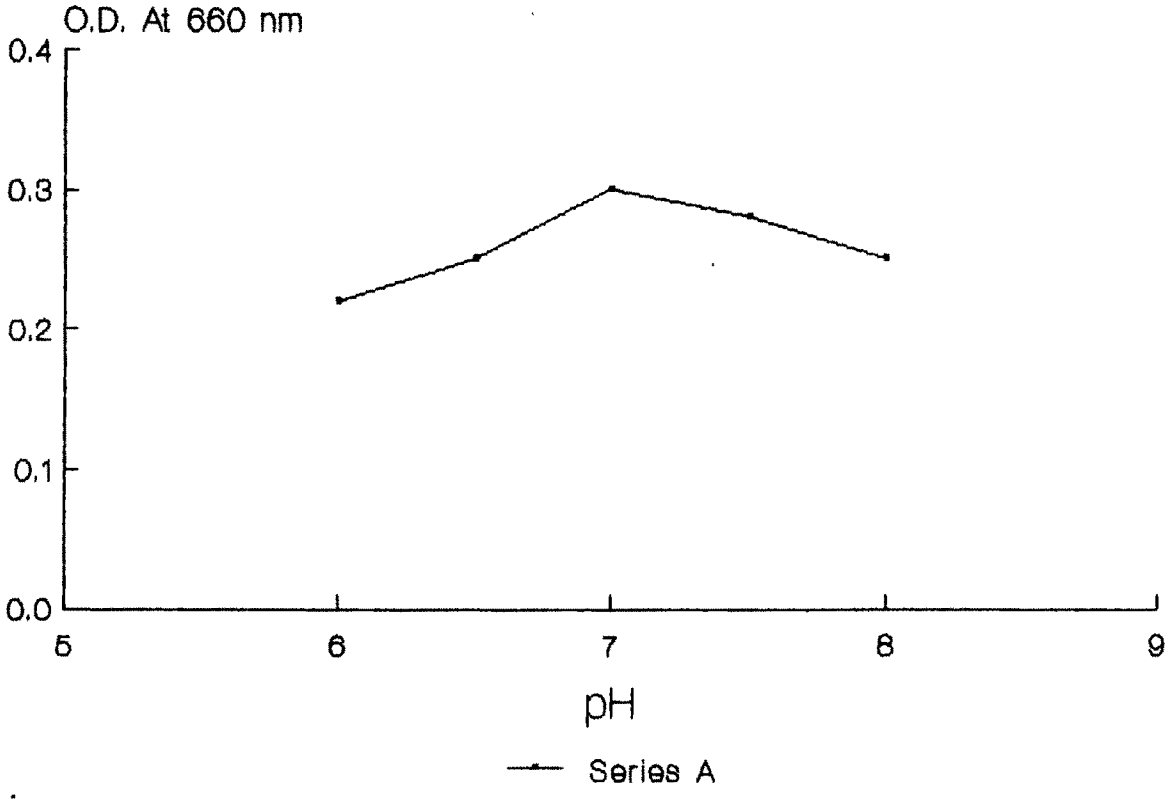
b. It is also possible that unlike acrylonitrile and acetonitrile which are small lipophilic molecules, acrylic acid being charged may not be permeable to the cell and may require transport mechanism.

Since other acids such as succinate, acetate etc. are utilized, it is possible that acrylic acid may not share the same transport mechanism. However, acrylonitrile is utilized as a sole source of carbon and nitrogen indicating the existence of enzyme machinery to further metabolize acrylic acid within the cell, hence transport of acrylic acid can be viewed as a major obstacle.

The optimum pH for growth of *Arthrobacter* was seen to be between 6.5 and 8.0 (Fig. 12) and temperature between 25 and 40 ^{o}C. The optimum time for growth of the organism was found to be between 48 and 96 hr.

Arthrobacter sp. I9 isolated by Yamada *et al.* (1979) was the only other organism known to utilize acrylonitrile as a sole

Fig. 12 Growth of Arthrobacter BC1 at diff pH on acrylonitrile (0.2%) as a sole source of carbon and nitrogen



source of carbon and nitrogen. There is no other report regarding the microorganisms which can utilize acrylonitrile as a sole source of carbon and nitrogen. *Arthrobacter sp. I9* also showed maximum growth at 0.2% v/v of acrylonitrile and has similar features. This organism (*Arthrobacter I9*) metabolized acrylonitrile directly to acrylic acid and ammonia without the formation of the intermediate amide. However, we could also not detect amide in the culture filtrate at any time during growth. The route of metabolism can be determined in controlled study using resting cells or by studying the nature of these enzymes in cell free extracts. Further experiments were designed with this in mind.

3.3 Studies on acrylonitrile breakdown by whole (resting) cells of *Arthrobacter BC1*

Intact cells are good models for the study of metabolism of various compounds. Nitrile hydrolysis has been studied quite extensively using intact cells (Miller and Gray, 1982). These hydrolysis reactions are almost quantitative and they provide a convenient way to investigate the specificities of the enzymes involved in the *in vivo* breakdown together with any linked transport system. These studies also help us to evaluate the efficiency of breakdown and to optimize conditions for maximal expression of the enzymes concerned. Another useful aspect of these studies, is that this organism can be evaluated for its

capacity to bioconvert nitriles to amides and acids which are of commercial importance.

From these considerations and also to establish the metabolic fate of nitriles, resting cells were used to determine the breakdown of nitriles. Table 11 shows the rate of nitrile and amide hydrolysis by resting *Arthrobacter BC1* cells as judged by the amount of ammonia produced when nitrile or amide were provided as substrates. It can be seen that the rate of ammonia production was consistently greater from amide as compared to that of nitriles. This indicates that the rate of amide hydrolysis was more than the rate of hydration of nitrile. This is consistent with the observation made by others that in this reaction the step of nitrile hydration (assuming that it is a two step enzymatic reaction) is rate limiting (Miller and Gray, 1982; Asano *et al.*, 1982).

In the case of *Arthrobacter BC1* we had so far not established whether acrylonitrile hydrolysis was mediated by a single enzyme or by two enzymes. According to the prevailing hypothesis saturated aliphatic water soluble nitriles such as acetonitrile are broken down to the corresponding acid and ammonia via the formation of amide as an intermediate. On the other hand, unsaturated aliphatic nitriles, such as acrylonitrile and aromatic nitriles, such as benzonitrile, are directly converted to the corresponding ammonia without the

Table 11 Breakdown of nitriles and amides by Arthrobacter BC1

Substrate	activity
Acrylonitrile	0.100
Acetonitrile	0.172
Acrylamide	0.180
Acetamide	0.180

Results are a mean of 3 observations.

1 activity unit = mol of ammonia lib/min

formation of any amide as in the case of the organism *Arthrobacter I9* (Yamada *et al.*, 1979) which utilizes acrylonitrile as a sole source of carbon and nitrogen*N. rhodochrous NCIB 11216* which utilizes benzonitrile .

In order to determine the fate of acrylonitrile breakdown in resting *Arthrobacter BC1* cells, a time dependent study with acrylonitrile as a substrate was conducted. It can be seen from Fig. 13 that 100 mM acrylonitrile disappeared from the medium within 30 min. It was interesting to note the accumulation of acrylamide in the medium from the 10th min. onwards. During this process no acid was detectable till 30 min. It is clear from these results that acrylonitrile is first hydrated to acrylamide and then hydrolyzed to acrylic acid and ammonia. In a similar study conducted with acetonitrile it aws seen that acetonitrile breakdown to acetic acid and ammonia proceeded via formation of the intermediate acetamide (Fig. 14).

The results of these studies seemed to be contrary to the earlier hypothesis that unsaturated and saturated nitriles did not follow the same course in their metabolism. However, a stoichiometric conversion of the substrates and products were not observed. Moreover ammonia was detectable in the media from 5th min. onwards indicating the breakdown of the amide formed. Thus the results can be explained in two different ways

Fig. 13 Utilization of acrylonitrile by whole Arthrobacter cells

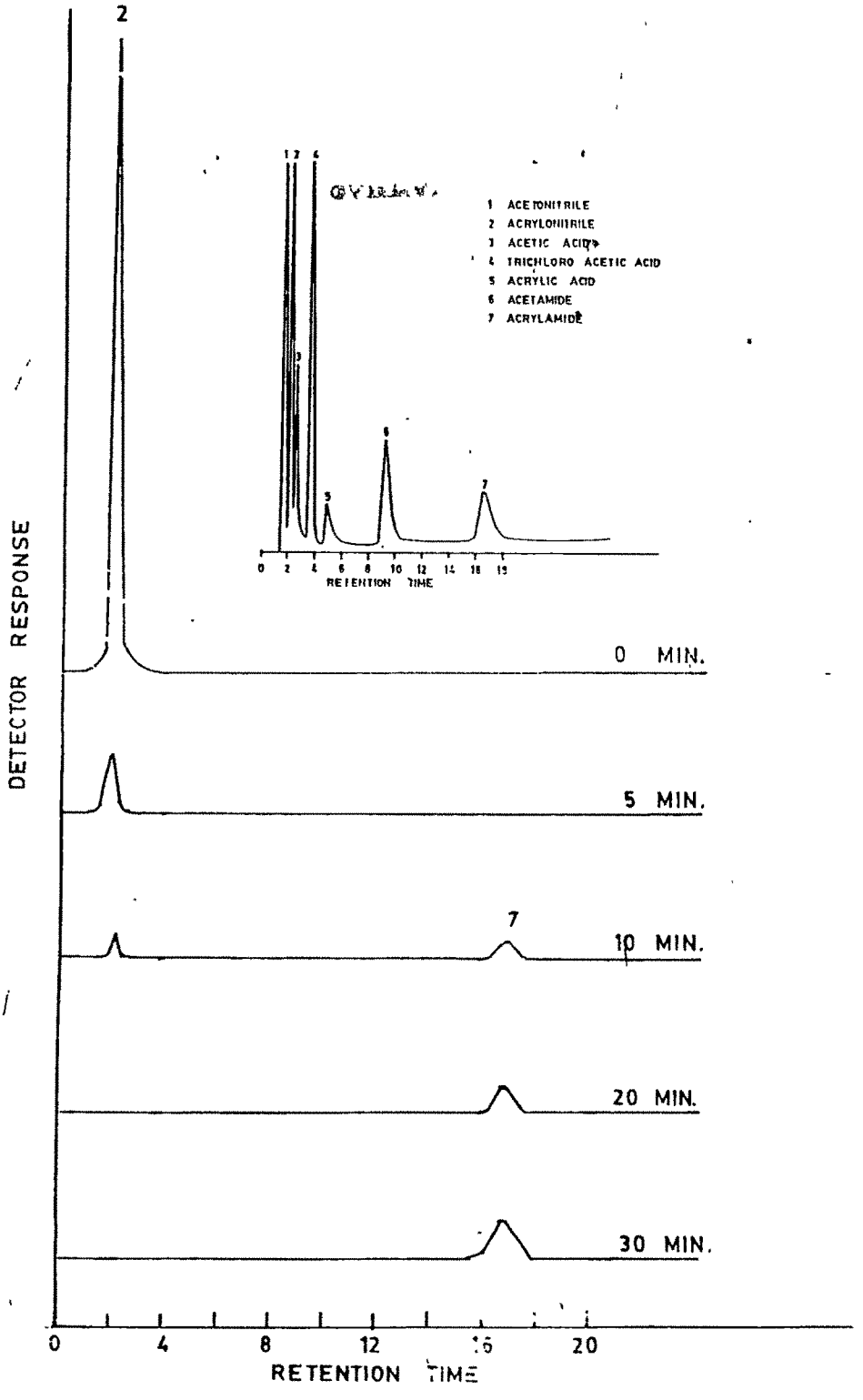
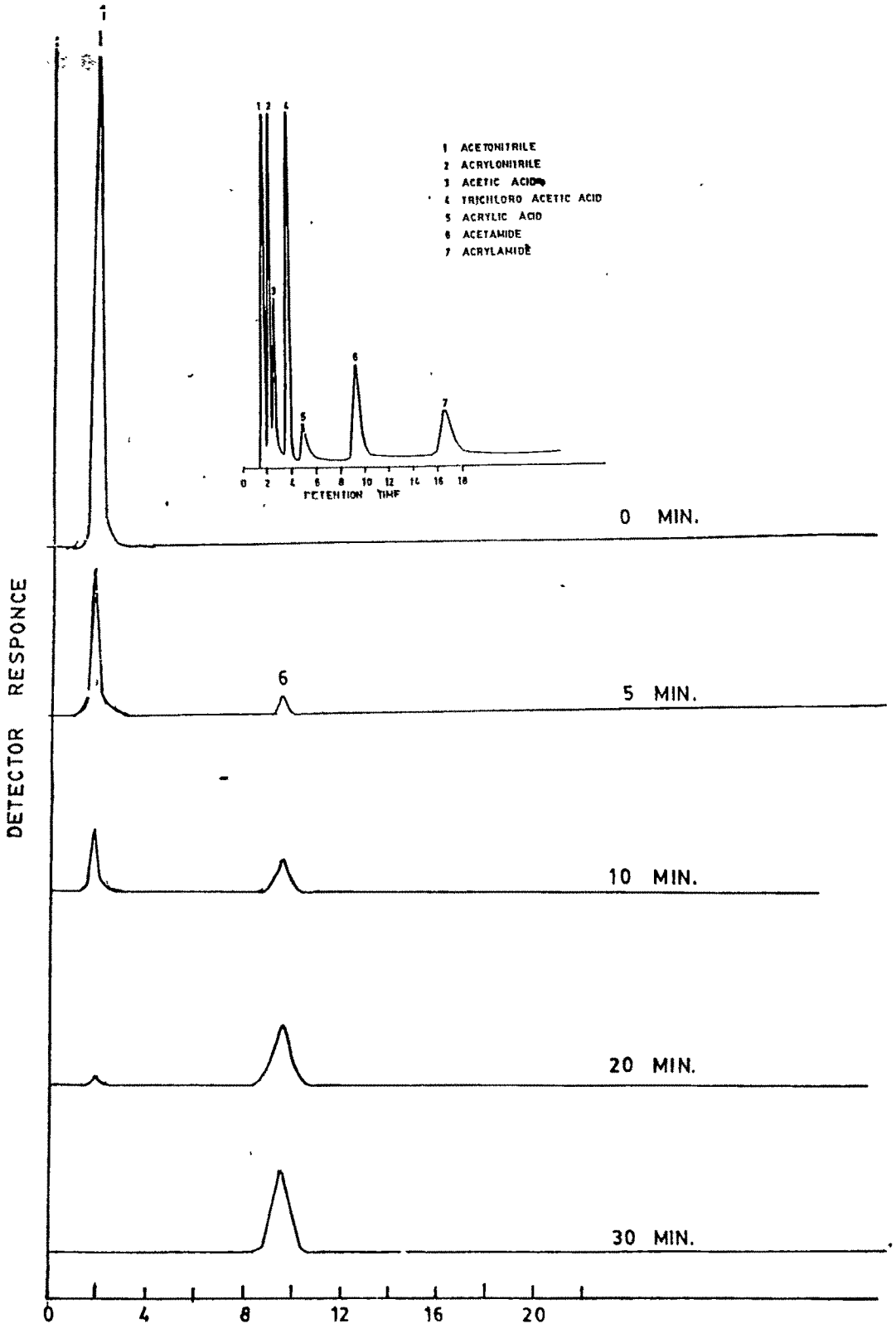
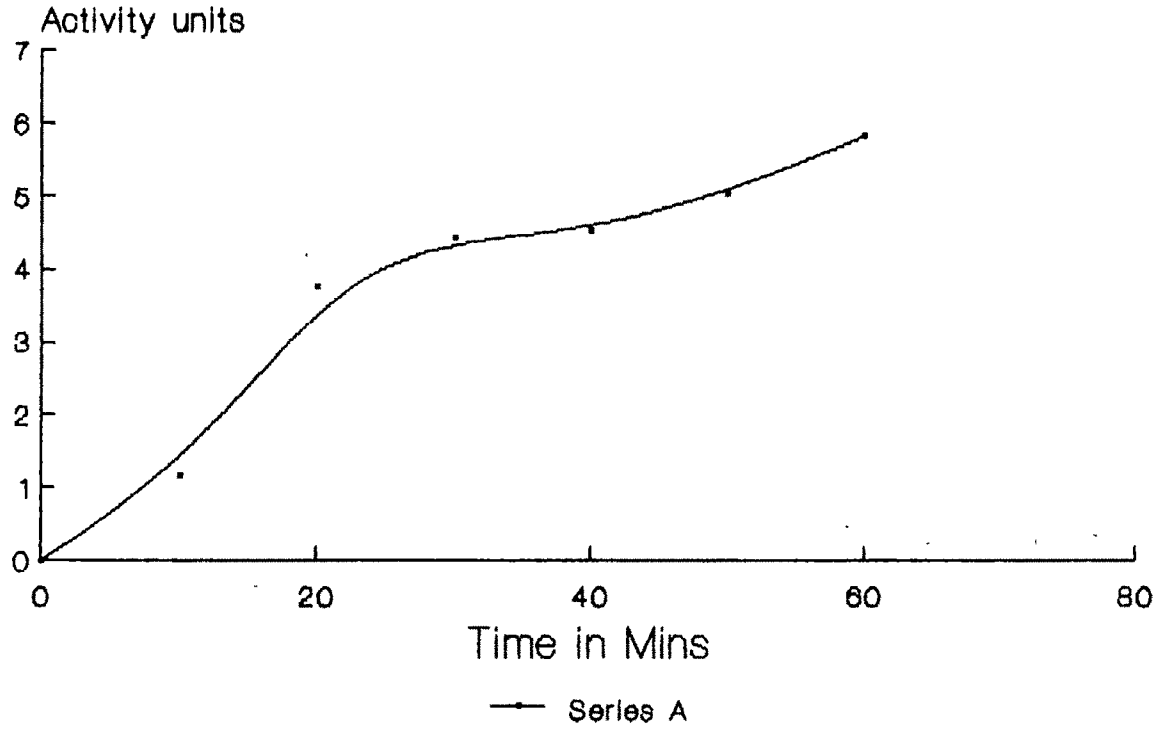


Fig. 14 Utilization of acetonitrile by whole Arthrobacter cells



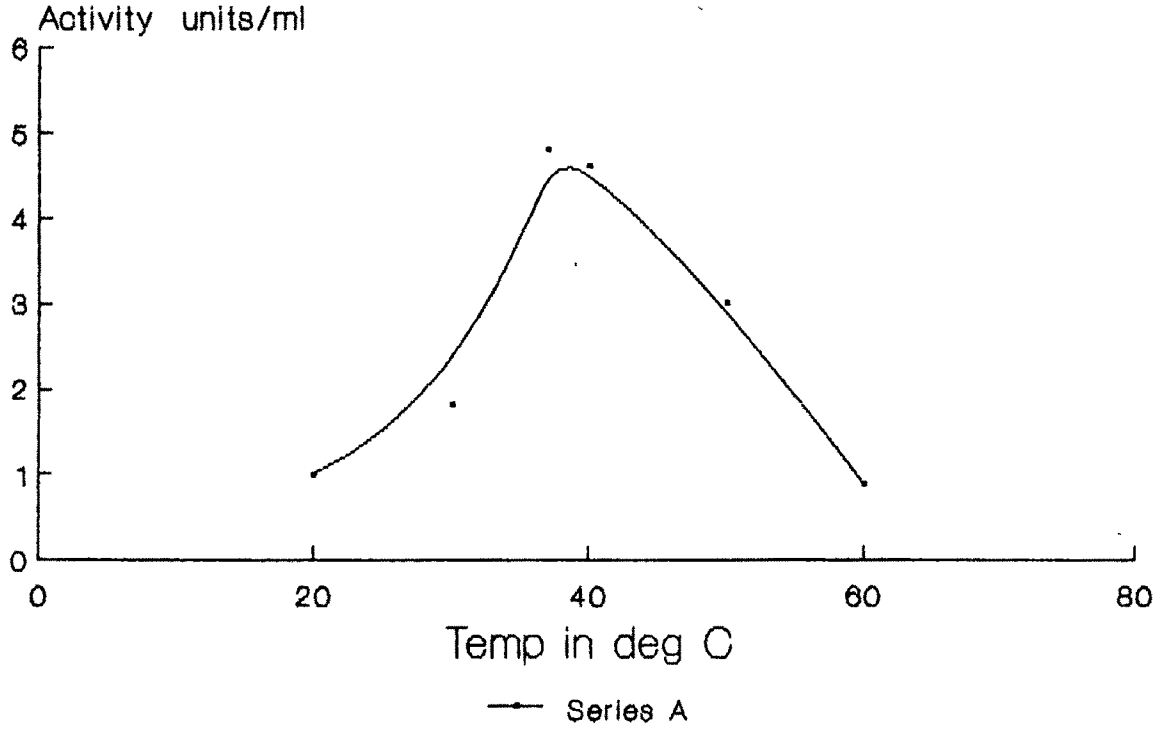
EM-1599

Fig. 15 Effect of time on acrylonitrile utilization by whole cells of Arthrobacter BC 1



Units μmoles of ammonia lib / ml
Cono. of acrylonitrile - 10 mols

Fig. 16 Effect of temperature on nitrile utilization by whole cells of Arthrobacter BC 1



Unit - μmoles of ammonia lib/min
aorylonitrile conc. 10 mM

a. Either this organism metabolizes nitriles, irrespective of the type, through the formation of an intermediary amide.

b. This organism has both the enzyme systems i.e. to convert nitriles to amide and then to acid and ammonia and also to convert nitriles to acid and ammonia with out the formation of the intermediate amide. This can be established only after studying, in detail, the enzymes involved, *in vitro*.

