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The development of a novel strategy for the microbial treatment of acrylonitrile effluents

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Abstract

Effluent from the manufacture of acrylonitrile is difficult to biodegrade. It contains nine major organic components: acetic acid, acrylonitrile, acrylamide, acrylic acid, acrolein, cyanopyridine, fumaronitrile, succinonitrile, and maleimide. A range of bacteria have been isolated that can grow on, or convert all of the organic components of effluent from the manufacture of acrylonitrile. These bacteria can be used as the basis of a mixed culture system to treat the effluent. The bacteria were utilised in batch and continuous cultures to degrade a synthetic wastewater containing acrylonitrile, acrylamide, acrylic acid, cyanopyridine and succinonitrile. The mixed microbial population was adapted by varying the growth rate and switching from continuous to batch and back to continuous growth, to degrade these five compounds as well as acrolein, fumaronitrile and maleimide.

Abbreviations: BOD – Biological Oxygen Demand, COD – Chemical Oxygen Demand, T_D – Doubling Time, ppm – parts per million, HPLC – High Pressure Liquid Chromatography, GLC – Gas Liquid Chromatography

Introduction

The chemical manufacture of acrylonitrile is via the direct oxidation of propylene in the presence of ammonia, a process known as ammoxidation (Wittcoff & Rueben 1980). The wastewater from this process contains a complex mixture of organic nitriles, amides and acids. Tables 1a and 1b show the gross composition and major organic components of effluent streams from a typical acrylonitrile manufacturing plant. Both effluent streams contained high concentrations of reduced organic material (as measured by the Chemical Oxygen Demand, COD) and an idea of the scale is given by the flow rates (0.9 and 0.57 m^3 min^{-1}). This waste effluent cannot be released into the environment due to its toxicity and high Biological Oxygen Demand (BOD) and COD values. Disposal is usually by chemical means or by deep well injection. This investigation has concentrated on developing an economic and effective microbial method to degrade the components in this effluent, with the aim of completely removing the toxic materials and minimising the residual BOD and COD. The major organic components of the acrylonitrile effluent are succinonitrile, fumaronitrile, 3-cyanopyridine and acrylonitrile, together with acrylamide, maleimide, acrolein, acrylic acid and acetic acid. Although other impurities can also be present, these compounds comprise over 90% of the total carbon present.

The microbial metabolism of nitriles has been investigated in detail over the past twenty years. Aliphatic nitriles are usually catabolised in two stages, via conversion to the corresponding amide and then to the acid plus ammonia by a nitrile hydratase and

Table 1. Typical acrylonitrile (AN) aqueous waste.

(a) Gross composition.

Component	mg/l	
	Stream 1	Stream 2
Ammonia nitrogen	22,000	160
Kjeldhal nitrogen	46,600	180
BOD_5	9,000	3,000
Total organic carbon	11,000	4,300
COD (chromate)	22,000	10,000
Chloride	100	3
Dissolved solids	98,100	3,880
Suspended solids	140	7
Total solids	98,200	
Volatile Dis. (solvd. solids)	96,200	3,200
Volatile suspended solids	97	6
Sulfate (SO_4)	76,000	58
Cyanide total	1,000	450
pH	4.7	5.8
Flow (kg h^{-1})	27,000	29,000
Flow (litres min^{-1})	900	573