In the intact cells, the optimum pH for the breakdown of nitriles and amides were found to be between 6.5 and 7.5. The breakdown of nitriles showed a linear increase upto 30 min. after which the hydrolysis continued at a lower rate (Fig. 15). The decreased rate of nitrile breakdown could indicate a possible effect of amide accumulation on the rate of nitrile hydrolysis. The optimum temperature for the breakdown of nitrile was found to be between 35-40 ^{o}C (Fig.16).

These studies would enable us to attempt the use of *Arthrobacter* cells for the treatment of the ACN/ACR waste waters. The waste waters also contain heavy metals, so in this case studies regarding the ability of this organism to sustain the heavy metals present in these waste waters has to be evaluated. With this perspective, the ability of *Arthrobacter BC1* cells to hydrolyze nitriles in the presence of a few heavy metals was studied. Table. 12 shows the effect of heavy metals.

Table 12 Effect of heavy metals on the hydrolysis of nitriles by whole Arthrobacter BC1

Heavy metals	Residual activity (%)
Control	100
Hg	50
Cu	83
Co	67
Pb	77
Mn	74
Zn	84
As	44
Ag	50

activity = μ moles of ammonia lib/min.

Conc of heavy metal used = 1 PPM

3.4. Studies with cell free extract of *Arthrobacter BC1* cells

The first enzyme to be purified and characterized was nitrilase from barley leaves (Thimann and Mahadevan, 1956). Since then lot of work has been done on the enzymes involved in the metabolism of natural and synthetic nitrile and to a large extent the metabolism of nitriles is well understood (Nagasawa and Yamada, 1989). The enzyme involved in the hydration of nitrile to amide is aliphatic nitrile hydratase (also called as nitrilase by few). The second enzyme being amidase which hydrolyses the amide formed to acid and ammonia (Acyl amide amido hydrolase E.C.3.5.1.4). On the other hand Nitrilase which catalyses the conversion of unsaturated nitriles such as acrylonitrile directly to acid and ammonia has been detected in *Arthrobacter I9* (Bandyopadyay *et al.*, 1986).

It has been conclusively proved that these enzymes are different with respect to their substrate specificities, inducers etc. In the case of the *Arthrobacter BC1* , it was seen that saturated and unsaturated nitriles shared the same fate and the metabolism of both type of nitriles proceeded via the formation of the intermediary amide. This indicates the possible presence of two enzyme system for the metabolism of acrylonitrile, quite different to that in *Arthrobacter I9* (Bandyopadyay *et al.*, 1986). Further experiments were designed in order to establish that two enzymes were involved and to

characterize these enzymes *in vitro*.

In most of the organisms the enzymes nitrile hydratase and amidase have been showed to be cytoplasmic (Asano *et al.*, 1980; Collins and Knowles, 1985; Arnaud *et al.*, 1977; Nagasawa *et al.*, 1986). Keeping this in mind, the conditions for the optimum recovery of enzyme by sonic disruption was standardized. It was observed that the cells disrupted with great difficulty. Sonication for 30 min. with 5 min. burst and 2 min. intervals with glass powder gave the maximum recovery of the enzyme in soluble form (supernatent of centrifugation at 25,000g). More than 50% of the enzyme activity was still located in the cell debris Table 13. This could be due to two possible reasons :

a. The enzymes could be membrane bound.

b. The cells are not disrupted totally leading to the activity from whole cells.

It is also postulated that nitrile hydratase could be membrane bound (Collins and Knowles, 1985). If the enzyme was membrane bound, it is also possible that amidase is soluble and this gives an provides an opportunity to locate nitrile hydratase and amidase differentially and show that these enzymes are indeed two separate enzymes. In order to do so the pellet from 22,000 g centrifugate was treated with detergents like sodium dodecyl sulfate, sodium deoxy cholate and Triton X-100. During

Table 13: Localisation of activities of nitrile hydratase and amidase

	Fraction	nitrile hydratase U / ml	amidase U/ ml
Crude homogenate		0.097	0.124
Centrifugation at 25,000 g	Supernatent	0.046	0.093
	Pellet	0.071	0.097
Supernatent from 25000 g } centrifuged at 105,000 g }	Supernatent	0.040	0.093
	Pellet	0.000	0.000

* Unit = μ mols of ammonia lib/min
Results are an avrage of at least 3 measurements

these procedures it was observed that the activity from the pellet was decreasing. Unfortunately corresponding increase in enzymatic activity in the supernatent was not observed (Table 14). The possible reason being that detergents may tend to disrupt the quaternary structure of the enzyme leading to decrease in the catalytic activity. It has also been observed that *Coryneform* group of bacteria are resistant to sonic disruption and and even on prolonged sonication, only about 50% of the cells are disrupted (Dolin, 1950). Thus it can be concluded that, the activity in the pellet, is due to the undisrupted and partially disrupted cells in the pellet. From these experiments, it can be concluded that the enzyme was almost entirely present in the soluble fraction and not bound to the membrane.

It has been observed that majority of enzymes nitrilases, nitrile hydratases and amidases contain thiol group in their active sites. During sonication, addition of EDTA and DTT helped in preserving the enzyme activity possibly by protecting these groups from getting oxidized, although their addition in the assay system during the reaction did not improve the enzyme activity. Mercaptoethanol was also tried, but routinely DTT or cystine was used, as 2-mercaptoethanol interfered strongly with ammonia estimation.

Experiments were then carried out to determine the kinetic

TABLE: 14 Effect of detergents on activities of nitrile hydratase and amidase

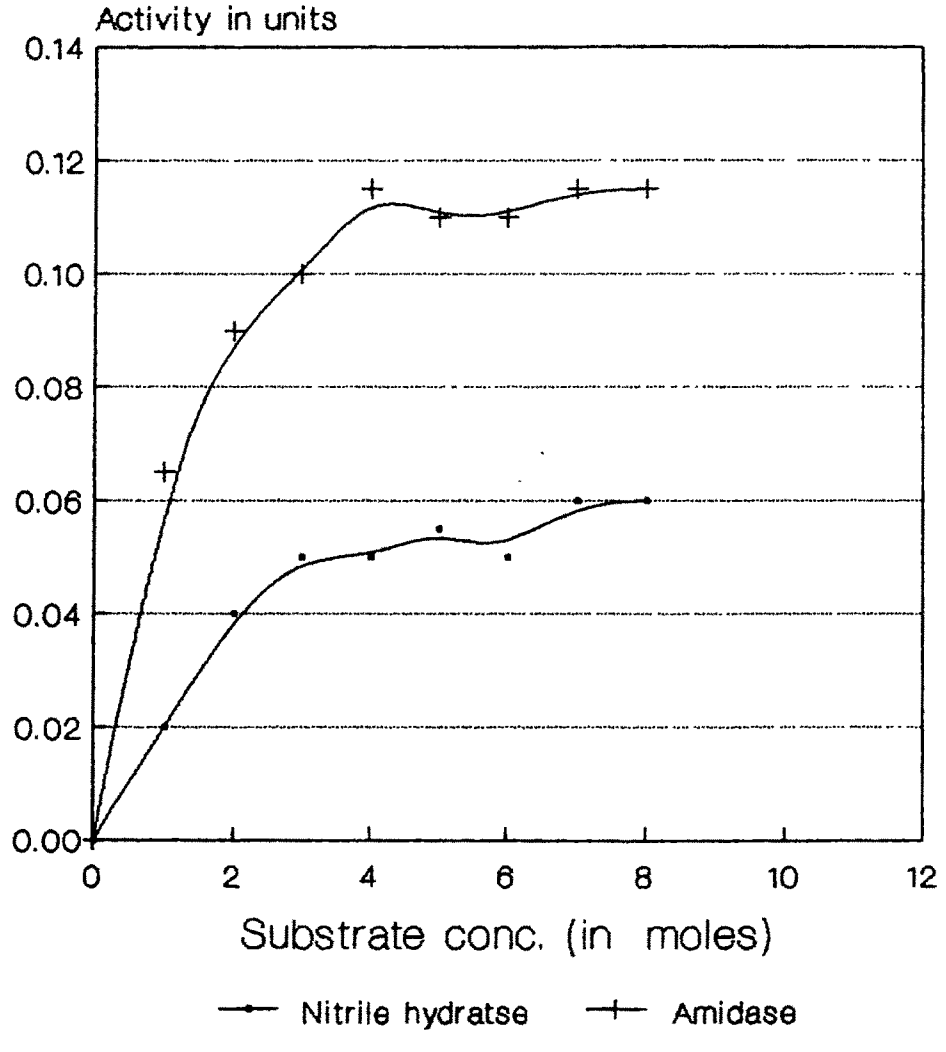
Treatment	Whole sonicate		Supernatent		Pellet	
	nitrilase	amidase	nitrilase	amidase	nitrilase	amidase
Cells (Control) No detergent added	3.556	4.520	1.814	2.451	2.067	2.379
+Triton X-100 (0.05%)	3.840	5.080	1.853	2.510	2.300	2.730
+ SDS (0.05%)	3.120	4.000	1.814	2.515	0.897	1.423
+ Sod. Deoxy Cholate (0.05%)	2.240	3.120	1.384	2.086	0.527	0.956

* Unit = μ mols of ammonia lib/min
Activity is in total units
Results are an avrage of at least 3 measurements

properties of these two enzymes i.e. nitrile hydratase and amidase. In earlier experiments we were not able to differentially locate the enzymes. Therefore, it is of interest to find out whether the kinetic properties of these enzymes differed; if so it could be said that nitrile hydratase and amidase are two independent enzyme systems. Fig. 17 shows the substrate concentration curve of both, nitrile and amide hydrolyzing enzymes. The V max. of the amide hydrolyzing enzyme was consistently greater than that of the nitrile hydrolyzing enzyme but the optimum substrate concentration for both the enzymes was similar, indicating a possible similarity in Km. The optimum pH for both the enzymes were 7.0 and did not show much deviation in activity over a range of 6.5-7.5 pH (Fig. 18). The activity, however, sharply declined, below pH 6.0. while above pH 8.0 the activity showed a decline but, could not be accurately measured, as Tris buffer seemed to interfere with the estimation of ammonia . It was interesting to observe that the optimum temperature for nitrile and amide hydrolyzing enzymes were at 60 ^{o}C (Fig. 19). Similar observations have been made in *Arthrobacter 19* nitrilase (Bandyopadhya *et al.*, 1986). The optimum time of incubation was found to be 45 min. similar as in the case with whole cells the hydrolysis continued even after 45 min. but the rate was lower (Fig. 20).

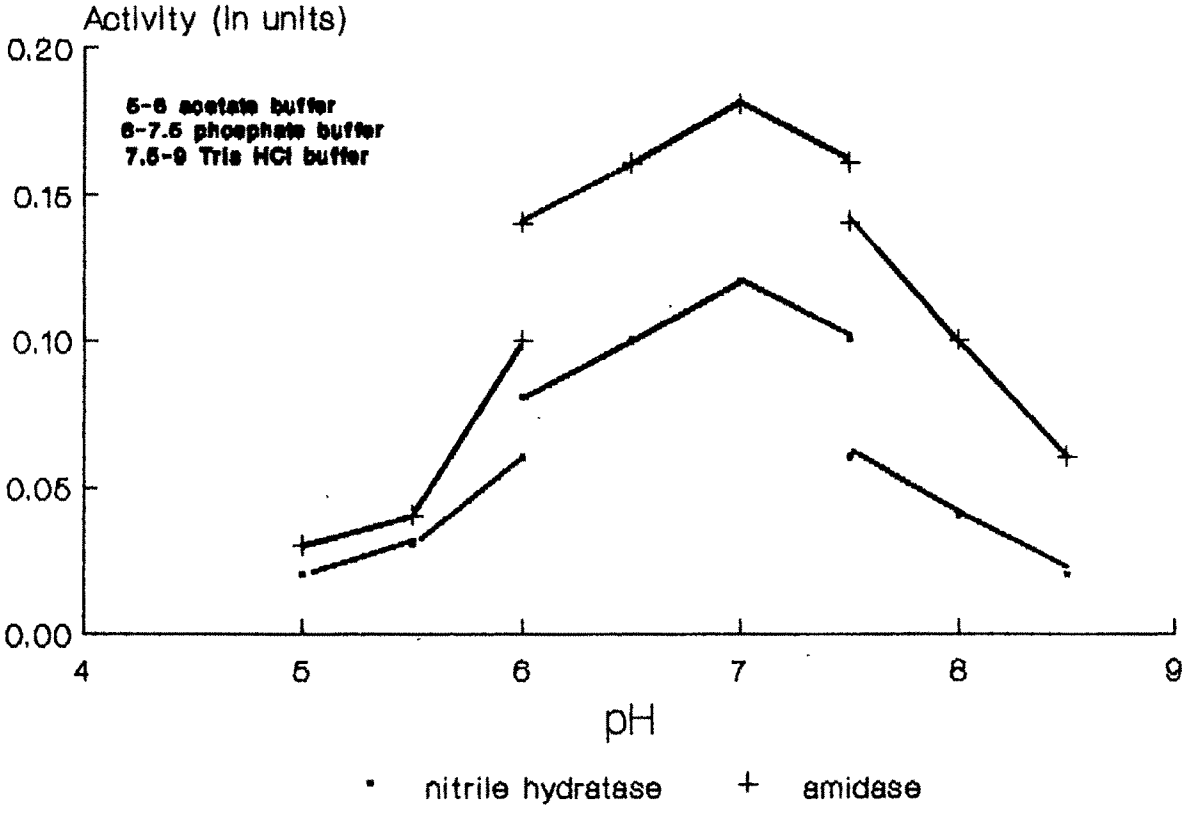
These enzymes nitrilase and amidase were found to have a broad

Fig.17 Effect of substrate concentration on the activities of nitrile hydratase and amidase



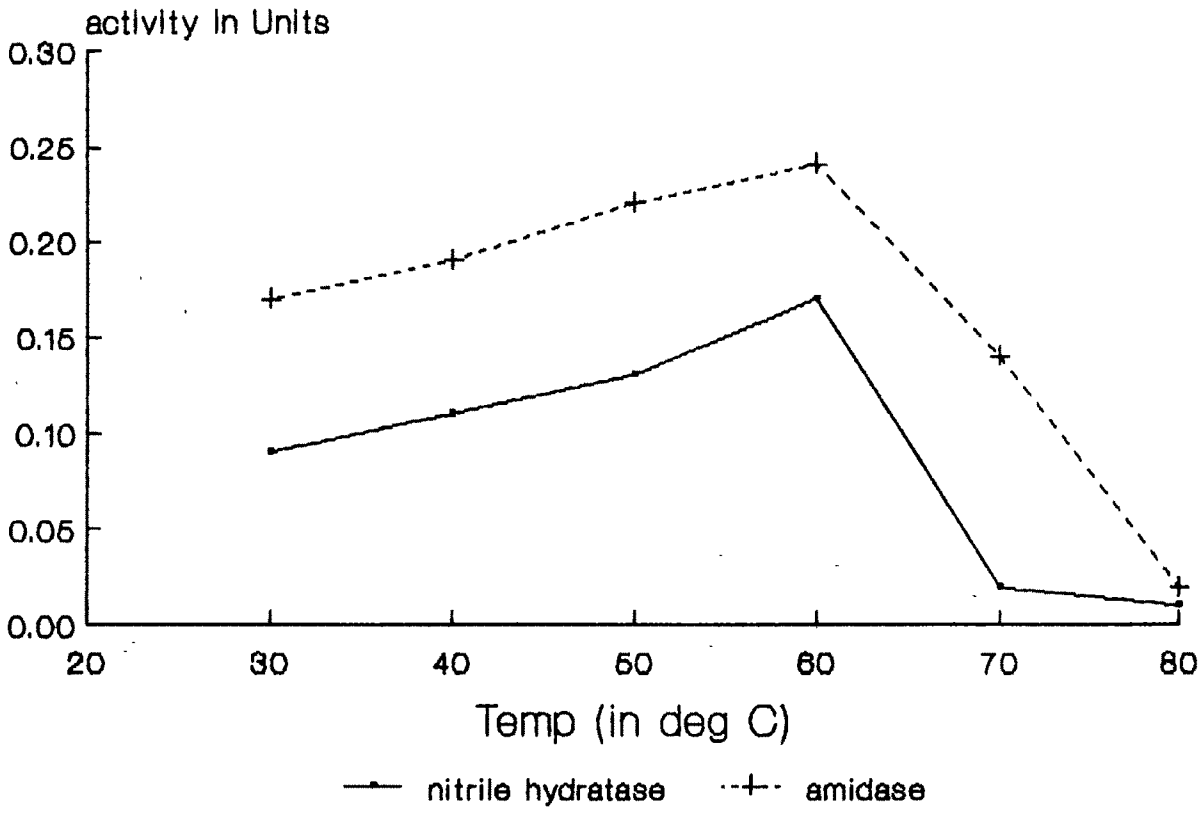
unit = μ moles of ammonia lib/min

Fig. 18 Effect of pH on the activities of nitrile hydratase and amidase



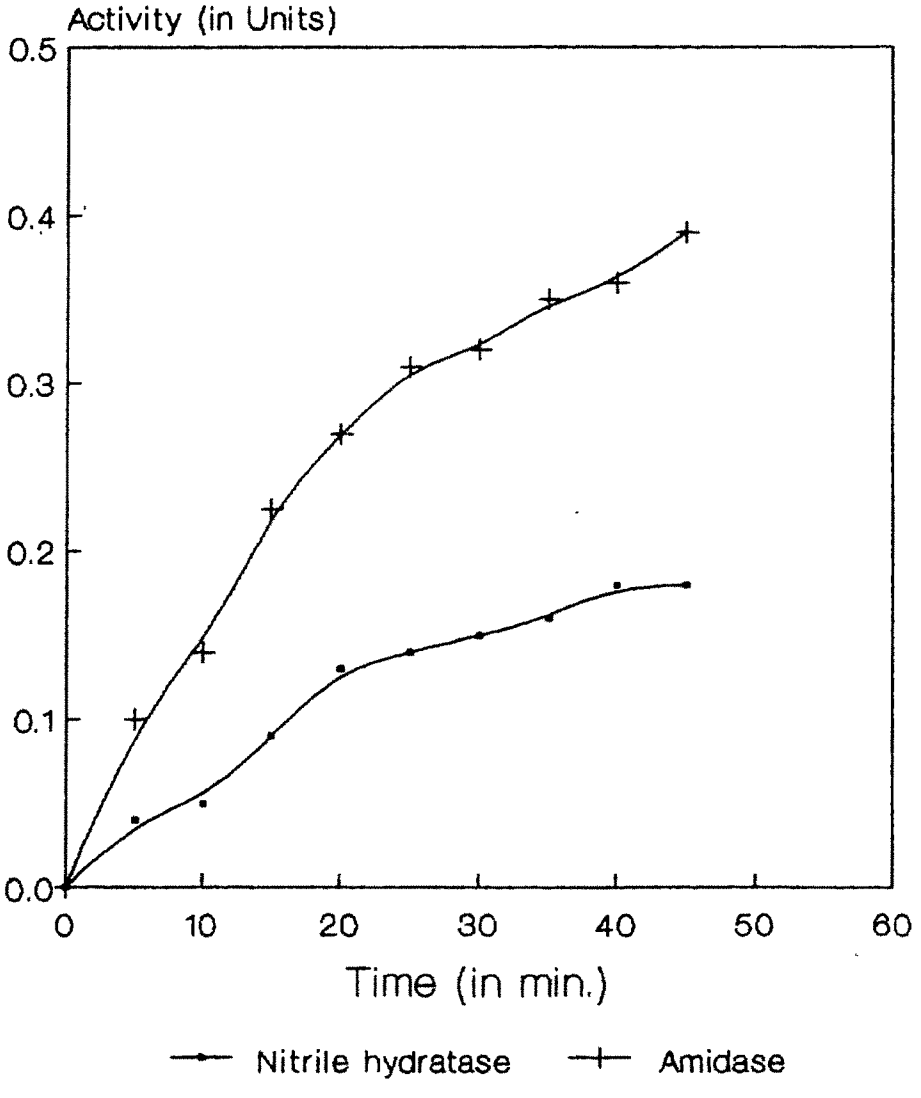
unit = moles of ammonia lib/min

Fig. 19 Effect of temperature on the activities of nitrile hydratase amd amidase



unit-μmoles of ammonia lib/min

Fig.20 Effect of time on the activities of nitrile hydratase and amidase



Unit = μ moles of ammonia lib

Table 15 Activity of Enzymes On Various Substrates

Substrate	Sp Activity
Acrylonitrile	0.105
Acetonitrile	0.110
Propionitrile	0.110
Acetamide	0.140
Acrylamide	0.132
Glutamine	0
Aspargine	0
Urea	0.0071
B- Cyano alanine	0.000

Sp act = μmoles of amm.lib./min/mg protn

substrate specificity and could act on acetonitrile, acrylonitrile and propionitrile whereas amino-propionitrile, and ß-cyano alanine were not good substrates (Table 15). Natural substrates for the enzymes involved in the breakdown of xenobiotic compounds are still not well understood. It is known that metabolism of natural organonitriles exist in plants and microorganisms (Knowles, 1976). *Pseudomonas sp. 13* is known to produce a nitrile hydratase that converts ß-cyanoalanine to asparagine (Yanese *et al.*, 1985). The enzyme in *Arthrobacter BC1* did not act on ß-cyanoalanine, indicating that it must be a different enzyme. In the case of amidases which act on naturally occurring amides, *e.g.* Asparginase, glutaminase, urease, are known for a long time. In this case the enzyme amidase from *Arthrobacter BC1* could not liberate ammonia from any of these amides. It will be interesting to identify the natural substrates of these enzymes in order to fully understand the evolution of the enzymes involved in not only in nitrile metabolism but also in the other enzymes which act on such xenobiotic substrates.

UV spectra of the enzyme preparation showed a characteristic protein absorption at 280 nm. It is known that certain nitrile hydratases, exhibit absorption at 720 nm, due to prosthetic group iron, and at 415 nm due to another cofactor, pyrrole quinoline quinone (PQQ) (Nagasawa *et al.*, 1987; Siguira *et al.*,

1988). PQQ is a putative cofactor used by nitrile hydratases. Such absorption peaks were, however, not obtained in the enzyme from *Arthrobacter BC1* . Not all nitrile hydratases contain heme and PQQ. The nitrile hydratase of *Rhodococcus rhodochrous J1* contain cobalt (Nagasawa *et al.*, 1986). The enzyme preparation from *Arthrobacter BC1* has a characteristic pink color. We could not however establish the presence of any cofactor or prosthetic group.

In all the above experiments ammonia was the parameter measured as the index of either nitrile or amide hydrolysis. Under these conditions the activity of amide hydrolyzing enzyme was many fold higher than that of nitrile hydrating enzyme (judged by the formation of acrylamide by GC or HPLC). Thus endogenous amidase was used in the coupled assay to estimate the activity of the nitrile hydratase. The cell free extracts of *Arthrobacter BC1* showed the presence of ß-lactamase, which is also a type of amidase. The organism *Arthrobacter BC1* was also found to be resistant to ß-lactum type antibiotics such as penicillin, ampicillin, cephalosporin etc. It is quite likely that this enzyme is either closely related to acrylamidase or possibly the same enzyme. Acrylamidase also showed acyl tranferase activity, characteristic of penicillinases. Recently, it has been reported that caprolactum is a good inducer of nitrile hydratase in *Rhodococcus rhodochorus N774*

(Nagasawa *et al.*, 1990). It will be interesting to investigate the similarity between lactamases and amidohydrolases involved in nitrile metabolism.

3.5 Induction of nitrile hydratase and amidase with various carbon and nitrogen sources.

It has been known that majority of the enzymes obtained from various bacteria involved in nitrile metabolism by nature are inducible, In all the cases nitriles and amides were found to be the most favorable inducers. Further experiments were performed in order to determine the inducibility of these two enzymes in *Arthrobacter BC1*.

Table 16. shows the activities of the nitrile hydratase and amidase enzymes when the organism *Arthrobacter BC1* was grown on various nitriles and amides as a sole source of carbon and nitrogen or when grown as a source of nitrogen only, in the presence of a other carbon source. Experiments were also carried out to see the effect on these enzymes, when acrylonitrile was provided as a carbon source only along with ammonium sulfate as a nitrogen source. In accordance with earlier reports, nitrile and amide were seen to be good inducers for both enzymes. Acrylamide, acetamide, and acetonitrile were able to induce both enzymes required for the breakdown of nitriles indicating a broad inducer specificity. However, activities of these two enzymes could not be

Table 16 Effect of carbon and nitrogen sources on the induction of nitrile hydratase and amidase

Substrate	nitrile hydratase (Sp. act)	amidase (Sp. act.)
Acrylonitrile (ACN) (0.2% v/v)	0.182	0.387
Acrylamide (ACM) (0.2% w/v)	0.150	0.230
Acetamide (ACTM) (0.2% w/v)	0.135	0.270
Glucose (0.2%) ACN (0.2% ") (24 hrs)	N.D	N.D
Glucose (0.2%) ACN (0.2% ") (48 hrs.)	0.028	0.083
Glucose (0.2%) ACM (0.2% w/v)	ND	ND
Glucose (0.2%) ACTM (0.2% w/v)	0.330	0.450
Glucose (0.2%) Amm. Sulph (0.2%)	ND	ND
Succinate (0.2%) ACN (0.2% v/v)	0.327	0.375
Succinate (0.2%) Amm Sulph (0.1%)	ND	ND
Acetate (0.2%) ACN (0.2% v/v)	0.463	0.583
Acetate (0.2%) Amm. Sulph 0.2%	ND	ND
ACN (0.2%) amm. Sulph. (0.2%)	----------No growth----------	

Unit = μ mols of ammonia lib/min
Sp act.= Units/mg protein
Results are an avrage of at least 3 measurements
ND - Not detectable

effectively checked with benzonitrile as an inducer or an enzyme substrate. Benzonitrile being sparingly soluble in water, assay of these enzymes was difficult.

As shown in Table 16. the activities of both these enzymes were only detectable after 48 hr. when the organism was grown in basal salt medium containing glucose as a carbon source along with acrylonitrile as a nitrogen source. This could be because glucose itself, or its metabolites are involved in the repression of these enzymes. This seem to be in line with the observations made during the experiments relating to ammonia production during growth of this organism when glucose and acrylonitrile were provided as a carbon and nitrogen sources respectively. On the contrary when acetonitrile was substituted for acrylonitrile as a nitrogen source such repressive effects were not observed. This could be explained as a result of more easier assimilation acetic acid. On the other hand, acrylic acid, the end product of acrylonitrile degradation, is known to be not easily utilizable, and its accumulation could cause deleterious effect on the organism. The easily utilizable metabolites of glucose, would obviously, interfere with further utilization of acrylic acid, and it would lead to slower utilization of acrylic acid. The accumulation of acrylic acid, thus could lead to the repression of these enzymes nitrile hydratase, and amidase, ultimately this results in the reduced

nitrile breakdown and less accumulation of acrylic acid. Thus, it can be concluded that acrylic acid is most probably the repressor of these enzymes. Similar conclusions can be drawn from the Table 16. When glucose is provided with acrylamide, it reduces the activity of these enzymes, but it does not affect the activity in the case of acetamide.

The results of these experiments, also, clearly indicate that ammonia produced as an end product of nitrile hydrolysis has a definite repressive effect on both the enzymes. Total loss of the enzyme activities were observed when the organism was grown in glucose, succinate and acetate as a carbon source and ammonium sulfate as a nitrogen source. Further, no growth resulted when the organism was inoculated in a medium containing acrylonitrile and ammonium sulfate. This is due to the fact that repression of these enzymes by ammonia will prevent the breakdown of acrylonitrile. Hence, carbon source will not be available to the organism for growth. When glucose was supplemented in the same medium, activity of these enzymes was seen to be restored. This result can be explained by the fact that, during growth the levels of ammonia would be depleted in the medium, and it could reverse the repression of these enzymes. consequently the enzymes would then become susceptible to induction by acrylonitrile.

It has long been known that the enzymes; nitrilase, nitrile

hydratase and amidase are subject to induction and repression. *Brevibacterium sp. R-312* is known to possess a constitutive, broad substrate specific nitrilase (Jallageas, 1978) and an inducible amidase. The nitrile hydratase is also known to be inducible in *Arthrobacter J1* (Asano *et al.*, 1980). The inducers in this organism being acetonitrile and acetamide. Acetate, which repressed the formation of nitrile hydratase, did not affect the amidase activity indicating a difference in inducer specificities. Contrary to this, acetate did not induce the formation of acetonitrilase or acetamidase in *N. rhodochrous* (Collins and Knowles, 1986). In this case acetamide was seen to induce both the enzymes.

The organism *Arthrobacter 19* which hydrolyzed acrylonitrile directly to acrylic acid and ammonia also possessed an inducible nitrilase (Bandyopadyay *et al.*, 1986). An amidase which had no role to play in the metabolism of acrylonitrile was also coinduced in this organism. The role of the amidase was not clear.

Microorganisms are known to produce different types of amidases. A mutant strain of *Brevibacterium R 312, Brevibacterium A4* known to have lost the acylamide amido hydrolase (EC 3.5.1.4 acrylamidase) activity, but was observed to produce another amidase which acted on a amino amides converting them sterospecifically to a amino acids. This enzyme

was different than the enzyme involved in the hydrolysis of acrylamide (Kieny-L`Homme *et al.*, 1981). The amidase coinduced in *Arthrobacter I 9* could also have different properties and may be participating in a different reaction. However, attempts have not been made to verify the properties of this amidase.

In case of *Rhodococcus sp.* (Miller and Gray, 1982) acrylonitrile and acrylamide were good substrates for the nitrile hydratase and amidase. They were formed when the organism was grown on acetonitrile, whereas, acrylonitrile and acrylamide were unable to induce these enzymes. It was speculated that acrylic acid could be toxic to the cell and/or that acrylamide was a repressor of the enzyme amidase. Similarly in the case of *N. rhodochrous* (Linton and Knowles, 1987) it was observed that acrylonitrile and acrylamide were poor sources of carbon/nitrogen and also poor inducers. However *in vitro,* the enzyme was able to act on acrylonitrile and acrylamide as substrates.

From the above reports, it can be concluded that the nitrilase and amidase induced by acetonitrile may very well act on acrylonitrile and acrylamide. This indicates that the regulation of these enzymes are at the genetic level. On the contrary, the only known enzyme induced by acrylonitrile, is nitrilase which does not convert acrylonitrile to acrylamide,

but directly breaks it down to acrylic acid and ammonia. This indicates the possible deferential genetic locus of these enzymes.

The enzymes of *Arthrobacter BC1* , seemed to exhibit contrary behavior as compared to the enzymes reported from other organisms. In this case nitrile hydratase and amidase are seen to be induced by acrylonitrile and acrylamide. It can be seen that, in order to have the efficient utilization of acrylonitrile as a carbon and nitrogen source the machinery for further utilizing acrylic acid is imperative. It is, however, postulated that the amidase and nitrile hydratase may be part of a single operon, sharing the same regulatory apparatus. (Collins and Knowles, 1986). The genetic level evidence for the possible existence of such an operon structure was first reported in *Brevibacterium R 312* (Jean-Francois Mayaux *et al.*, 1990).

In the case of *Arthrobacter BC1* the acrylic acid metabolizing enzymes should also be induced in sequence with the nitrile hydratase and amidase, thus making this organism capable of utilizing acrylonitrile as a sole source of carbon and nitrogen. Whereas in case of other organisms, genetically, the metabolism of acrylic acid may be linked to a nitrilase system and not a nitrile hydratase; amidase system as found in the

case of *Arthrobacter I9* (Bandyopadhyay *et al.*, 1986).

Conclusive evidence for such a mechanism can be obtained only after examining the genes responsible for both these enzymes and elucidation of their structure. By this it could conclusively be proved that nitrilase and nitrile hydratase/amidase are genetically different.

Another distinct possibility worth considering is that the reaction of nitrilase may be the effect of both nitrile hydratase and amidase being present together and functioning simultaneously. Acrylamide which is formed during the hydration of acrylonitrile may remain bound to the enzyme and hence, may not be detectable as free amide. Such a possibility can be conclusively established by isolating the protein bound acrylamide. Alternatively it can be demonstrated that in no stage, acrylamide, free or bound, is produced thus showing the reaction of nitrilase as being totally different from nitrile hydratase and amidase.

From our experiments, we can postulate that the genes responsible for nitrile metabolism especially in the case of *Arthrobacter BC1* , will consist of structural genes, coding for the synthesis of nitrile hydratase, amidase and acrylic acid metabolizing enzymes and would be a part of a single operon.

3.6 Purification of Nitrile hydratase and Amidase:-

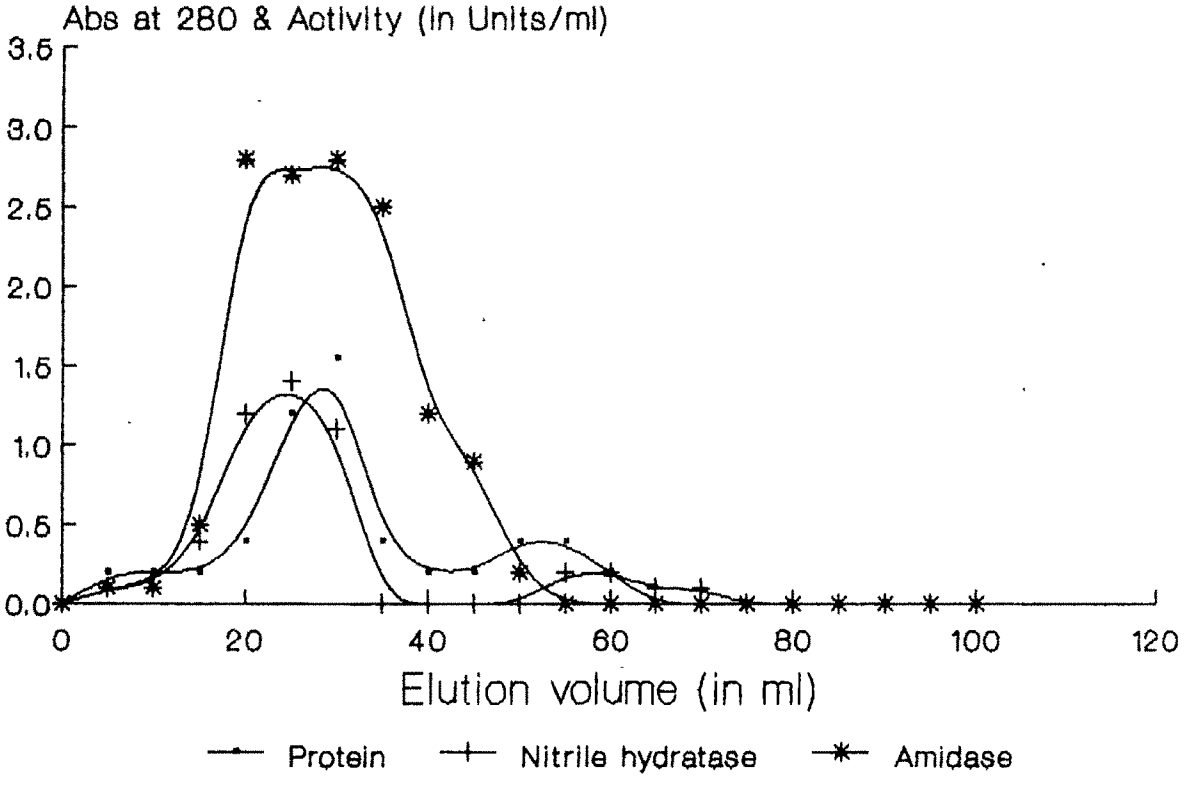
Nitrile hydratase has been purified and characterized from *P. chlororaphis B23* (Nagasawa *et al.*, 1987), *Brevibacterium R312* (Nagasawa *et al.*, 1986) and *Rhodococcus sp. N774* (Endo and Wantanbe, 1988). Little is known about the amidase which participate in these reactions (Nagasawa and Yamada, 1989). The amidase from *Arthrobacter J1* has been purified and characterized (Asano *et al.*, 1982). Nitrilases have been earlier purified from barley leaves (Thimann and Mahadevan, 1964), ricine nitrilase from *Pseudomonas* (Sugiura *et al.*, 1988) and benzonitrilase from *Nocardia* species and *Fusarium solani* (Harper, 1976; 1977a; b). Nitrilase from *Arthrobacter I9* which was the only known organism to grow on acrylonitrile as a sole source of carbon and nitrogen (Bandyopadhyay *et al.*, 1986) has also been purified. As mentioned earlier, the organism showed the presence of nitrile hydratase and amidase. However, it was not possible to show any differences in its localization, kinetic properties or with respect to inducer specificities. There by it had become impereive to purify these enzymes and prove their independent existence. Even though acetaldehyde is a konwn inhibitor of amidase (Amarant *et al.*, 1988) unfortunately, the attempts to inhibit amidase resulted in the simultaneous inhibition of nitrile hydratase.

Cell free extract was prepared as described in materials.

Sequential ammonium sulfate precipitation was then performed, 0-20%, 20-40%, 40-60%, 60-80%, and 80-100%. It was observed that both the enzymes precipitated at 40-60% saturation of ammonium sulfate. The resultant precipitate, after suspending in 50 mM KDE buffer, was passed through Sephadex G-50 column this was initially done for the purpose of desalting as in all the enzyme reactions, ammonia was being measured. Some amount of purification was observed even in Sephadex G-50. Ammonium sulfate precipitate was dialyzed in order to remove the excess ammonia. However it was observed that, dialysis overnight resulted in the loss of nitrile hydratase activity. Therefore to achieve rapid desalting and simultaneous purification, the Sephadex G-50 column was made longer. In this procedure effective desalting and purification was obtained. Fig. 21 shows the elution profile of these two enzymes in Sephadex G-50 columns.