an amidase, respectively (Nagasawa & Yamada 1989; Thompson et al. 1988). Benzonitrile and related aromatic nitriles (Harper 1976, 1977a, 1977b) and a heterocyclic nitrile (Hook & Robinson 1964; Robinson & Hook 1964) are hydrolysed directly to the corresponding acids and ammonia via a nitrilase; little free amide is formed and for production of the acid there is no requirement for amidase activity. Acrylonitrile and acrylamide have been demonstrated to act only as a nitrogen source for growth of a number of nitrile degrading bacteria, whereas Yamada et al. (1979) isolated an *Arthrobacter* species that could utilise acrylonitrile as the sole source of carbon and nitrogen. Acrylic acid is a potent inhibitor of β-oxidation (Thijsse 1964). Microorganisms can however utilize acrylic acid, and it has been found as an intermediate in the fermentation of propionate by *Clostridium propionicum* (Sinskey et al. 1981). 3-Cyanopyridine has been reported to act as a substrate for the production of nicotinamide by *Brevibacterium R312* (Commeyras et al. 1977). It has also been shown to be a substrate for the nitrilase enzyme of a *Nocardia* sp. NCIB 11215 when grown on benzonitrile (Harper 1985). There is direct conversion of cyanopyridine to nicotinic acid by a strain of *Nocardia rhodochrous* (Vaughan et al. 1988). The various pathways of nicotinic acid metabolism have been reviewed by Shukla (1984). Kuwahara et al. (1980a) described an *Aeromonas* sp. that degrades succinonitrile as a source of nitrogen, simultaneously hydrolysing the two terminal nitrile groups. These authors have also described succinonitrile metabolism by the fungus *Fusarium solani* (Kuwahara et al. 1980b). Fumaronitrile is an unsaturated dinitrile which exhibits broad spectrum toxicity. The use of fumaronitrile as a bacteriocide was patented by Rohm and Haas (1967). Fumaronitrile (0.05% w/v) was added to an emulsion containing a copolymer of ethyl acrylate, methyl acrylate and itaconic acid and a non-ionic emulsifier. The emulsion was inoculated with bacteria and incubated at 25° C. No bacterial growth was observed over a period of six months. Fumaronitrile has also been patented as an antiseptic for metal-cutting fluids (Watanabe et al. 1974). Fumaronitrile (50 ppm) was added to an oil water mixture containing 4.7×10^4 bacteria ml^{-1}. After seven days storage at 30° C no viable organisms were found in the mixture. Acrolein is a highly volatile aldehyde that has been used widely as a herbicide, to control submerged aquatic weeds in irrigation channels. The loss of acrolein from water by volatilization has been studied by Bowmer et al. (1974). The reaction of acrolein with water has been reviewed by Geyer (1962). The primary reaction is reversible hydrolysis to give β-hydroxypropionaldehyde which is less volatile than acrolein.

Many of the major toxic components of the effluents from acrylonitrile manufacture might therefore be expected to be readily biodegraded (e.g. acrylonitrile, acrylamide, acetic acid, cyanopyridine and succinonitrile) but various other components could be more recalcitrant (e.g. maleimide, fumaronitrile and acrolein). Furthermore, the presence of a range of these compounds could exert an inhibitory effect on the development of activated sludge systems and, indeed, such acclimated systems have not been obtained by the gradual exposure to the acrylonitrile effluent. In pure culture the adaptation of a microorganism to degrade a novel substrate, for example a xenobiotic, requires extensive mutation and the fortuitous acquisition of a novel metabolic pathway (Cain 1984). However, with a mixed culture the organisms can act in concert, combining their catabolic potential to mineralise compounds which the individual community members are unable to achieve. It is the aim of the work presented in this publication to develop a microbial system to degrade acrylonitrile waste effluents. Initially microorganisms have been sought which degrade or transform the major individual components of the waste effluents.

Table 1. (b) Major organic components of the AN effluent.

	Structural formulae	Stream 1 average mg/l	Stream 2 average mg/l
Acetyldehyde	CH_3CHO	28	5
Acrolein	$CH_2 = CH\text{-}CHO$	52	36
Acetic acid	CH_3COOH	1310	2323
Acrylic acid	$CH_2 = CH\text{-}COOH$	1827	786
Acrylamide	$CH_2 = CHO\text{-}CONH_2$	821	49
Acrylonitrile	$CH_2 = CH\text{-}CN$		
Cyanopyridine		620	65
Fumaronitrile	$NC\text{-}CH = CH\text{-}CN$	794	66
Maleimide		1818	91
Succinonitrile	$NC\text{-}CH_2\text{-}CH_2\text{-}CN$	231	2380

These have been used to develop a mixed microbial population that is able to degrade a synthetic acrylonitrile waste effluent.

Methods

Organisms

All organisms apart from *Nocardia rhodochrous* strain LL100-21 (DiGeronimo & Antoine 1967) and *Brevibacterium R312* (Arnaud et al. 1976a) were obtained by enrichment culture (Krieg 1981). All were grown on minimal medium consisting of M-9 salts (Miller 1972) with ammonium salts omitted plus 1 ml trace metals $litre^{-1}$ (Bauchop & Elsdon 1960) and a range of nitriles, amides and acids as carbon and nitrogen, carbon, or nitrogen sources supplied at a concentration of either 25 mM or 10 mM. Where indicated the medium was supplemented either with glucose (10 mM) as the source of carbon or NH_4Cl (20 or 10 mM) as the source of nitrogen. Growth was in either 100 ml medium in 250 ml conical flasks or 500 ml medium in 2 l conical flasks incubated at 25° C in an orbital shaker (250 rev/min).