Although we were not able at this stage to separate the two enzymes, nitrile hydratase and amidase, a considerable purification was obtained (Table 17). Amidases are generally known to be of smaller molecular weight 25-30 KD (Bandyopadhyay *et al.*, 1986; Carver *et al.*, 1989) as compared to nitrile hydratases which has many subunits and is larger (Table 5). Thereby, it was anticipated that these enzymes would get separated in Sephadex G-75 wherein nitrile hydratase would be

Fig.21 Elution profile of nitrile hydratase and amidase on Sephadex G-50



Unit - μmols of ammonia lib/min
column dimension 1.8X23 cms

Table:17 Purification of nitrile hydratase and amidase

	Total activity (units)		Sp. Activity (Units)		Protein (mg)	Fold		Recovery (%)	
	NH	AMD	NH	AMD		NH	AMD	NH	AMD
Crude extract	2.788	9.533	0.136	0.466	20.5	---	---	---	---
Ammonium sulphate	---	---	---	---	9.87	---	---	---	---
60 % Saturation									
Pooled active fractions									
a. Sephadex G-50	2.410	8.500	0.310	1.015	7.76	2.3	2.3	87	89
b. Sephadex G-75	1.940	7.135	1.66	2.46	1.17	12.2	5.3	70	75

NH - nitrile hydratase
AMD - amidase

Unit = μmols of ammonia lib/min

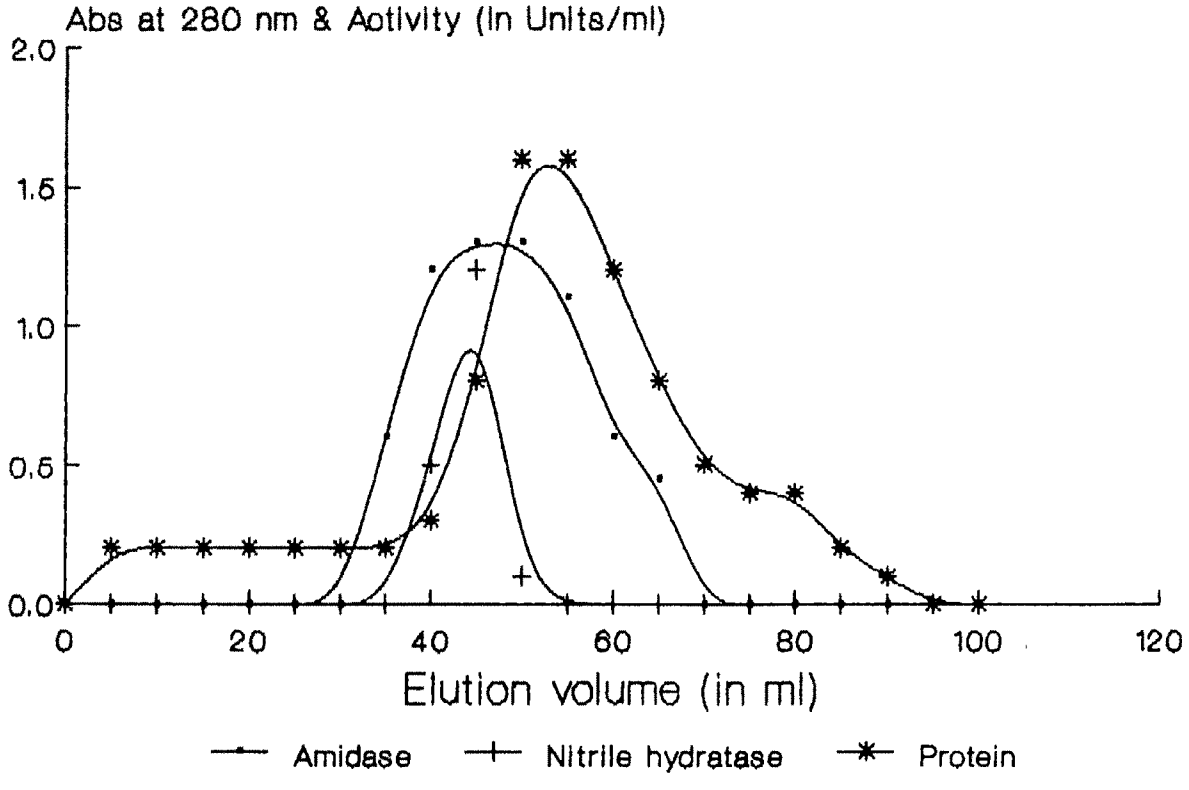
Specific activity = units /mg protein

eluted in the void volume and amidase would filter through. Fig. 22 shows the elution profile of nitrile hydratase and amidase in Sephadex G-75. Even at this stage these enzymes were co-eluted, but the protein was available at a considerable level of purity. Table 17 shows the outcome of all the purification steps.

Inspite of the fact that about 12.5 fold purification was obtained from crude extract still the enzymes could not be quantitatively separated in all these protocols. Therefore, in a separate preparation the ammonium sulfate precipitated and desalted enzyme was loaded into DEAE cellulose. It was observed that (Fig. 23) most of the enzyme remained bound to the column. It was then eluted with a linear gradient of 0-1M NaCl. When the fractions were assayed, only the amidase activity was seen with considerable purity, while the nitrile hydratase activity was not detectable. Similar results were obtained with DEAE Sephadex (Fig. 24).

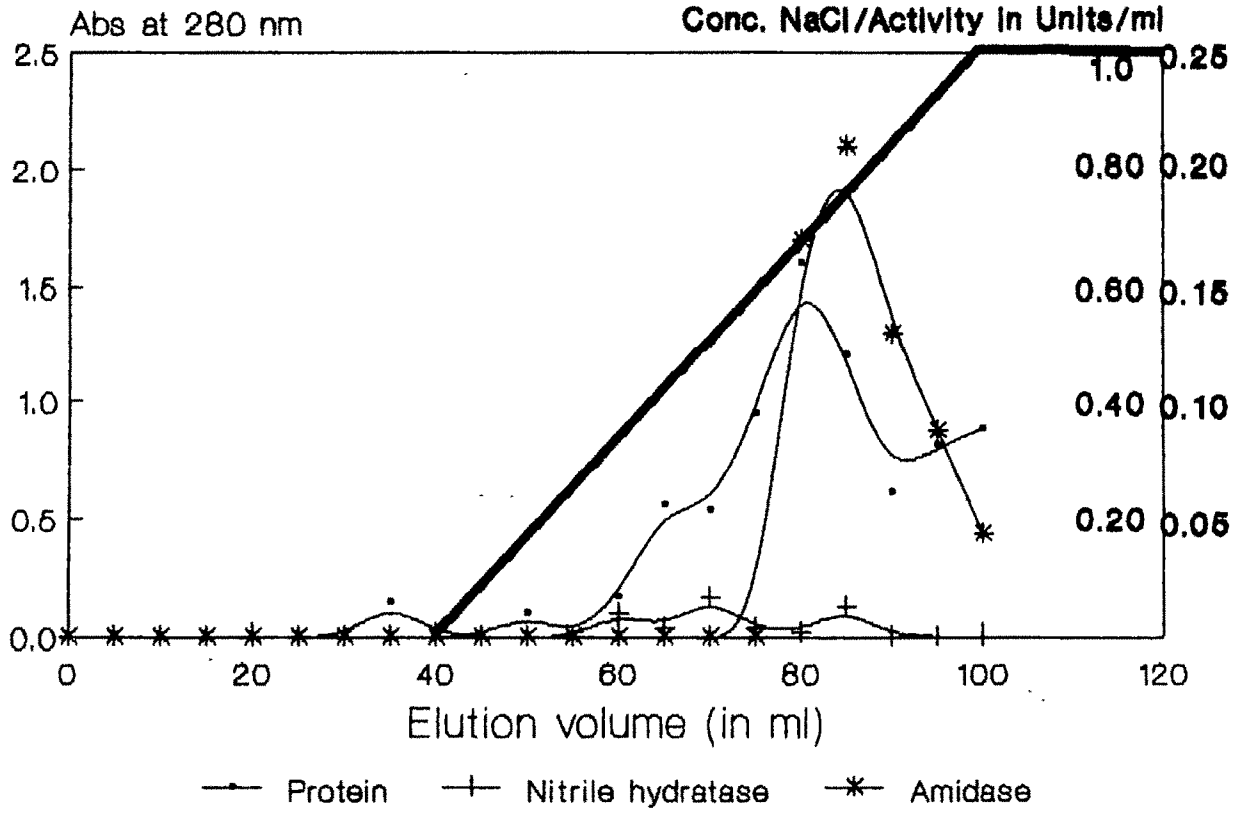
Subsequently attempts were made to separate these enzymes on Sephadex G-200. The crude enzyme was prepared, precipitated with ammonium sulfate, and then passed through sephadex G-50. Fig. 25 shows the elution profile of these enzymes in sephadex G-50 column. The column used in this case was longer than the earlier one (column dimensions 2.2X40 cms). It was observed that the active protein eluted from fractions 15 to 20. Earler

Fig. 22 Elution of nitrile hydratase and amidase on Sephadex G-75



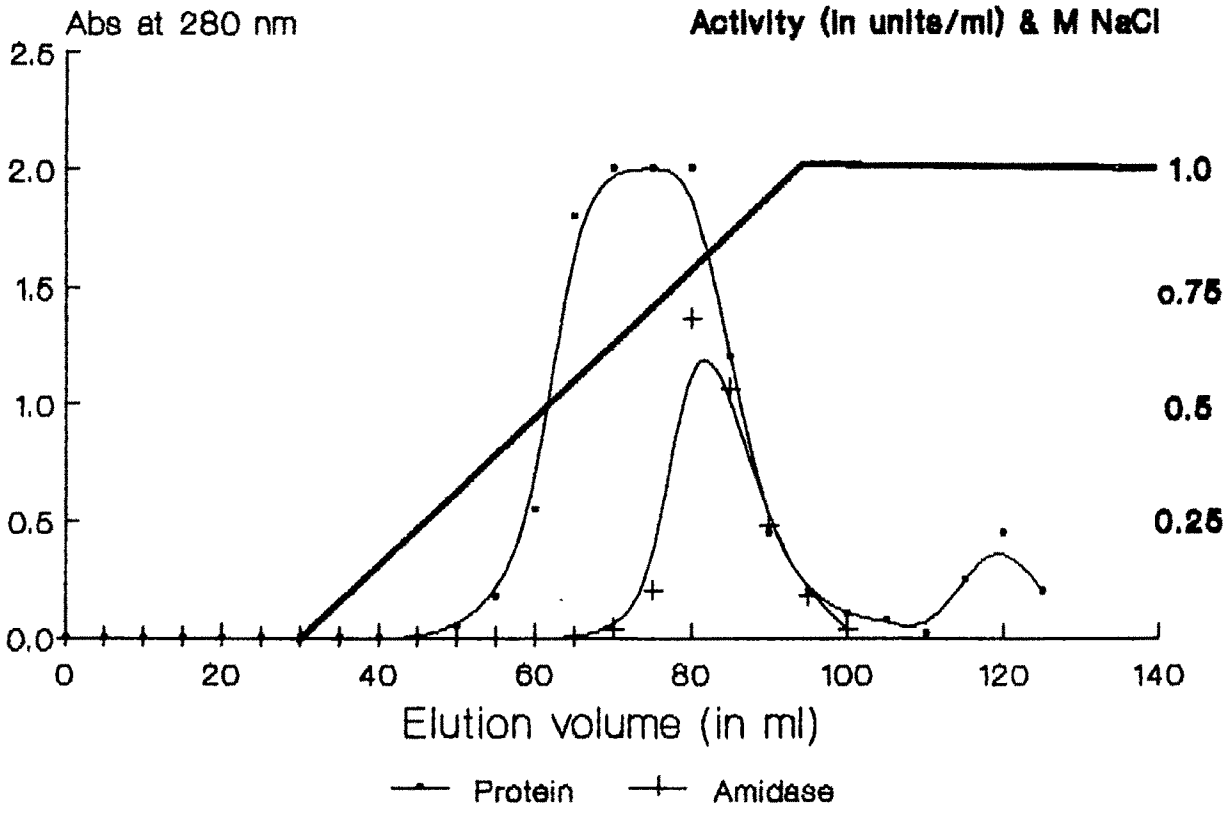
Unit - μmoles of ammonia lib/min
Column dimension 2.2X45 Cms.

Fig. 23 Elution of nitrile hydratase and amidase on DEAE cellulose



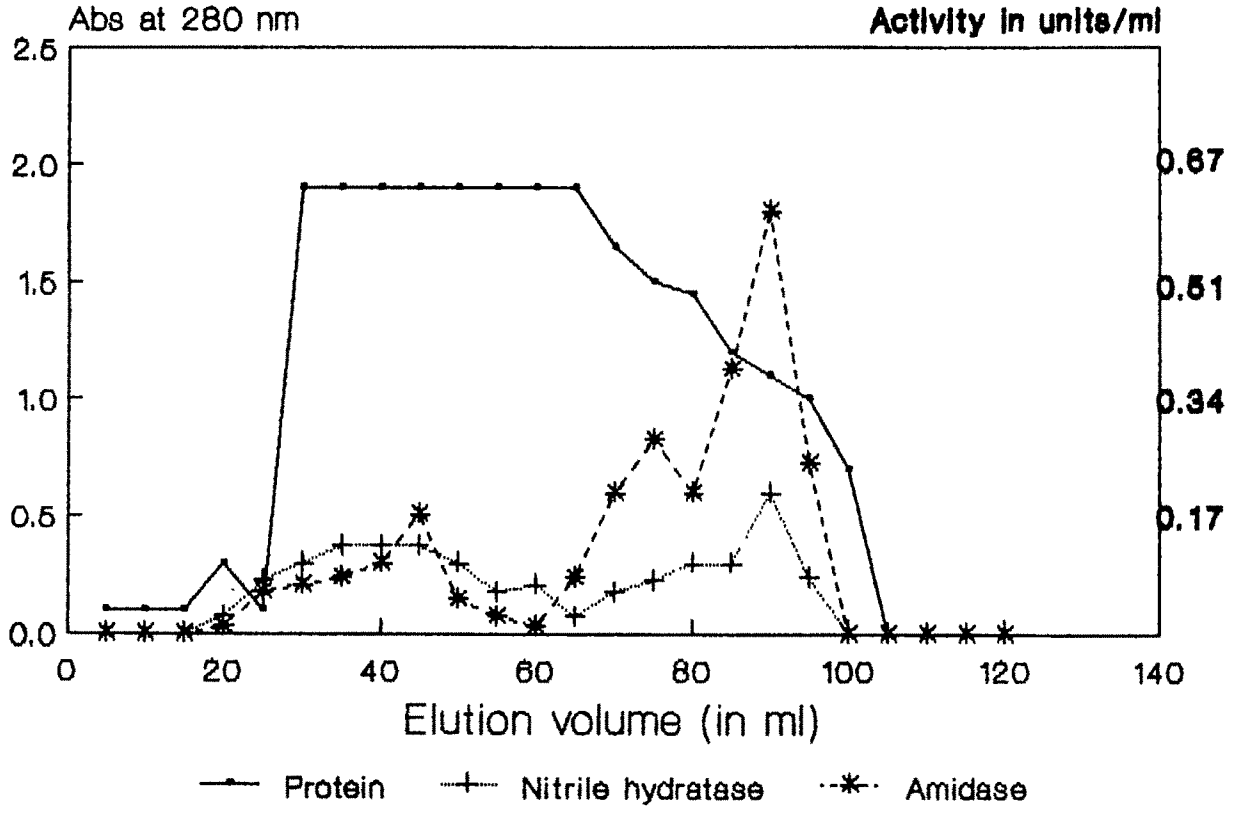
Unit-μmoles of ammonia lib/min

Fig. 24 Elution of nitrile hydratase and amidase on DEAE Sephadex



Unit - μ moles of ammonia lib/min

Fig. 25 Elution profile of nitrile hydratase and amidase on Sephadex G-50



Unit-μmoles of ammonia/ml/min

fractions had high protein but it showed considerably low activity of the enzymes. This purification step had resulted in extremely low recovery of protein approx. 12% (Table 18). The fractions which showed low activity were pooled together (Fractions 4 to 14) and assayed and it was seen that the endogenous ammonia production (ammonia liberated in the absence of substrates, nitrile or amide). The enzyme preparation was dialyzed overnight at 4 ^{o}C against KDE buffer in order to remove any free ammonia. The results are shown in Table 19. From these results it is quite clear that the enzyme produced ammonia during the reaction as indicated by the control which was terminated prior to the start of the reaction. The ammonia production could only occur if the enzyme had some substrate either nitrile or amide bound to it.

To obtain a better yield of the enzyme the organism was grown with 0.2% v/v acrylonitrile overnight. After 24 hr. 0.2 % acrylonitrile was added again and the organism was allowed to grow for another 12 hr. after which the cells were harvested. This addition of high amount of substrate could have saturated the cells with acrylonitrile. As the enzyme preparation was already subjected to sephadex G-50 and dialysis, the chances for the presence of any free substrates are extremely low. Moreover, it was observed that when this enzyme preparation stored at 4 ^{o}C it produced ammonia, indicating that the enzyme

Table: 18 Purification of nitrile hydratase and amidase

	Total activity (units)		Sp. Activity (Units)		Protein (mg)	Fold		Recovery (%)	
	NH	AMD	NH	AMD		NH	AMD	NH	AMD
Crude extract	5.056	4.815	0.040	0.038	126	---	---	---	---
Ammonium sulphate	---	---	---	---	56	---	---	---	---
60 % Saturation									
Pooled active fractions									
a. Sephadex G-50	.688	1.632	0.0688	0.1632	10	1.8	4.8	14	34
b. Sephadex G-200	.621	0.9540	0.674	1.035	0.921	17.0	27.0	12	20

NH - nitrile hydratase
AMD - amidase

Unit = μ mols of ammonia lib/min

Specific activity = units/mg protein

Table: 19 **Binding of substrate to enzyme**

	SUB BLANK 1	SUB BLANK 2	NITRILE HYDRATASE (Units)	AMIDASE (Units)
1. Un dialyzed (Control)	2.111 (105.6%)	1.999 (100%)	2.444 (123%)	2.666 (133%)
2. Enzyme dialyzed for 24 hrs. at 4 °C against KDE buffer (100 mM)	1.644 (200%)	0.822 (100%)	1.644 (200%)	2.111 (257%)
3. Dialysed enzyme kept at 4 °C for 24 hrs	1.999 (110%)	1.822 (100%)	1.999 (110%)	2.055 (112%)
4. Enzyme dialysed for 6 hrs. at 40 °C against KDE buffer (100 mM	0.088 (100%)	0.088 (100%)	0.088 (100%)	1.022 (1161%)

Unit = μmols of ammonia lib/min

1 - Blank contained only buffer and enzyme extract and was terminated at the end of incubation time (30 Min.)

2 - blank contained only buffer and enzyme extract and was terminated prior to start of the reaction and allowed to incubate till the end of incubation time (30 Min.)

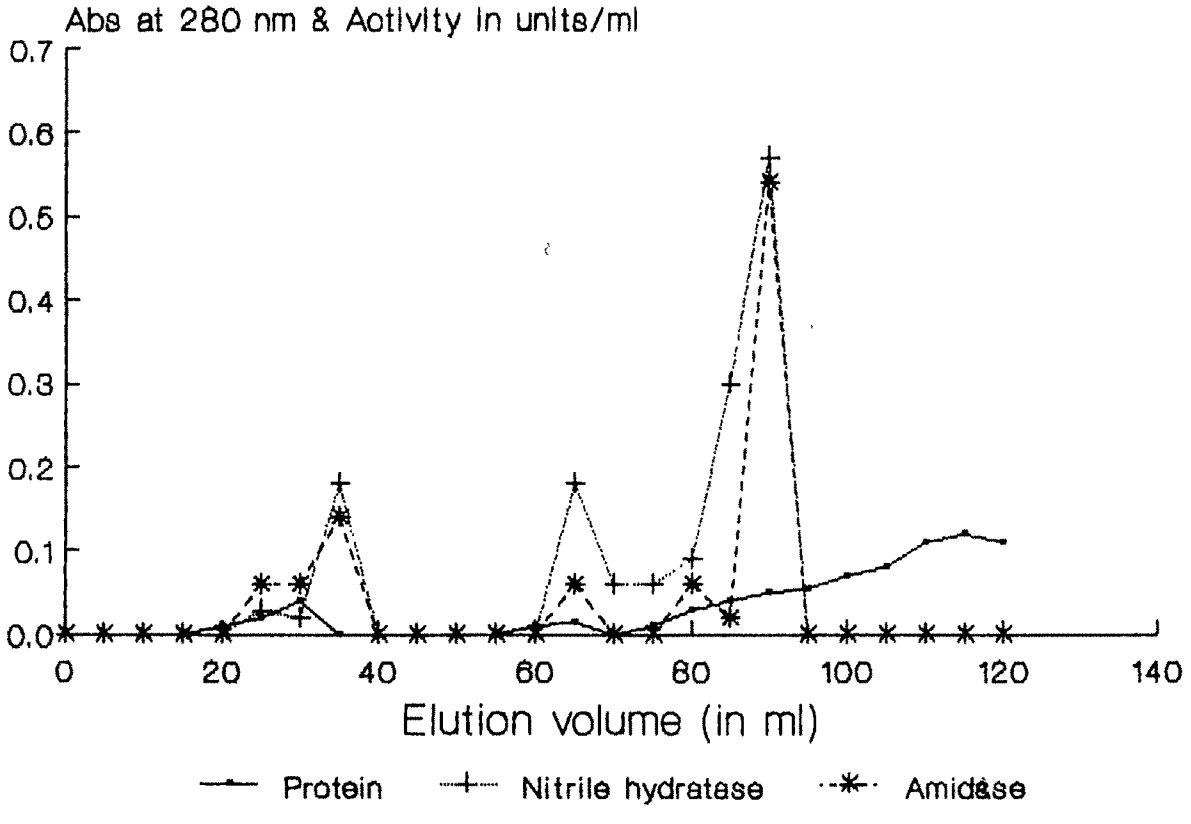
* - Values in prarenthesis indicate the activity as percent compared to ^{1} blank value

was catalyzing even at temperatures as low as 4°C. In order to remove all the bound substrates this enzyme preparation was dialyzed for 6 hr. at 40°C. The resultant preparation showed amidase activity but not of nitrile hydratase. It was presumed that during the course of dialysis at elevated temperatures the nitrile hydratase was inactivated.

The fractions 15 to 20 were concentrated by precipitation with ammonium sulfate, dialyzed and chromatographed on sephadex G-200 column. Even in this case the activity of both nitrile hydratase and amidase were not separated and they eluted as a single protein peak indicating that they were similar (Fig. 26).

In order to achieve some more degree of purification, and to determine the approximate molecular weight, the enzyme was subjected to high performance size exclusion chromatography. Fig. 27 shows the elution profile of this enzyme in TSK SW 3000 column. There were 3 peaks at retention times of 4.6 min., 7.8 min., and 10.4 min. The peak at 7.6 and 10.4 contained the amidase activity and nitrile hydratase activity could not be detected. Fig. 28 shows the elution profile of crude extract, ammonium sulfate precipitate and active fraction no. 3 from Sephadex G-50 eluant in TSK SW 3000 amidase had a peak at 10.4 min. whereas the amount of protein was extremely low. However nitrile hydratase could not be detected. Fig. 29 shows the

Fig. 26 Elution profile of nitrile hydratase and amidase in Sephadex G-200



Unit-μmoles of ammonia lib/min

HIGHSPEED SIZE EXCLUSION PROFILE OF ENZYMES ON TSK SW-3000

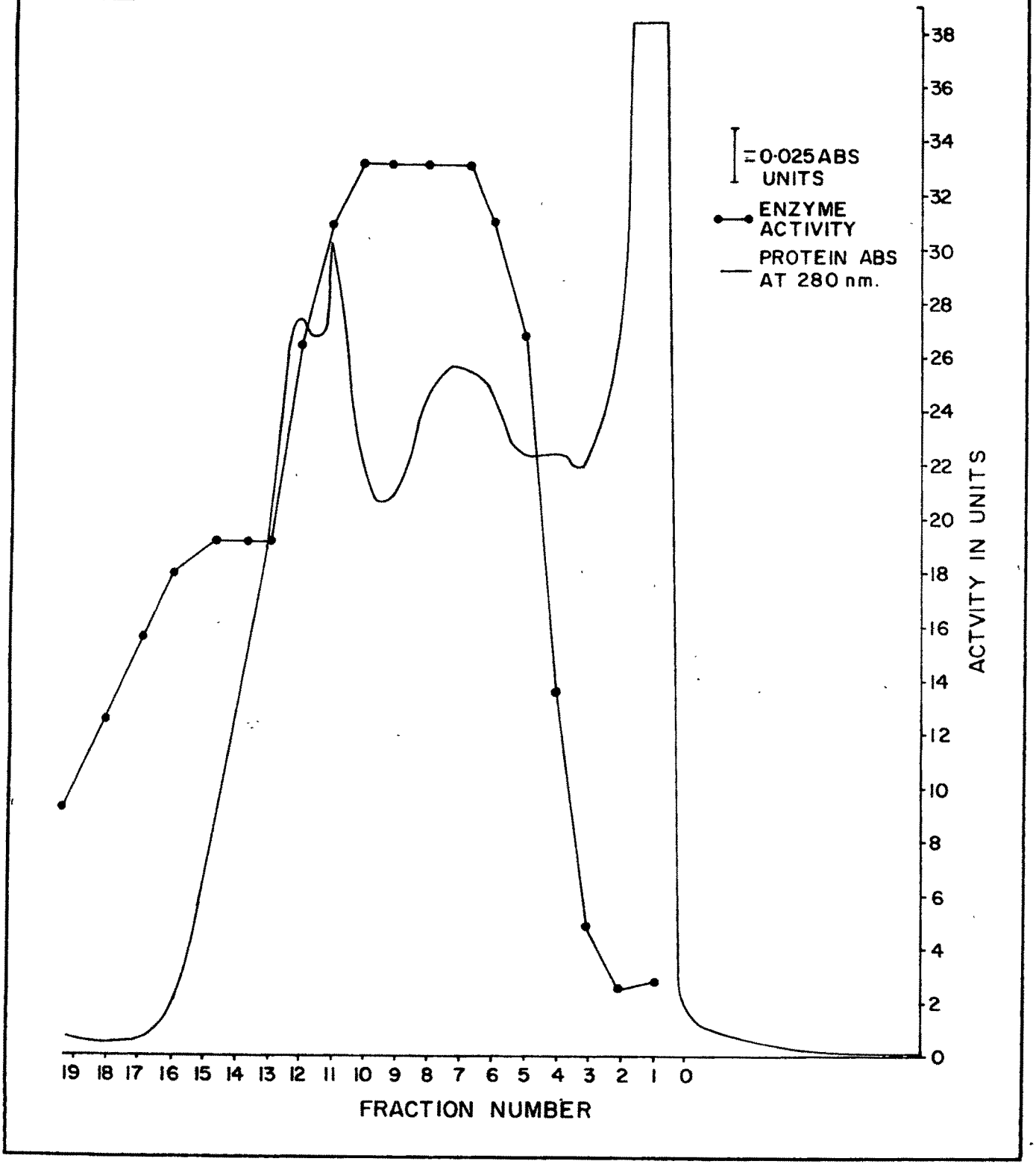


FIG. 28

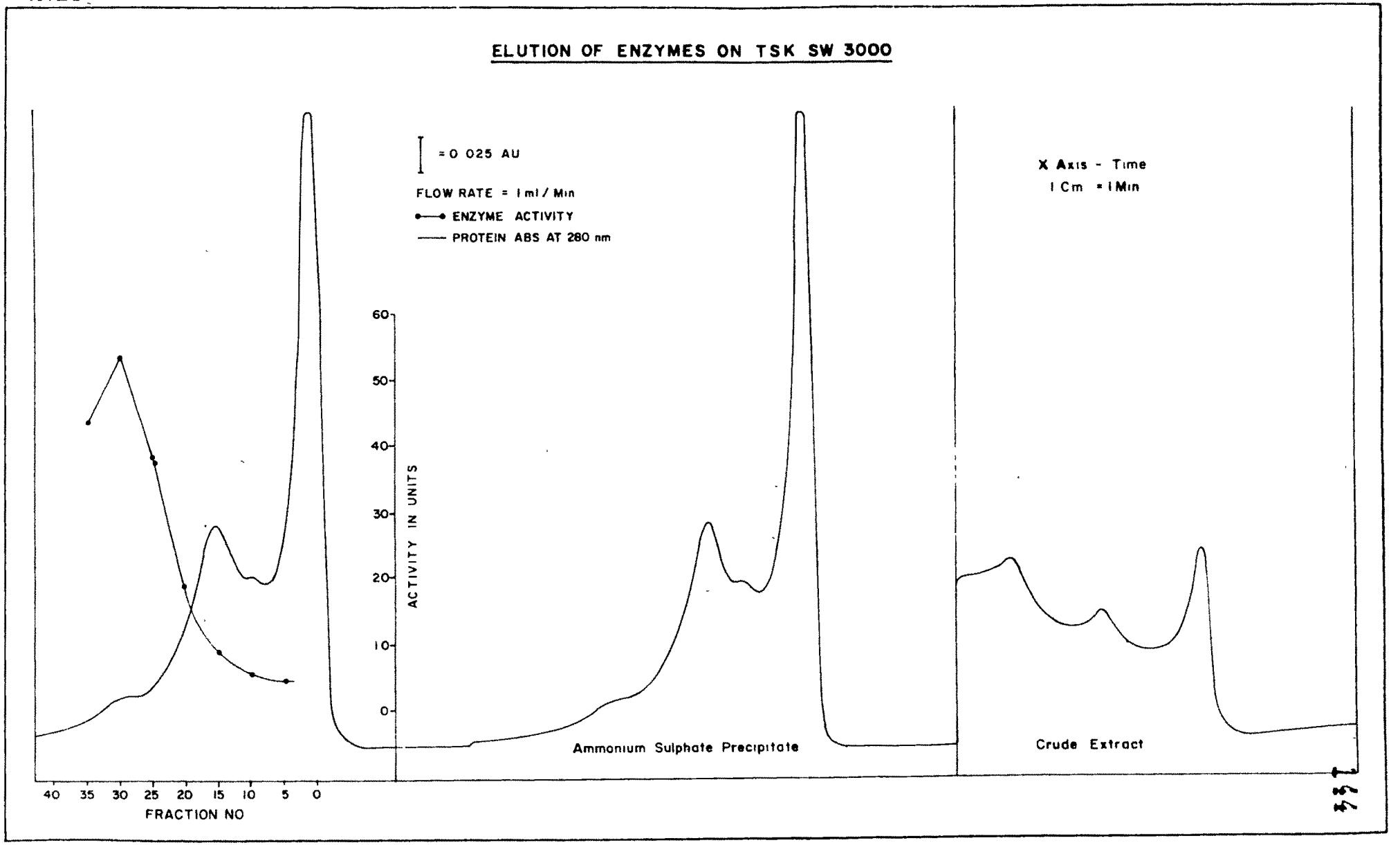
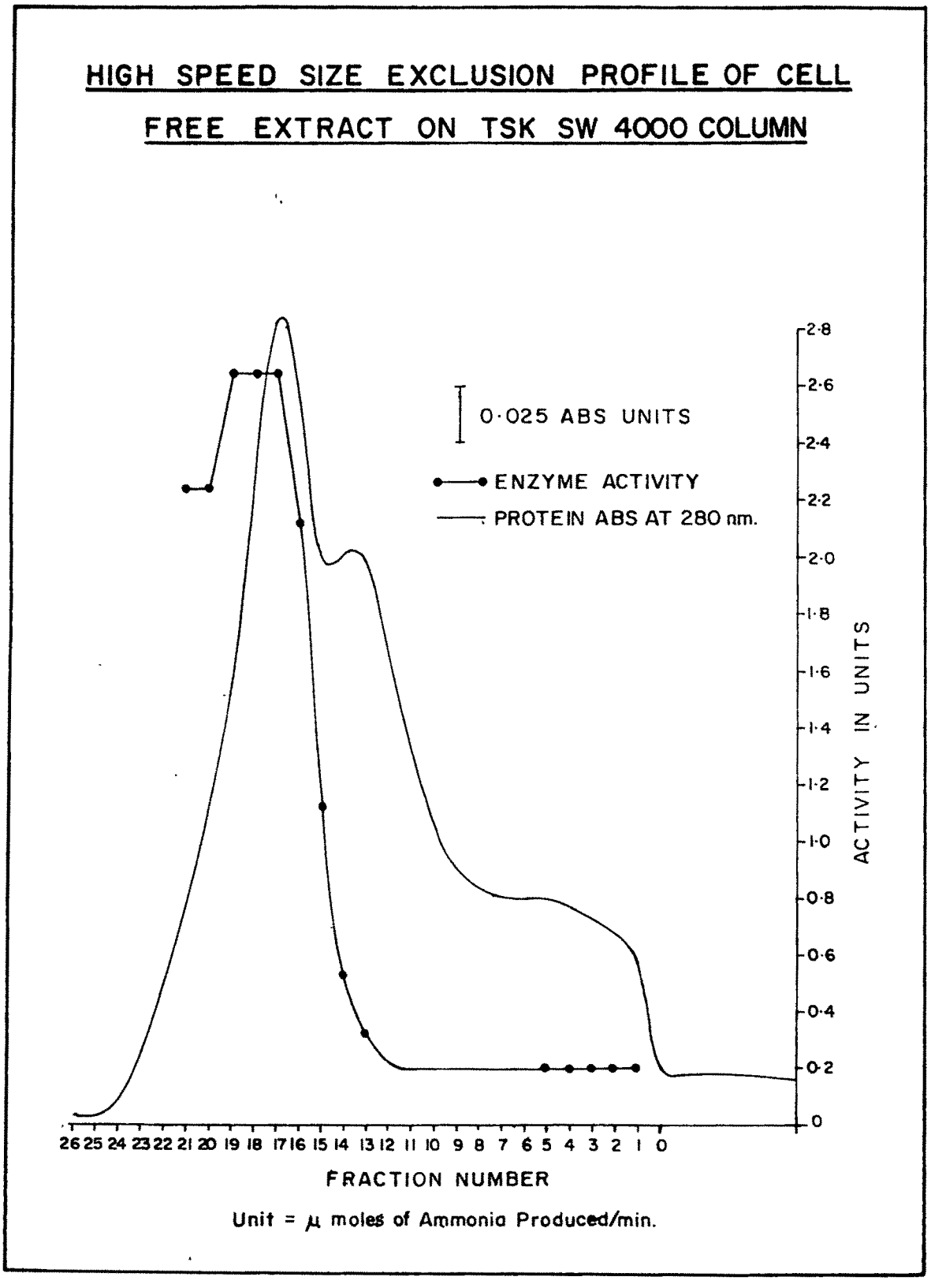

ELUTION OF ENZYMES ON TSK SW 3000
= 0 025 AU
FLOW RATE = 1 ml / Min
ENZYME ACTIVITY
PROTEIN ABS AT 280 nm
X Axis - Time
1 Cm = 1 Min
ACTIVITY IN UNITS
60
50
40
30
20
10
0
40 35 30 25 20 15 10 5 0
FRACTION NO
Ammonium Sulphate Precipitate
Crude Extract

FIG.29


HIGH SPEED SIZE EXCLUSION PROFILE OF CELL FREE EXTRACT ON TSK SW 4000 COLUMN
0·025 ABS UNITS
ENZYME ACTIVITY
PROTEIN ABS AT 280 nm.
2·8
2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
ACTIVITY IN UNITS
26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0
FRACTION NUMBER
Unit = μ moles of Ammonia Produced/min.

elution profile of amidase in TSK SW 4000 column. In *Arthrobacter sp. 19,* amidase which apparently had no role in the metabolism was detected and was of approximately the same molecular weight as enzyme reported here.

Substrate induced time dependent aggregation of 12 subunits of the enzyme nitrilase was reported in *Nocardia rhodochrous NCIB 11216* (Harper, 1977). In general, nitrile hydratases have also been seen to have elaborate subunit structures (Table 5). In the present study it has been seen that amidase had approximate molecular weight of about 30,000 and nitrile hydratase presumably higher when the substrate was bound to the enzyme. Later experiments with whole cells showed that acrylamide production from acrylonitrile was much higher at 4°C than the breakdown of acrylamide leading to the accumulation of acrylamide at 4°C. These observations and earlier reports show that nitrile hydratase can form aggregates and can also bind strongly to either acrylonitrile or acrylamide. Thus, in this organism, it is possible that acrylonitrile induces the aggregation of smaller subunits to become an active nitrile hydratase. The smaller subunits may independently possess amidase activity. In the case of *Arthrobacter I9* (Bandyopadyay *et al.*,1986) a similar mechanism could explain role of the amidase found. Substrates of both enzymes could remain bound to the enzyme till the end. This will result in no detectable form

of amides making the enzyme reaction seem as though it is a single step reaction.

In order to determine the induction of aggregation of subunits, by acrylonitrile, the extract was incubated with acrylonitrile for various periods of time, and passed through TSK SW 3000. The results of these experiments colud not clearly indicate such a phenomenon. It is likely that longer exposure to acrylonitrile is required to cause this effect.

Fig. 30 & 31 show the protein profile of crude extract, ammonium sulfate/Sephadex G-50 and Sephadex G-75 eluant on TSK SW 2000. It can be seen that a considerable purification has been observed in these steps. It can also be seen that Sephadex G-50 fractions showed the removal of a good amount of other proteins. The enzyme preparation, in native PAGE, showed three bands of acyltranferase activity. Which is the characteristic feature of amidases. However, we could not conclusively show that this enzyme was homogeneous in SDS-PAGE.