Enrichment culture

The isolation of microorganisms capable of degrading the substrates was carried out using classical enrichment techniques in liquid culture (Cook et al. 1983). The inocula were obtained from several locations: soil; activated sludge from Canterbury sewage treatment works (homogenised prior to use to disrupt microbial flocs); sediment and water from the Great Stour river near Canterbury. The river water samples were filtered and resuspended at 4% of the initial volume. The substrates were added at a concentration of 25 mM as the sole source of carbon and nitrogen; in the cases of acrylic acid and acrolein, the medium was supplemented with 20 mM NH_4Cl. Acrolein reacted with ammonium chloride, so sodium nitrate was used as the nitrogen source when acrolein was the carbon source. Where growth was observed in the primary enrichment flask an aliquot was subcultured in identical media. This procedure was repeated twice. The microorganisms present in the fourth stage flasks were subcultured on solid media (nutrient agar) and pure cultures obtained by subsequent transfers on this media. The ability of pure cultures to degrade the substrates was checked by growth on solid media (minimal medium plus the relevant substrate (25 mM) plus 2% (w/v) minimal agar) and in liquid culture (minimal medium containing 25 mM substrate).

Determination of growth substrates and ammonia

Samples of growth medium (1.5 ml) were centrifuged for 5 min in a microcentrifuge to remove bacteria. The cell-free medium was examined for the concentration of the growth substrate by either gas liquid chromatography (GC) or high pressure liquid chromatography (HPLC). A gas chromatograph equipped with a flame ionisation detector was used with glass columns (1.5 m × 4 mm i.d.) packed with either Poropak Q, mesh 80–100, or Poropak PS (Waters Associates). The

detector was at 250° C, the column at 200 or 210° C, the injector was at 200° C, and the carrier gas was nitrogen at 50 ml min^{-1}. Reverse phase HPLC was performed using a Partisil-10 ODS 3 C_{18} (4.6 × 250 mm) column. Detection was performed at 220 nm. Resolution was by various ratios of an A:B solvent mixture. Solvent A contained 5 ml isopropanol and 1 g H_3PO_4 made up to 1 litre in water, and B contained 5 ml isopropanol, 1 g H_3PO_4 and 750 ml methanol, plus water to 1 litre. Quantitative measurements were obtained by comparison with a standard curve of known concentrations of the test compounds. Samples were analysed for ammonia concentration by mixing with 0.5 ml nitroprusside reagent and 0.5 ml of alkaline hypochlorite reagent was added (Fawcett & Scott 1960). After mixing, the solution was incubated at 35° C for 15 min and the absorbance measured at 570 nm. Ammonium chloride was used as a standard.

Hydrolysis of fumaronitrile, maleimide and acrolein

Bacteria were harvested in the mid or late exponential phases of growth (OD_{610} 0.7–1.0) by centrifugation at 23,000 g for 10 min at 25° C, washed twice in Na_2HPO_4/KH_2PO_4 buffer (60 mM, pH 7.2) and resuspended to give 1.5 to 3 mg dry wt. Dry weight was related to absorbance (610 nm) by drying samples (50 ml) overnight in an oven at 105° C. Substrate (fumaronitrile, maleimide or acrolein) was added to the bacterial suspension in phosphate buffer and aliquots were taken at intervals. The bacteria were removed by centrifugation, and the supernatant analysed for residual substrate and ammonia production.

Taxonomy

Identification procedures were performed using API identification kits (API System SA, Montalieu Vercen France) and a variety of supplementary tests (Cowan & Steel 1965).

Apparatus

The chemostat used was an L H Fermentation Ltd. (Slough) 500 series fermenter. It was used as a 1 litre single-phase single-pass homogenous reactor conforming to the operational requirements of complete mixing. A working volume of 750 ml was used, the temperature was maintained at 25° C by a heater/temperature sensor control unit (model 503), and the dissolved oxygen was controlled at near saturation by automatic control of impeller speed by a model 509 oxygen controller linked to the agitator (502D). Aeration was provided by a model 504 air controller. The pH was maintained between pH 7.1–7.2 by the automatic addition of sulphuric acid (1 M) using a pH electrode (Ingold) connected to a model 505 pH controller. The medium was delivered to the reactor by a Gilson Minipuls 2 pump Gilson France SA, Villiers le Bel, France). A pressurised anti-growback device was used to prevent growth in the feed line.