3.7 Genetics of Nitrile hydrolysis in *Arthrobacter BC1*

Recently, much attention has been focused on the molecular mechanisms involved in the microbial degradation of toxic chemicals. However, little is known about the molecular mechanism involved in the biodegradation of nitrile compounds. It has been postulated that nitrile hydratase and amidase are

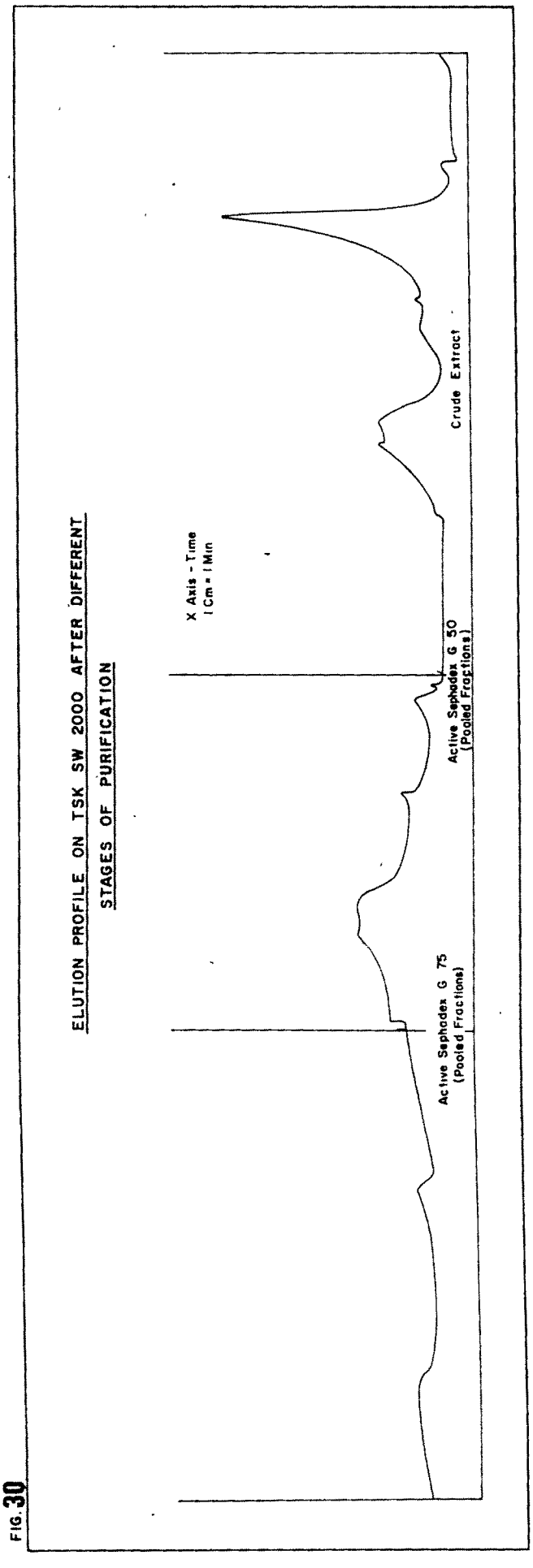
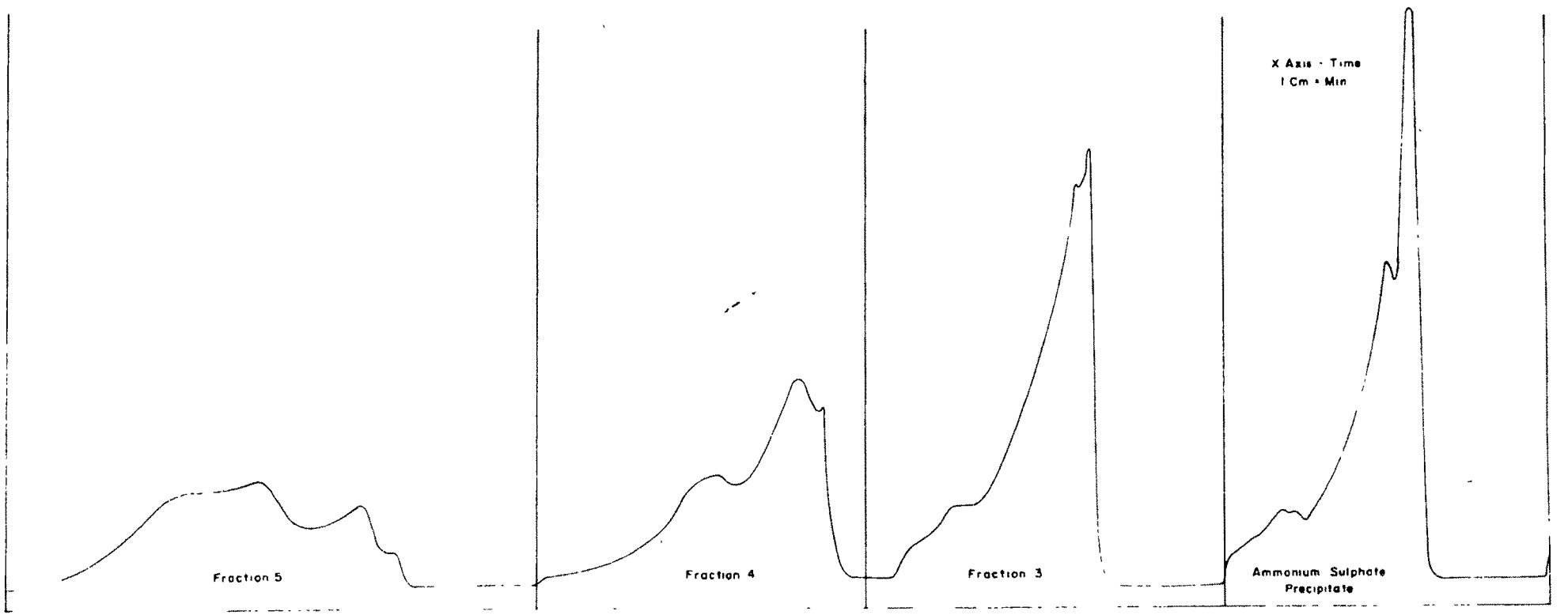

ELUTION PROFILE ON TSK SW 2000 AFTER DIFFERENT STAGES OF PURIFICATION
X Axis - Time
1 Cm = 1 Min
Crude Extract
Active Sephadex G 50
(Pooled Fractions)
Active Sephadex G 75
(Pooled Fractions)

FIG. 30

FIG 31

PROFILE OF FRACTIONS FROM DIFFERENT STAGES OF PURIFICATION IN TSK SW 2000



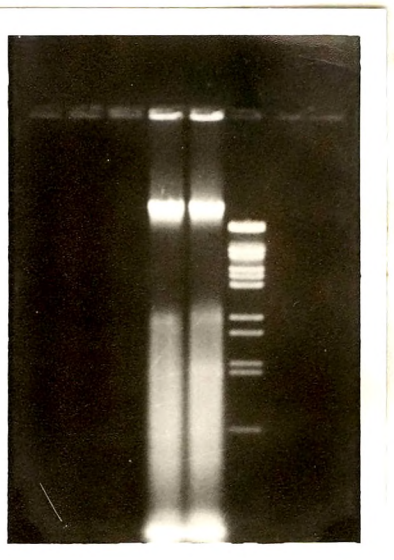
structural genes having the same operon regulation (Collins and Knowles, 1983). Further it was observed that *in Pseudomonas sp.13*, by curing experiments, the genes responsible for assimilation of n-butronitrile was located on the chromosome (Yanese *et.al.*, 1985). The organism *Pseudomonas. sp. 13* contained plasmids, but curing the organism of plasmids, still retained the ability to assimilate butronitrile. Plasmids were also observed in *K. ozanae* which had the ability to degrade bromoxynil, a herbicide, and an aromatic nitrile (McBride *et al.*, 1986). The organism produced bromoxynil specific nitrilase which converted bromoxynil directly to the corresponding acid and ammonia without the formation of amide. The 2.6 Kb gene locus was cloned in E. coli using a pUC 19 vector (Stalker and Mcbride, 1987). Further the enzymes from the cloned cells were compared to the wild type, and the corresponding gene of about 1212 bases was sequenced. The nucleotide sequence was in good agreement with the protein composition. *Azotobacter sp.* isolated in our department contained a plasmid coded nitrile hydratase activity and amidase coded by the chromosomal DNA (Goud, 1987). Recently the gene responsible from an enantiomeric selective amidase was also cloned from *Brevibacterium R 312* and it was seen that it was coupled to nitrile hydratase whose structural gene was 73 bp down stream and they probably were part of a single operon.

Apart from these studies, there has been no report on the genetic locus of the metabolism of nitriles. The genetic studies are of importance as the enzymes could then be obtained in large quantities. Also isolation these genes and its behavior in other hosts can be evaluated and regulation of the genes could be studied in detail.

From these considerations, it was interseting to determine the organization of the genes and their localization correponding to these enzymes. In addition, cloning of these genes would also help to understand the nature of the enzymes and their regulation. It would also be interesting to find different hosts which could express these genes and aquire the ability to convert acrylonitrile to acrylic acid, but may not be able to utilize acrylonitrile as a sole carbon and nitrogen source.

In order to achive these objectives, initial studies were carried out to determine the location of these genes in *Arthrobacter BC1*. Since, molecular genetics of *Arthrobacter* is not known experiments have been carried out to standardize the conditions suitable for plasmid isolation. Many methods were evaluated. The profile after rapid lysis with Triton X-100 and binding the DNA with glass powder using Geneclean™ did not indicate the presence of any extrachromosomal genetic elements. The next method was using lysozyme and SDS (Fig. 32). In this there was a large band at about 15Kb corresponding to the

Fig. 32 Plasmid profiles of Arthrobacter BC1 by alkaline lysis method



Lane 1 & 2 Arthrobacter DNA
Lane 3 Std. lambda DNA digested with Bst E II

standard Bst E II digest. There were faint bands at about 5 Kb and 3 Kb. which were confirmed to be high molecular weight RNA as they disappeared when treated with RNAse. In order to ascertain whether the 15 Kb was a plasmid or fragments of chromosomal DNA, the DNA was subjected to restriction analysis. Digestions were performed with EcoRI and Hind III (Fig. 33 a,b). There was no sign of digestion for 30' and on prolonged digestion a smear was produced indicating the possibility of the bands being chromosomal fragments, unlike plasmids which produce discrete bands.

As the methods used did not yield any extrachromosomal elements, method of Wheatcroft and Williams (1981) was attempted, since it had demonstrated the presence of plasmids in *Pseudomonas putida* which were not detectable by other methods. Fig. 34 shows the profile when the cells were lysed by this method. It was interesting to observe that the DNA preparation by this method indicated the presence of DNA bands faintly visible at about 5 Kb as compared to standard Bst EII digested Lambda DNA. These bands were not RNA as they were resistant to RNAse treatment. This method of lysis was evaluated with two controls standard plasmid pUC 19 as a positive control and a strain E. coli HB101, which does not possess any plasmids, as a negative control (Fig. 35). It can be seen that this method was efficient in the preparation of

Fig. 33 Restriction digestion of DNA obtained by alkaline lysis

Fig. 33a

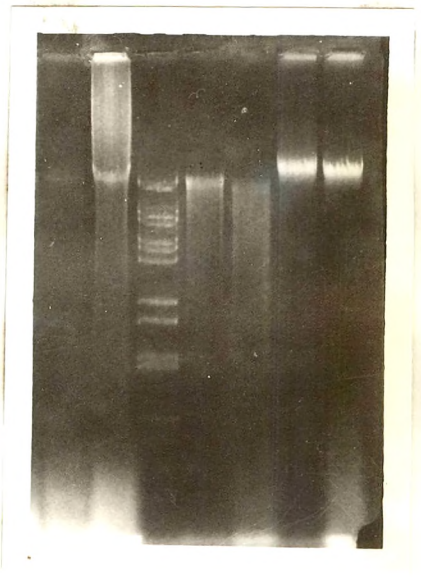


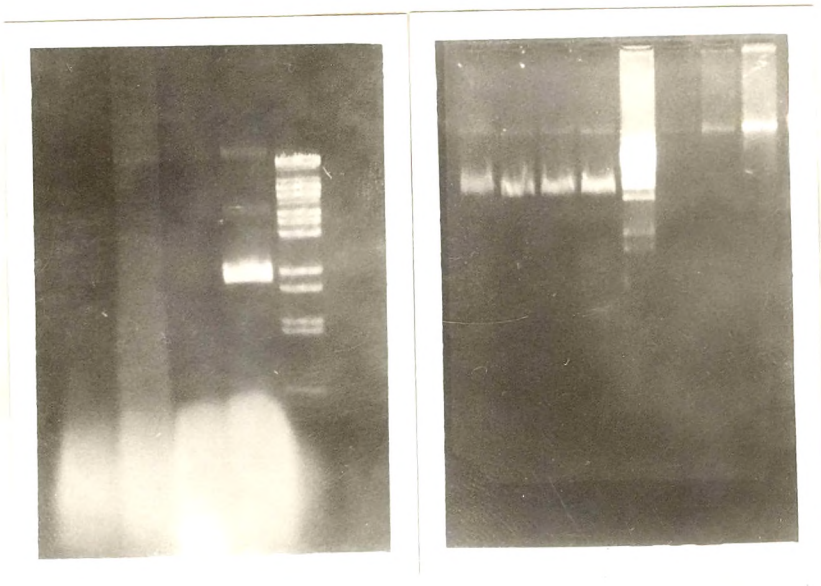
Fig. 33b

Lane 1 Std. lambda DNA
digested with Bst EII
2 Arthrobacter DNA
digested with Hind III
3 Arthrobacter DNA
digested with EcoRI
4 Arthrobacter DNA
treated with RNase
5 Arthrobacter DNA
control

Fig. 34 Plasmid profile of Arthrobacter by method of Wheatcroft and Williams

Lane 1&2 Arthrobacter DNA
Lane 3 lambda DNA digested with Bst EII

Fig. 35 **Plasmid profiles of control organisms by the method of Wheatcroft and Williams**



Lane 1	E. coli HB101
Lane 2	E. coli containing pUC19 Plasmid
Lane 3&8 lambda	DNAdigested with Bst EII
Lane 4-7 E. coli	XL1 containing bluescript plasmid

plasmids and also produced almost very little contamination due of chromosomal DNA.

As this method was found effective, this was further used to prepare plasmids under different growth conditions, in order to produce the optimum amount of these plasmids. The organism was grown in Luria broth, containing 0.2% acrylonitrile and in basal salt medium containing glucose and 0.2% acrylonitrile. Fig. 36 a,b shows the plasmid profiles of this organism when grown under such conditions. The organism showed entirely different plasmid profile under different growth conditions. It can be seen from Fig. 36a that plasmids were amplified when grown in LB and LB containing ACN, indicating the possibility of increased copy number of plasmids. It was also observed that this organism when grown on LB and LB with ACN did not show the activities of the enzyme nitrile hydratase and amidase, making the correlation between these plasmids and metabolism difficult. An entirely different plasmid profile was observed when the organism was grown in BSM containing 0.2% ACN (Fig. 36 b). The three bands could indicate the superhelical, nicked circular, and covalently closed circular forms of the plasmid DNA. This organism however showed the activities of both enzymes nitrile hydratase and amidase indicating the possible link between this plasmid and metabolism.

The lysate was centrifuged on CsCl /EtBr density gradient

Fig. 36 Plasmid profiles of Arthrobacter under different growth conditions

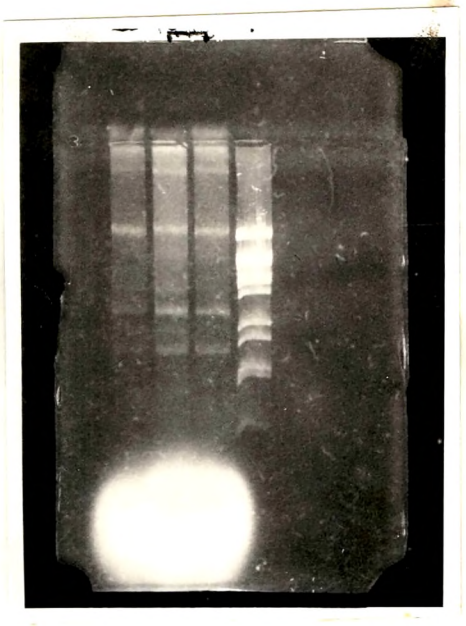


Fig. 36a

Lane 1 Arthrobacter grown in L.B.
Lane 2&3 Arthrobacter grown in L.B. + ACN
Lane 4 Lambda DNA digested with Bst EII

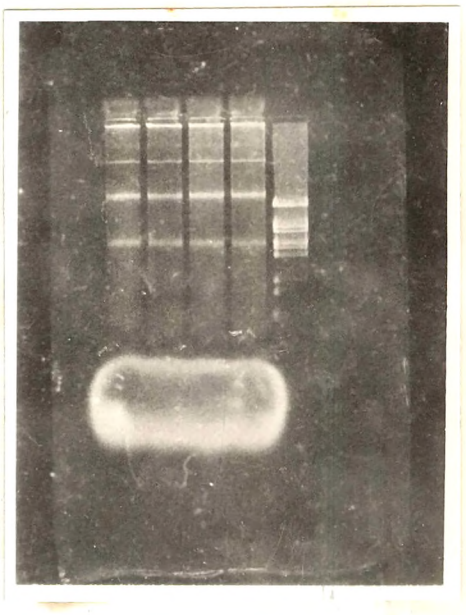


Fig. 36b

Lane 1-4 Arthrobacter grown in BSM containing glucose and ACN
Lane 5 lambda DNA digested with Bst EII

centrifugation and this showed two bands. The lower plasmid band was eluted, EtBr removed, and DNA precipitated with 98% ethanol. This plasmid was digested with EcoRI, Hind III, and Not I but conclusive evidence could not be obtained as the plasmids were resistant to digestion carried out for longer times.

In an attempt to establish the relation between plasmid and metabolism, curing studies were undertaken. Different curing methods were tried with acridine orange, acriflavine, and SDS. The colonies were then replica plated on to LB, LB. ACN, BSM ACN, BSM acrylamide plates. It was difficult to observe cured colonies as the growth in minimal plates containing acrylonitrile itself was small and curing could not be established. It was also observed that this organism was resistant to many antibiotics, especially those of the ß-lactum group, (Table 20) and also produced ß-lactamase. Assuming that the ampicillin resistance was conferred by plasmid, ampicillin resistance was used as a marker to score cured colonies. The results are shown in Table 21. The ampicillin sensitive colonies initially did not grow in BSM containing acrylonitrile or acrylamide, indicating that these factors may reside in the plasmid. On subculturing, it was seen that the organism regained the ability to grow on acrylonitrile. It was also observed that this cured strain did not contain plasmids but

Table 20 Antibiotic sensitivity of Arthrobacter BC1

Antibiotic	Min. Inhib. Conc.
Tetracycline	< 100
Kanamycin	1000
Ampicillin	600
Penicillin V	1000
Penicillin G	1000
Streptomycin	600
Cephalosporin	1000
Erythromycin	200

Conc. as μgm/ml& grown in Nutrient media

Table 21 Growth of cured colonies of Arthrobacter BC1

Cured colonies	L amp Agar	BSM ACN Agar	BSM ACRM Agar	L agar
A 3/5	-	-	-	+
B 1/1	-	-	-	+
C 2/5	-	-	-	+
C 2/1	-	-	-	+
D 1/6	-	-	-	+
D 2/4	-	-	-	+

Curing agent, acridine orange

retained the ability to grow on nitriles. This could be explained as the plasmid was not responsible for nitrile hydrolysis and may be involved in conferring some other phenotype such as antibiotic resistance or tolerance. Further work is necessary to conclusively establish any relationship between plasmid and the capacity to utilize acrylonitrile by this organism. Isolation of these plasmids and transferring them to other hosts will enable us to establish the link, if any, between the plasmid and metabolism. This will also enable us to clone the gene responsible for nitrile hydratase and amidase. It is possible that gene may code for more than one structural gene and a regulatory region. Moreover the reason for the differential plasmid profile under different conditions will also be of importance.

Another interesting aspect is to evaluate the capacity of this *Arthrobacter BC1.* to accommodate broad host range plasmids of *Pseudomonas* origin e.g. pKT230, pKT 231. It would help transform and to express *Pseudomonas* TOL genes in *Arthrobacter.* This will be of enormous importance as the capacity of *Pseudomonas sp.* in biodegradation of xenobiotics is well established. *Arthrobacter sp., Coryneform* group of bacteria, in general, are known to be dominant in nitrile degradation and in biotransformation. In effect transfer of genes within *Pseudomonas and Arthrobacter* could lead to construction of

bacterial strains with enhanced biodegradative capacities. Since *Arthrobacter BC1* was resistant to a variety of antibiotics, appropriate screening markers were not available for selecting transforments after tranformation with pKT 230 & pKT 231. Therefore, transformation procedure for *Arthrobacter BC1* remains to be developed.

3.8 Bioconversion of acrylonitrile to acrylamide and acrylic acids:-

Nitriles are widely used in organic synthesis and as precursors for compounds such as amides and organic acids. However, chemical conversion of nitriles poses several disadvantages as reactions require strongly acid or basic conditions. Energy consumption is high and toxic substances like hydrogen cyanide are produced. Currently attention is focused on the use of biocatalysts, i.e. cells and enzymes, bring about such conversions. The advantages of the bioconversion process are:-

a) The use of low salts and other compounds

b) The pH and temperature conditions of the reaction are not severe.

c) Does not produce toxic by-products.

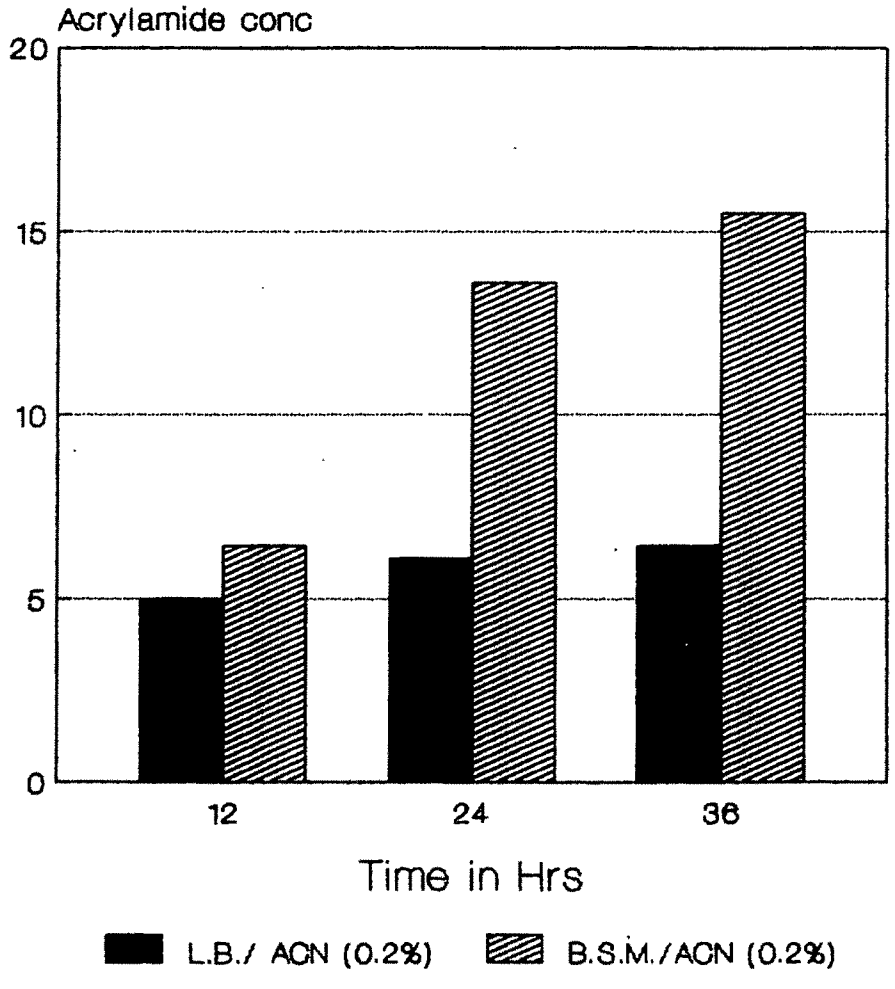
d) They are specific and produce optically pure compounds.

Such studies were initiated by Prof. Galzy and his colleagues in France with the organism *Brevibacterium sp. R312* (Bui *et*

al., 1982) and were able to patent the process. In Japan, Nitto Chemical Co. along with Kyoto University, have developed a process of using poly acrylamide gel entrapped cells of *Pseudomonas chlororaphis* to convert acrylonitrile to acrylamide. This process is already producing about 6000 tons of acrylamide. (Nagasawa *et al.*, 1987). *Arthrobacter BC1*, showed rapid accumulation of acrylamide and acetamide, during the course of whole cell studies. In order to evaluate the capacity of this organism for bioconversion, attemptes were made to use whole cells and cells trapped in acrylamide beads. *Arthrobacter BC1* was grown in different media (e.g., minimal media containing acetonitrile and acrylonitrile, LB containing acrylonitrile, minimal media containing glucose and acetonitrile, etc.).

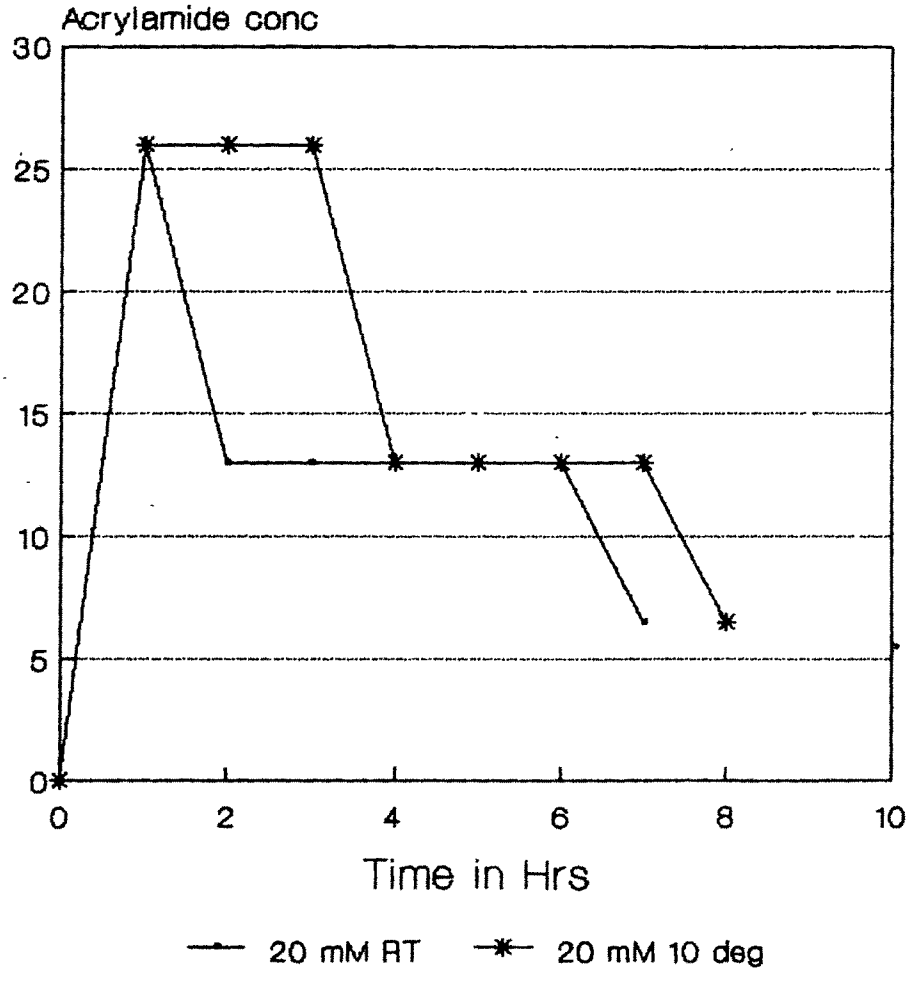
We observed that the cells grown in glucose and acetonitrile gave very good bioconversion (Fig. 37). The bioconversion was more quantitative between 10-20 ^{o}C. At higher temperatures, the acrylamide formed tends to be broken down faster to acrylic acid and ammonia as seen in Fig. 38a&b. The ratio of cells to substrate also seemed to influence the bioconversion rate. Fig. 39 shows the effect of cell concentration on the accumulation of acrylamide. It can also be seen that cell concentration also influences the breakdown products Fig. 40 It is quite clear that acrylonitrile greatly reduces the breakdown of the

Fig 37Effect of growth medium on the accumulation of acrylamide by Arthrobacter sp. BC 1 cells



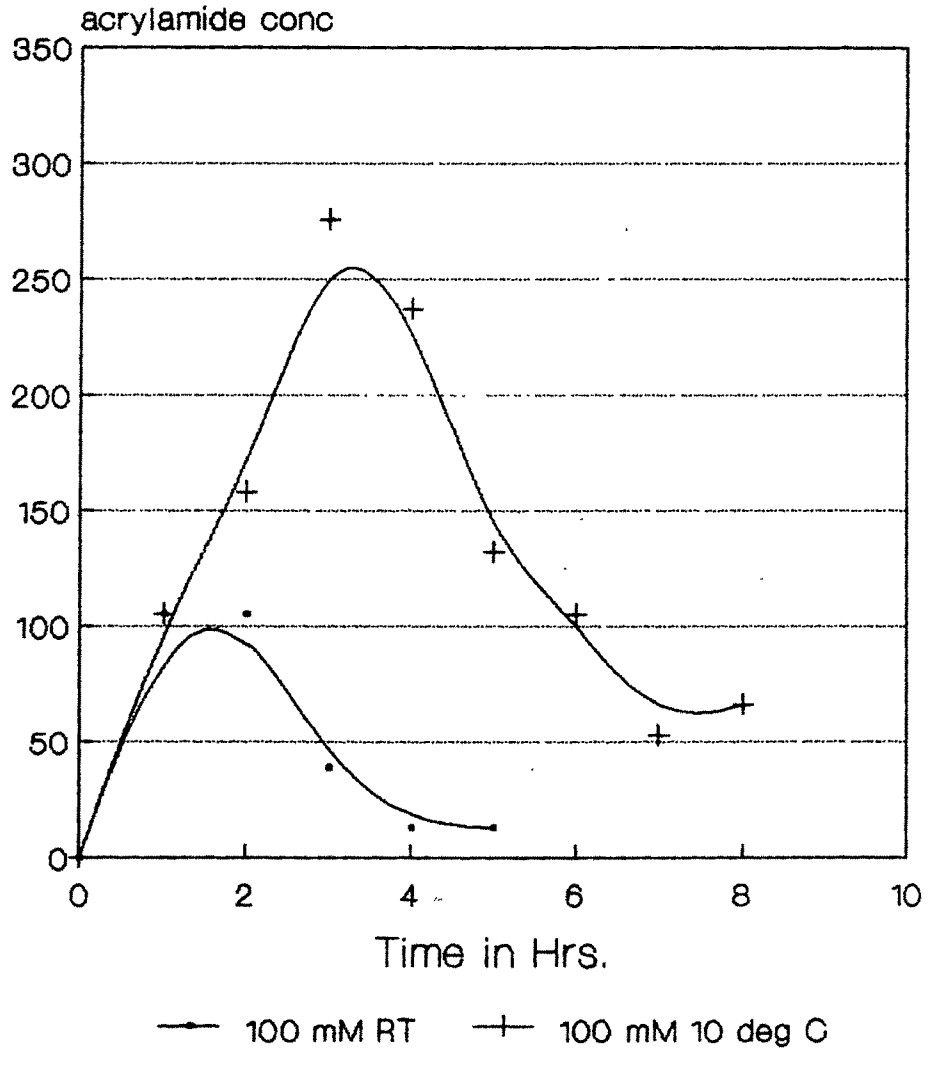
Values in milli molar
conc of acrylonitrile 100 mM
Temp 10 deg C

Fig 38 Effect of Temp. and acrylonitrile conc. on the accumulation of acrylamide by Arthrobacter BC1 cells



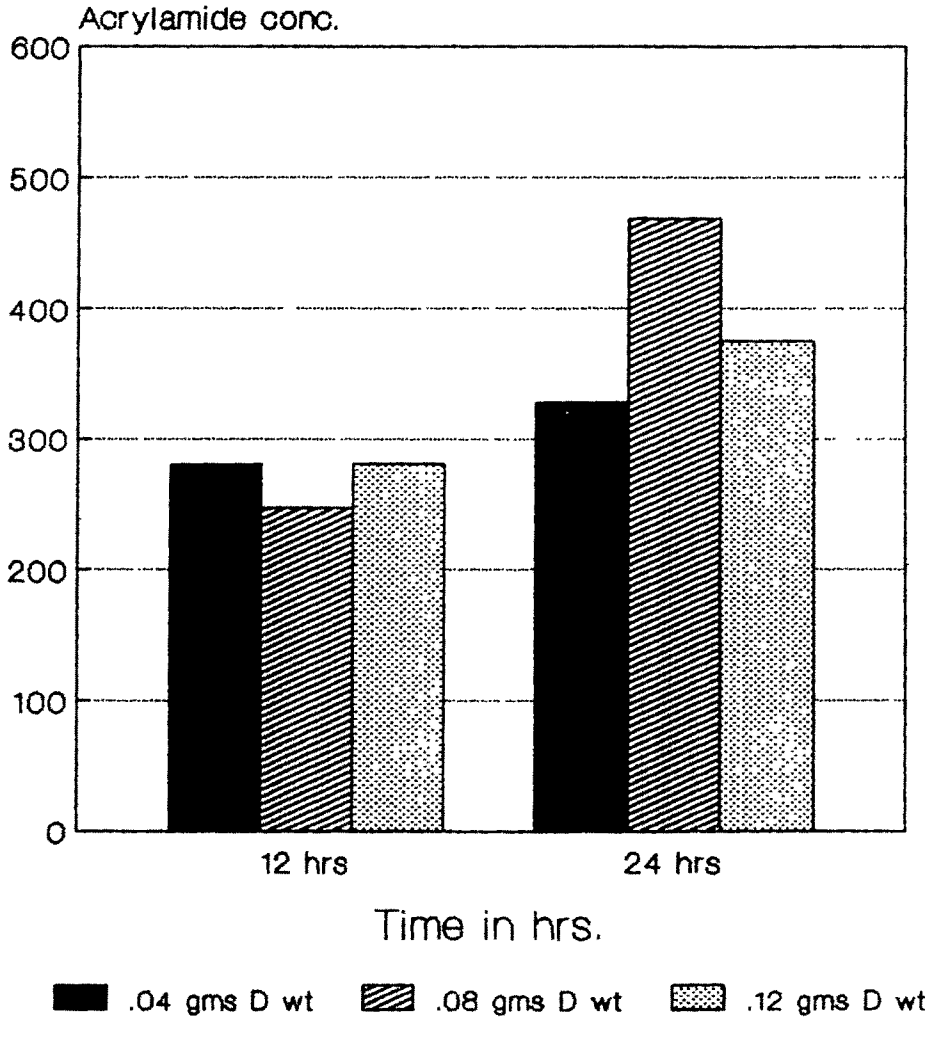
Values in n molar
conc of Acrylonitrile 20 & 100 mM
Temp room temp and 10 deg C

Fig.38b Effect of temp on the accumulation of acrylamide by Arthrobacter BC 1 cells



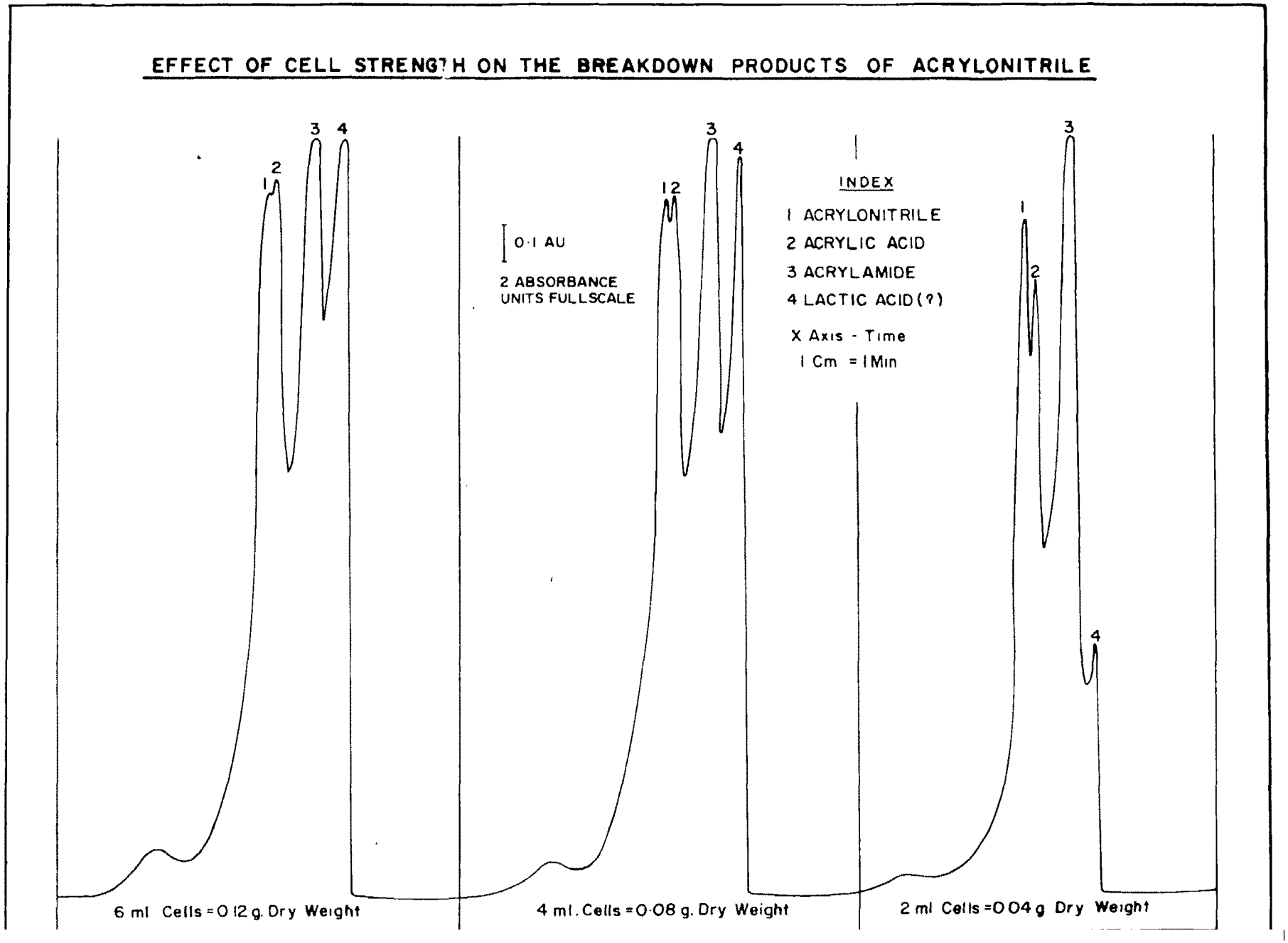
Values in n molar
Temp Rt & 10 deg C

Fig.39 Effect of cell density on acrylamide accumulation by Arthrobacter BC1 cells



values in mM
Acrylonitrile conc. 400 mM
Temp 10 deg C

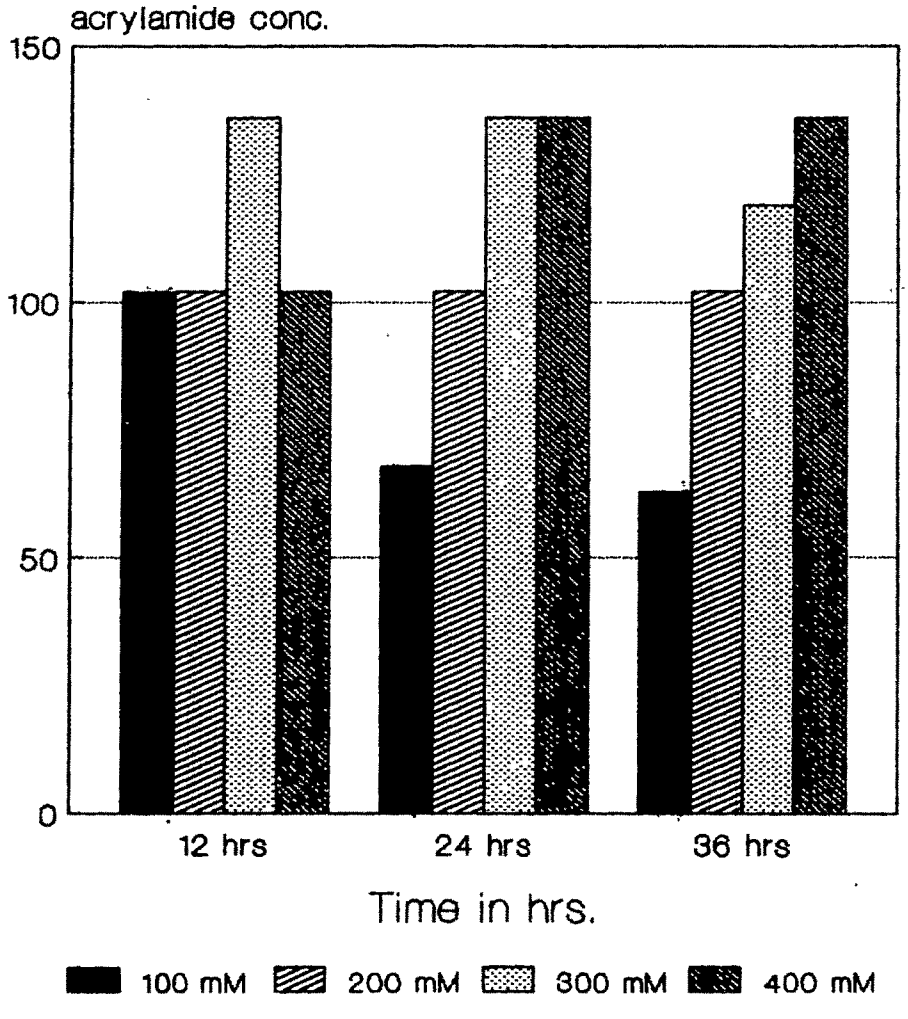
FIG.40


EFFECT OF CELL STRENGTH ON THE BREAKDOWN PRODUCTS OF ACRYLONITRILE
0·1 AU
2 ABSORBANCE UNITS FULLSCALE
INDEX
1 ACRYLONITRILE
2 ACRYLIC ACID
3 ACRYLAMIDE
4 LACTIC ACID (?)
X Axis - Time
1 Cm = 1 Min
6 ml Cells = 0·12 g. Dry Weight
4 ml. Cells = 0·08 g. Dry Weight
2 ml Cells = 0·04 g Dry Weight

acrylamide formed during the process. From Fig. 41 it can be seen that higher concentrations of acrylonitrile in the medium, favored amide accumulation by reducing its breakdown. The inhibition of amidases by acrylonitrile has been reported (Masteracci *et al.*, 1984). On depletion of the acrylonitrile from the medium acrylamide breakdown increases indicating the necessity of maintaining a particular concentration of acrylonitrile in the medium in order to minimize the breakdown of the acrylamide. The effect of acrylonitrile concentration on the breakdown products can be seen in Fig. 42.