Results

Growth of laboratory strains of bacteria

Two nitrile metabolising microorganisms previously studied in our laboratory, *Nocardia rhodochrous* LL100-21 (Collins & Knowles 1983) and *Brevibacterium R312* (Miller & Knowles 1984), were tested for their ability to degrade each of the eight major problem substrates found in the acrylonitrile effluents (Table 1b). The capability of these organisms to metabolise the compounds as both the carbon and nitrogen source, or as either the carbon or nitrogen source was investigated. Both organisms displayed a similar growth specificity at a substrate concentration of 20 or 25 mM, being able to utilize acrylamide, acrylonitrile, succinonitrile and 3-cyanopyridine as the nitrogen source in the presence of an additional carbon source (i.e. 10 mM glucose). Neither bacterium was able to use these compounds as the source of carbon or carbon plus nitrogen. In addition, neither organism was capable of utilising fumaronitrile, acrolein, acrylic acid or maleimide as the sources of carbon and/or nitrogen, as relevant. The initial enrichment study yielded a number of isolates. Microorganisms were obtained that grew on succinonitrile, acrylonitrile, acrylamide, cyanopyridine and maleimide as the sole source of carbon and nitrogen. Acrylic acid served as the source of carbon for two isolates. However, we were unable to obtain isolates by this initial simple enrichment technique that grew on fumaronitrile or acrolein.

Growth of isolates

The growth of each microorganism on its isolation substrate was determined by the absorbance at 610 nm (Table 2). The decrease in concentration of the growth substrate (nitrile, amide or acid) was measured, as

Table 2. Growth characteristics of the isolates on their enrichment substrates. Each substrate was used as the sole source of carbon and nitrogen, except for acrylic acid which was used as the sole source of carbon, with NH_4Cl as nitrogen source. All the isolates were grown at 25° C in shake flask culture supplemented with M9 minimal salts pH 7.2.

Substrate	Conc. of substrate (mM)	Organism	T_D (h)	Specific growth conc. rate (h^{-1})	Final NH_3 (mM)
Succinonitrile	25	SN-1	8.5	0.08	39.5
Succinonitrile	25	SN-5	6.5	0.11	39.5
Succinonitrile	25	SN-8	3.5	0.2	46.5
Acrylamide	25	AM-C	5	0.14	11.5
Acrylamide	25	AM-2	4.5	0.15	19
Acrylamide	25	AM-1(a)	4.5	0.15	17
3-Cyanopyridine	25	CYP-A	5.5	0.13	47.5
3-Cyanopyridine	25	CYP-B	5.5	0.13	47.5
Acrylic acid	25	AA-1	17	0.04	NA
Acrylic acid	25	AA-2	15	0.05	NA
Acrylonitrile	10	AN-2	2.5	0.28	8.25
Maleimide	10	MAL-B	46.8	0.015	8

T_D = doubling time (h). NA = not applicable.

was the appearance of ammonia. For complete utilisation of the mono-nitriles and amides (supplied at 25 or 10 mM) the maximal potential level of formation of ammonia was either 25 or 10 mM, respectively. For the dinitrile succinonitrile, and also for 3-cyanopyridine, which were supplied at 25 mM, the potential yield of ammonia was 50 mM. In fact, in each case, the maximal values of ammonia would be somewhat less than the theoretical maximum value due to incorporation of some of it into biomass. Except for maleimide, growth was relatively rapid, with a doubling time of less than 20 h and usually less than 8 h. In most cases the growth substrate was completely metabolised to carbon dioxide, ammonia and biomass within about 30 h.

Growth specificity of the isolates

An important part of this investigation was the growth and substrate specificity of each of the bacterial isolates, i.e. would bacteria isolated on one component of the effluent grow on or degrade other components of the effluent? A problem in developing a process could be the toxicity of some components of the effluent stream to bacteria potentially able to biodegrade other components of the system.

The growth specificity of the isolates on the other major components, and related compounds, of the effluent was therefore studied (Table 3). The majority of isolates, obtained on the more readily degradable compounds, were able to metabolise a range of compounds other than their isolation substrates. Five of the eight compounds under study were readily degraded by the isolates to carbon dioxide, ammonia and biomass. Of the other three compounds (maleimide, fumaronitrile and acrolein), one isolate (MAL-B) could biodegrade maleimide as the sole source of carbon and nitrogen but growth was slow, and none of the isolates metabolised fumaronitrile or acrolein.

Fumaronitrile toxicity

Fumaronitrile was tested as a biocide against a number of the isolates capable of degrading nitriles and amides (Table 4). Even at a concentration of 0.05 mM (about 4 ppm) fumaronitrile was toxic to growth of all the isolates. Only three of the bacteria showed any growth in the presence of 0.6 mM fumaronitrile. The toxicity of fumaronitrile to two laboratory strains of bacteria, *Escherichia coli* (Gram negative) and *Streptococcus faecalis* (Gram positive) was also demonstrated (Fig. 1). A fumaronitrile concentration of 0.1 mM complete-

Table 3. Growth specificity of isolates. The isolates were tested for their ability to grow on the other major components of the acrylonitrile waste effluents as sole carbon and nitrogen sources, or as the sole carbon source in the case of acrolein and acrylic acid.

Organism	Isolation substrate	Acrylamide	Acrylic acid	Succinonitrile	Cyanopyridine	Maleimide	Acrylonitrile	Fumaronitrile	Acrolein
SN-1	Succinonitrile	+	+	+	±	-	-	-	-
SN-5	Succinonitrile	+	+	+	-	-	-	-	-
SN-8	Succinonitrile	+	+	+	±	-	-	-	-
AA-1	Acrylic acid	+	+	-	-	-	-	-	-
AA-2	Acrylic acid	+	+	+	+	-	±	-	-
AM-2	Acrylamide	+	+	-	-	-	±	-	-
AM- C	Acrylamide	+	+	-	-	-	±	-	-
AM-1(a)	Acrylamide	+	+	+	±	-	-	-	-
CYP-A	3-Cyanopyridine	-	+	+	+	-	-	-	-
CYP-B	3-Cyanopyridine	+	+	+	+	-	-	-	-
AN-2	+	+	-	-	-	+	-	-	
MAL-B	Maleimide	-	-	-	-	+	-	-	-

(+) = good growth; (±) = some growth; (-) = not detectable growth.

Table 4. The toxicity of fumaronitrile.

Organism	Growth substrate	Days to visible growth fumaronitrile concentration (mM)		
		0.05	0.1	0.5
SN-1	Succinonitrile	3.5	–	–
SN-5	Succinonitrile	3.5	4.5	–
SN-8	Succinonitrile	7.5	8.5	–
CYP-A	Cyanopyridine	3.5	–	–
CYP-B	Cyanopyridine	4.5	5.5	5.5
AM-2	Acrylamide	4.5	–	–
AM-C	Acrylamide	3.5	4.5	–
AM-1 (a)	Acrylamide	3.5	4.5	–
AA-2	Acrylic acid	10	10	10
AN-2	Acrylonitrile	3.5	4.5	5.5

Without fumaronitrile there was visible growth after 0.5 to 1.0 days.
(-), no growth after 14 days.

ly inhibited the growth of both bacteria in nutrient broth.

Isolation of fumaronitrile-utilizing bacteria

As mentioned above, classical enrichment procedures using a wide range of conditions and potential sources of microorganisms failed to yield any microorganisms that were capable of utilizing fumaronitrile as a carbon and nitrogen source for growth or as a nitrogen source only. These experiments included use of soil samples which had been intermittently exposed to various nitrile compounds in the proximity of an acrylonitrile manufacturing plant. Repeated experiments failed to provide any fumaronitrile utilising isolates. As a result of these negative experiments an alternative approach was taken. A small scale (100 ml) continuous enrichment system was devised (Harder, Kuenen & Matin 1977). A mixture of activated sludge and soil was added to medium containing glucose (10 mM)