In these bioconversions a new peak was observed at 3 min. This peak was formed after acrylamide was broken down and acrylic acid was formed. This could be the product of acrylic acid metabolism. This peak was found to have the same retention time as lactic acid. Therefore the new peak could be lactic acid since it can be formed by the hydration of acrylic acid. This perhaps is the first report on the formation of lactic acid from acrylic acid. It was believed that acrylic acid is converted to propionic acid, propionyl CoA, malonyl CoA, succinyl CoA, and succinate to enter the TCA cycle. This points to the existence of an enzyme, acrylic acid hydratase, which can convert acrylic acid to lactic acid and lactic acid to pyruvic acid and acetyl CoA. This also adds to the observation in the induction experiments as to why glucose tends to repress

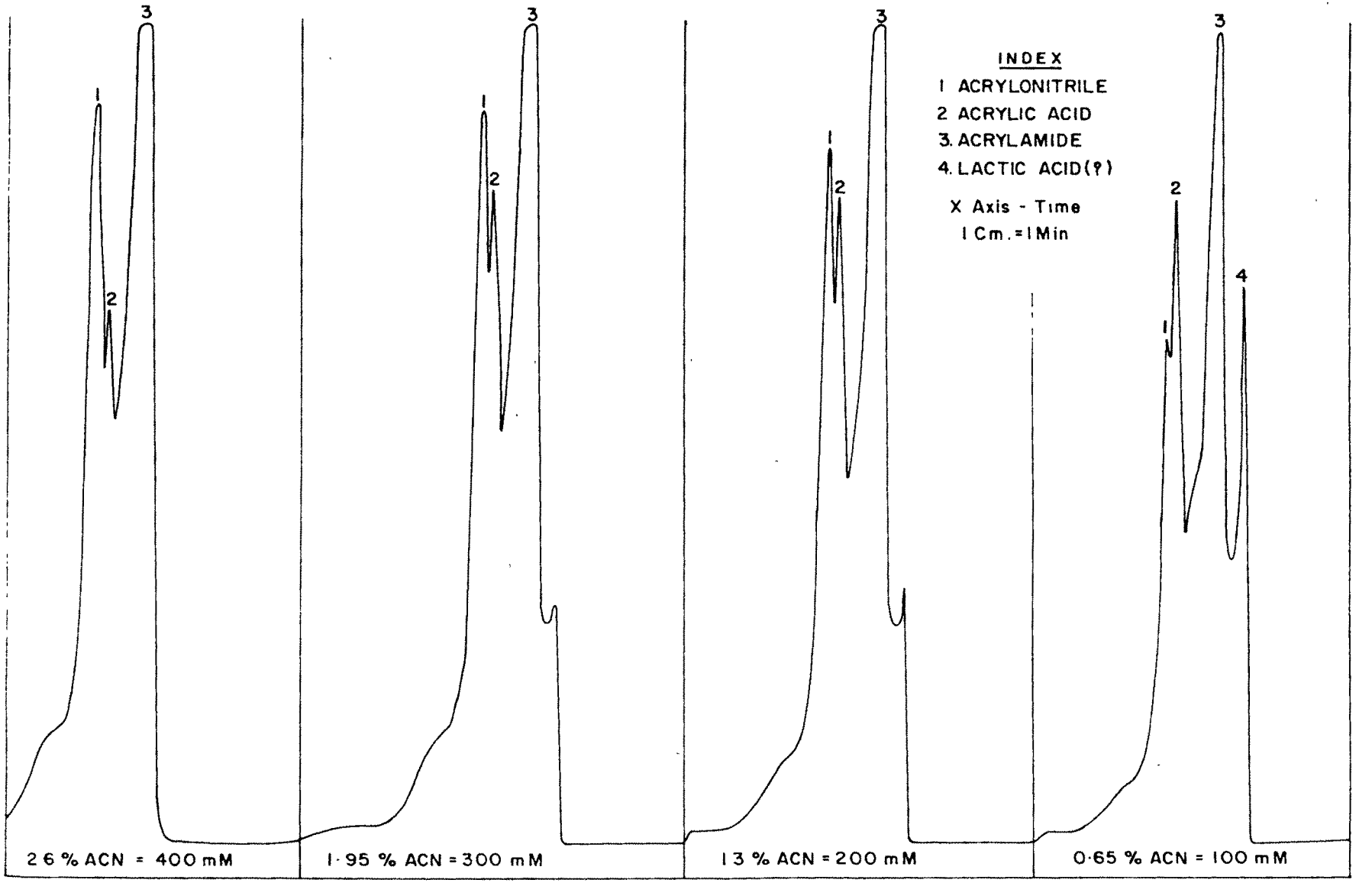
Fig 41 **Effect of Acrylonitrile conc on the accumulation of acrylamide by Arthrobacter BC 1 cells**



Values in mM
Temp 10 deg C

FIG. 42

EFFECT OF ACRYLONITRILE CONCENTRATION ON THE BREAKDOWN PRODUCTS OF ACRYLONITRILE

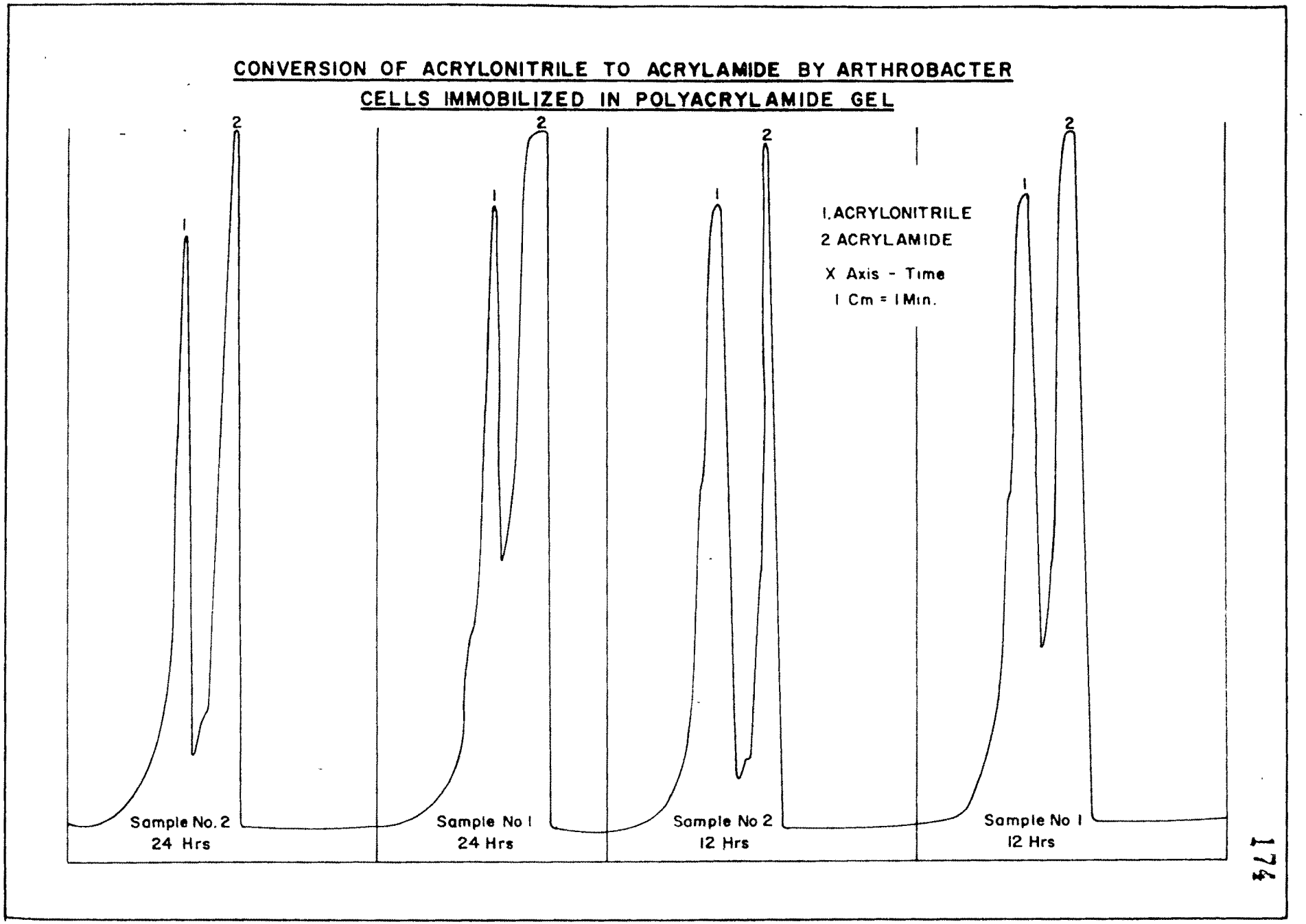


these enzymes.

In other cases, succinate was known to repress the enzyme, as succinate again is the end product of acrylate utilization. (Bandyopadhyay *et al.*, 1986). Succinate, however, did not in *Arthrobacter BC1* repress the enzymes, on the contrary increased its specific activity.

Fig. 43 shows the conversion of acrylonitrile to acrylamide by *Arthrobacter BC1* cells immobilized in polyacrylamide beads. It colud be seen that after immobilized cells could convert acrylonitrile to acrylamide. The experiments were performed in flasks wherein the beads tend to settle down affecting the homogeneity of the medium thereby reduces the efficiency of the conversion. Under these unfavorable conditions, 40-50% conversion in 24 hr was observed. Bioconversion procedure could be improved by employing a bioreactor and by maintaining various parameters like pH, time, temperature and substrate concentration. *Arthrobacter BC1* has unique capability of accumulating multiple products under the influence of different culture conditions like cell density, temperature, and acrylonitrile concentration. Lactic acid is found to accumulate only under static and anaerobic conditions, whereas under aerobic conditions in a shaker lactic acid would perhaps be rapidly utilized. Hence, this can become an extremely useful process for the synthesis of acrylamide, acrylic acid or lactic

FIG. 43


CONVERSION OF ACRYLONITRILE TO ACRYLAMIDE BY ARTHROBACTER CELLS IMMOBILIZED IN POLYACRYLAMIDE GEL
1. ACRYLONITRILE
2 ACRYLAMIDE
X Axis - Time
1 Cm = 1 Min.
Sample No. 2
24 Hrs
Sample No 1
24 Hrs
Sample No 2
12 Hrs
Sample No 1
12 Hrs

acid.

3.9 Assimilation of ammonia liberated during acrylonitrile breakdown by *Arthrobacter BC 1.*

It is seen that during the breakdown of acrylonitrile a large amount of ammonia is produced. Ammonia is the first stable product of nitrogen fixation and glutamate is the primary end product (Tyler, 1978). The assimilation of ammonia in microorganisms proceeds either via glutamate dehydrogenase (GDH) or glutamine synthetase (GS)/ glutamate synthase (GOGAT) pathway, depending on the type of organism and the ammonia concentration of the environment (Brown *et al., 1975;* Tyler, 1978). The GS/GOGAT pathway usually operates at low ammonia concentrations, reflecting the higher affinity of GS for ammonia whereas, GDH acts at higher concentrations. Alanine dehydrogenase has also been suggested to have ammonia assimilatory role (Aharonowitz and Friedrich, 1980). The regulation of GS GDH and GOGAT by ammonia is well established (Stadtman *et al.,* 1970). In *Azotobacter sp.* which utilizes acetonitrile, it was shown that with increase in ammonia level there was an increase in GDH activity and a decrease in GS/GOGAT activities. Table 22 shows that *Arthrobacter BC1* assimilates the ammonia formed by acrylonitrile breakdown predominantly by GDH pathway. It was observed that in *Arthrobacter fluorescens* high concentrations of ammonia

Table: 22 **Activities of ammonia assimilating enzymes in *Arthrobacter BC1***

Growth media	GDH (Units/ml)	GS (Units/ml)	GOGAT (Units/ml)
Glucose + Ammonium Sulphate	15	0.52	0.03
Acrylonitrile (0.2%)	20	0.33	0.02

Unit = μ moles transferred /min

inhibited GS and GOGAT but induced GDH (Cacciari *et al.*, 1986). The obsevation with *Arthrobacter BC1* is in close agreement with these results. Studies on GS, GDH and GOGAT at various ammonia concentrations will have to be done inorder to understand the regulation of these enzymes.

Conclusions

An organism *Arthrobacter BC1* was isolated from the petrochemical waste waters, which was capable of utilizing acrylonitrile as a sole source of carbon and nitrogen. The organism grew best at 0.2% V/V concentration of acrylonitrile at pH between 6.0 and 8.0, and at temperatures between 25 and 40 ^{o}C. The organism could effectively grow on other nitriles and amides either as a only source of carbon and nitrogen or as carbon or nitrogen source.

The whole cells of this organism converted acrylonitrile and other nitriles to the corresponding acid and ammonia through the formation of the intermediate amide contrary to the existing hypothesis.

Cell free extracts of this organism indicated the presence of two enzymes, nitrile hydratase and amidase, responsible for the metabolism. However, these enzymes had similar kinetic properties viz. Km, pH and temperature etc. The enzymes also exibited similar inducer specificities.

The enzymes showed similar elution profile in various chromatographic procedures and were quite inseparable. The enzymes suggested to possess the properties of substrate induced aggregation of subunits.

The organism possessed extrachromosomal genetic elements but a conclusive link between the and nitrile metabolism could not be established. Attempts to introduce broad host range plasmids like pKT 231 were also not successful due to the non availability of a suitable selection marker.

The organism showed excellent capacity to transform acrylonitrile to acrylamide, acrylic acid and lactic acid in both intact and immobilized cells. Biotransformation was favored under high acrylonitrile concentrations and at low temperatures.

SUMMARY OF THE WORK DONE ON THE METABOLISM OF ACRYLONITRILE BY BACTERIA ISOLATED FROM PETROCHEMICAL WASTE WATERS

Petrochemical industry is considered to be a backbone of any developing country, as it is a major source of many important chemicals used in plastics, dyes, drugs and fiber intermediates. The petrochemical industry is also the major source of environmental concern due to the toxicity of the chemicals found in its wastewaters. Biological treatment is extensively used to treat these wastes as it is cost effective, easy to operate and efficient in detoxification of majority of the xenobiotic compounds. Currently the major areas of interest are focused towards developing bacterial systems which could utilize multiple substrates efficiently. This approach could be achieved with the help of genetic engineering and biotechnology.

From this perspective, Department of Environment (DOE), Government of India, sponsored studies in the Department of Biochemistry, M. S. University of Baroda, Baroda, to identify the bacterial profile of the wastewaters of India's largest integrated petrochemicals production facility situated in Baroda. The study was initiated with the isolation and characterization of many species of bacteria, fungi and algae present in these waste waters. Of these, 15 bacterial species were found to be dominant and were capable of growing on

majority of the organic chemicals present in these waste waters. It was shown that mixed cultures could detoxify Dimethyl terephthalate (DMT) and Acrylonitrile/ Acrylate (ACN/ACR) plant wastes. The waste waters of ACN/ACR plant contains organic cyanides such as acrylonitrile, acetonitrile, etc., which are highly toxic and not easily biodegradable.

In the present study an organism, which could utilize acrylonitrile as a carbon and nitrogen source was isolated. This was identified and classified as *Arthrobacter BC1*. Many other organisms have been shown to utilize acetonitrile but organisms capable of utilizing acrylonitrile are scarce. Therefore, elucidating the details of the metabolism of acrylonitrile in *Arthrobacter BC1* was necessary before establishing its utility in waste treatment. The organism was found to grow optimally at 0.2% v/v concentration of acrylonitrile, at temperatures between 25 and 40 ^{o}C, within 48 hr., and at pH 6.5-8.0.

Resting cells of *Arthrobacter sp.* showed the sequential conversion of acrylonitrile to acrylamide and then to acrylic acid and ammonia. This is in contrast with the existing hypothesis that unsaturated aliphatic nitriles (acrylonitrile), aromatic nitriles (benzonitrile) and heterocyclic nitriles, are directly converted into their corresponding acids and ammonia, the reaction catalyzed by enzyme nitrilase. On the

other hand, saturated aliphatic nitriles (e.g. acetonitrile) are converted to their corresponding acids through the formation of an amide intermediate. This reaction is catalyzed by two independent enzyme systems nitrile hydratase and amidase.

Cell free extracts of *Arthrobacter BC1* exhibited both nitrile hydratase and amidase. Both these enzymes were remarkably similar in their kinetic properties. These enzymes possess broad substrate specificity with respect to nitriles and amides, the optimum pH was 7.0 and optimum temperature 60 ^{o}C,. These enzymes were induced by either nitrile or amide and repressed by ammonia. The enzymes also had similar elution profiles in Sephadex G-50; G-75 and G-200. The studies also indicated the possibility of substrate (acrylonitrile) induced aggregation of subunits. The organism also indicated the presence of extrachromosomal elements and exibited different plasmid profiles under different growth conditions. However, conclusive link between nitrile metabolism and extrachromosomal genetic elements could not be established by curing experiments. Further studies are required in order to establish the genetic basis of nitrile metabolism. Attempts to modify the *Arthrobacter BC1* by introducing broad host range plasmids such as pKT230 and pKT231 were not sucessful because a suitable slection marker was not avilable.

Industrial applications of nitrile bioconversions have been well known. This organism showed significant capacity to bioconvert acrylonitrile to acrylamide. It was observed in laboratory scale experiments that considerable amount of acrylamide accumulated in 12 hr. Lower temperaratures and high acrylonitrile concentrations favoured acrylamide accumulation both in intact cells and cells immobilized in acrylamide matrix. These results suggests that by carefully manipulating the acrylonitrile concentration, temperature, time etc., preferential accumulation of acrylamide, acrylic acid or lactic acid (which is the hydration product of acrylic acid), could be achieved in a suitably designed bioreactor.

The introduction of TOL genes in *Arthrobacter BC1* would enable us to treat these waste waters and also use the biomass thus formed to biotransform nitriles.