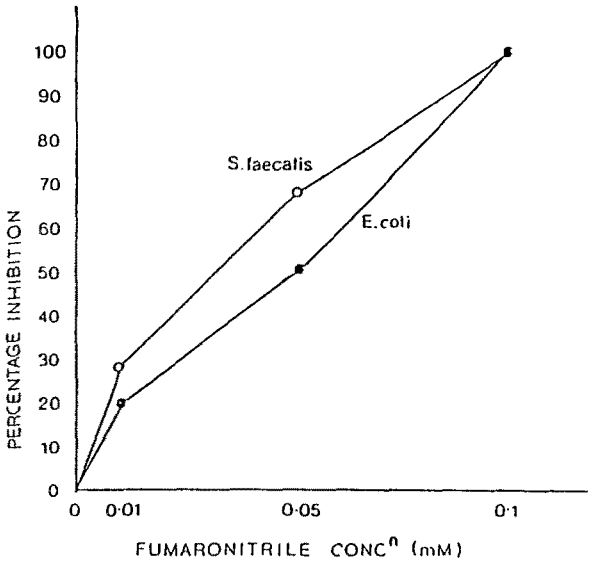

Fig. 1. Inhibition of growth by fumaronitrile. Two standard laboratory strains of *Streptococcus faecalis* and *Escherichia coli B* were grown in nutrient broth at 30° C in the presence of varying concentrations of fumaronitrile. The figure shows the percentage inhibition of the growth rate in the presence of fumaronitrile.

and ammonium chloride (5 mm) as the sole sources of carbon and nitrogen. The system was operated continuously at a dilution rate of 0.05 h^{-1}. The concentration of ammonium chloride was gradually decreased and the concentration of fumaronitrile, as a replacement nitrogen source, increased, until after a period of 4 months fumaronitrile (5 mM) had completely replaced the ammonium chloride. A stable population of four microorganisms was obtained. The mixed population utilized fumaronitrile (5 mM) as the nitrogen source. The mixed population was separated into individual microorganisms by serial dilution and plating onto nutrient agar. Each organism was tested for its ability to utilize fumaronitrile as a carbon and nitrogen source and as a nitrogen source only in minimal media liquid culture (with glucose (10 mM) as the carbon source). A range of fumaronitrile concentrations (0.5 to 5 mM) were tested. None of the pure isolates utilized fumaronitrile as a carbon and nitrogen source but an isolate was obtained that was capable of growth on glucose (10 mM) as the carbon source and fumaronitrile (1 mM) as the source of nitrogen. Higher concentrations of fumaronitrile were toxic to this organism.

Biotransformation of fumaronitrile

Although fumaronitrile was biocidal for the original range of isolates a number of the bacteria were tested for their ability to transform fumaronitrile (Table 5). Arnaud et al. (1976a, 1976b, 1977) isolated *Brevibacterium* strain R312 which has a non-specific nitrile hydratase. This bacterium, which had been grown on acetonitrile (20 mM) as sole source of carbon and nitrogen, was also examined for its ability to degrade fumaronitrile. Suspensions of harvested bacteria in phosphate buffer were tested for their ability to hydrolyse fumaronitrile even though they could not utilize it as a growth substrate. The disappearance of fumaronitrile was monitored using HPLC and by the release of ammonia (Table 5). Organism SN-8 (also isolated on succinonitrile) showed no hydrolysis, whereas SN-1 (also isolated on succinonitrile), FOF (the consortium of four bacteria from continuous enrichment on glucose plus fumaronitrile) and AN-2 (isolated on acrylonitrile) all displayed some hydrolysis of fumaronitrile, but did not convert all the added fumaronitrile, possibly due to either its toxicity or that of the hydrolysis products. *Brevibacterium* R312 and CYP-B (isolated on 3-cyanopyridine) displayed similar characteristics, but all the fumaronitrile was converted to another compound which accumulated in the medium. CYP-A (isolated on 3-cyanopyridine) completely degraded the added fumaronitrile more rapidly than the other organisms tested. The molarity of the ammonia released (10 mM) was equivalent to that of the fumaronitrile added (10 mM), indicating that only one nitrile group had been hydrolysed to a carboxyl group. The product was presumably either trans 3-cyanopropenoic acid or succinamic acid. The pure isolate capable of slow growth on low concentrations of fumaronitrile as a nitrogen source was not tested for its ability to degrade fumaronitrile due to the efficiency of CYP-A, and the much lower efficiency of the consortium of four bacteria, FOF, from which the slow growing isolate F1-A had been isolated. The rate of hydrolysis of fumaronitrile by CYP-A was compared to that of succinonitrile which, unlike fumaronitrile, can be utilized by this organism as a source of carbon and nitrogen for growth. The organism was grown on succinonitrile, harvested in late exponential phase and resuspended in two aliquots.

To one aliquot fumaronitrile (17 mM) was added and to the other succinonitrile (16 mM), and the rate of substrate disappearance and ammonia release monitored (Fig. 2). Surprisingly, hydrolysis of fumaronitrile

Table 5. The biotransformation of fumaronitrile.

Organism	Growth substrate	Initial rate of fumaronitrile conversion (μmoles mg dry wt^{-1} h^{-1})
SN-8	Succinonitrile	0
SN-1	Succinonitrile	13
FOF*	Nutrient broth	20
AN-2	Acrylonitrile	48
Brevibacterium R312	Acetonitrile	70
CYP-B	Succinonitrile	65
CYP-A	Succinonitrile	854

* Stable consortium of 4 bacterial isolates that is able to grow on glucose plus fumaronitrile. The organisms were harvested in mid or late exponential phase and resuspended in phosphate buffer (96 mM) at a concentration of 2 to 4 mg ml^{-1} dry wt. Fumaronitrile (10 mM) was added and the mixture shaken at 25° C, and samples being removed at intervals. The supernatant was assayed for fumaronitrile and ammonia.

Table 6. Bacterial isolates obtained in this study.

Organism code	Isolation substrate	Identification
SN-1	Succinonitrile (C + N)	*Alcaligenes* sp.
SN-5	Succinonitrile (C + N)	NI
SN-8	Succinonitrile (C + N)	*Pseudomonas* sp.
AM-1(a)	Acrylamide (C + N)	*Alcaligenes* sp.
AM-2	Acrylamide (C + N)	NI
AM-C	Acrylamide (C + N)	NI
CYP-A	3-Cyanopyridine (C + N)	*Pesudomonas* sp.
CYP-B	3-Cyanopyridine (C + N)	*Flavobacterium* sp.
AN-2	Acrylonitrile (C + N)	*Acinetobacter* sp.
AA-1	Acrylic acid (C)	NI
AA-2	Acrylic acid (C)	NI
F1-A	Fumaronitrile (N)	*Klebsiella pneumoniae*
SM-B1	Succinimide (C + N)	*Pseudomonas* sp.
SM-A2	Succinimide (C + N)	*Pesudomonas* sp.
MAL-B	Maleimide (C + N)	*Bacillus megaterium*
3CP-A	3-Cyanopropenoic acid (C + N)	*Pseudomonas* sp.
3CP-B	3-Cyanopropenoic acid (C + N)	*Achromobacter* sp.
AYA-A	Allyl alcohol (C)	*Pseudomonas* sp.
AYA-B	Allyl alcohol (C)	*Pseudomonas* sp.
AYA-C	Allyl alcohol (C)	*Pseudomonas* sp.

NI – not identified, C – carbon source only, N – nitrogen source only, C + N – sole source of carbon and nitrogen.

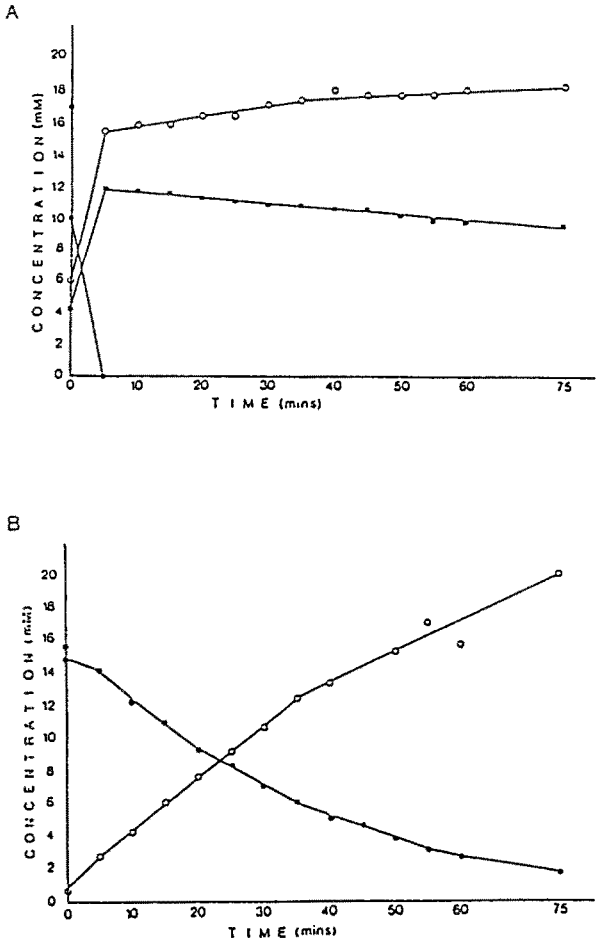

Fig. 2. Succinonitrile and fumaronitrile hydrolysis by bacterium CYP-A. (a) Fumaronitrile hydrolysis by microorganism CYP-A. • Fumaronitrile; o Ammonia; ■ 3-Cyanopropenoic acid. (b) Succinonitrile hydrolysis by microorganism CYP-A. • Succinonitrile; o Ammonia. Fumaronitrile and 3-cyanopropenoic acid were assayed by HPLC. Succinonitrile was assayed by GLC and ammonia was assayed colorimetrically.

by this organism was more rapid than the rate utilisation of succinonitrile. The initial product of fumaronitrile hydrolysis was subsequently slowly degraded with release of extra ammonia, presumably due to conversion to fumaric acid. As the initial ammonia release was equivalent to the conversion of only one nitrile group of fumaronitrile an attempt was made to isolate and identify the initial compound formed. A suspension of bacterium CYP-A was incubated with fumaronitrile, for a sufficient period to permit completion of the reaction. The bacteria were removed by centrifugation, and the supernatant acidified and solvent extracted. The solvent (dry diethylether) was removed by vacuum evaporation leaving a crystalline product. This compound was identified by mass spectrometry with reference to Gresseli (1973). A molecular ion peak of 97 was observed indicating that the compound was 3-cyanopropenoic acid.

Degradation of 3-cyanopropenoic acid

Since the original isolates were unable to grow on fumaronitrile but some of them were able to convert it to 3-cyanopropenoic acid, this compound was used as the substrate for an enrichment culture. 3-Cyanopropenoic acid (10 mM) was used as the sole source of carbon and nitrogen. Two bacterial isolates were readily obtained, both of which mineralised 3-cyanopropenoic acid to carbon dioxide, ammonia and biomass. By this approach, a system containing two microorganisms has been developed which might be capable of the mineralisation of the highly toxic dinitrile, fumaronitrile, to carbon dioxide, ammonia and biomass.

Isolation of acrolein-utilizing bacteria

Enrichment cultures were attempted with acrolein as the sole source of carbon. Despite using a wide variety of inocula and a range of different concentrations of acrolein, no microorganisms were obtained. The reactivity and volatility of acrolein (propenol, CH_2 = CHCHO) in aqueous culture medium was examined. Acrolein (25 mM) was added to a 250 ml conical flask containing sterile minimal medium (100 ml) and incubated with shaking (250 rev/min) at 25° C. After 24 hours only 34% of the added acrolein was detectable and after 45 hours less than 10%. The acrolein had either volatilized from the solution or been hydrated to β-hydroxypropionaldehyde (CH_2OHCH_2CHO), the latter being more probable as there was a similar rapid loss of detectable acrolein in sealed flasks. The incubation procedure was repeated, but in the presence of ammonium chloride (5 mM). After 16 hours only 10% of the added acrolein was detected and the medium had a distinct yellow colouration. Nitrate was therefore tested as a possible alternative nitrogen source for the enrichment cultures. Unlike ammonium chloride, this did not appear to react with the acrolein, but again no microbial isolates were obtained.

Degradation of acrolein

Due to the lack of success with acrolein in enrichment culture a different approach was taken. Allyl alcohol (2-propen-1-ol) was used as a sole carbon source for enrichment. This substrate was chosen because degradation of allyl alcohol is possibly via oxidation to acrolein and then to acrylic acid via alcohol dehydrogenase and aldehyde dehydrogenase. Three bacterial isolates were readily obtained that utilised allyl alcohol (25 mM) as the sole source of carbon. Each was tested for its ability to transform acrolein, the organisms having been grown on allyl alcohol, harvested in the exponential phase and resuspended in phosphate buffer. Acrolein (10 mM) was added to 10 ml of cell suspensions (1.5 to 3 (mg dry wt) ml^{-1}) in 250 ml sealed flasks and the flasks incubated at 25° C and shaken at 250 rev min^{-1}. All three organisms rapidly and completely transformed acrolein to acrylic acid within 2 hours. Thus by using these isolates acrolein could be transformed to acrylic acid, which was readily degraded by both these bacteria and the other isolates mentioned earlier in this study (Table 3).

Isolation of maleimide-utilizing bacteria

Only one microbial isolate was obtained by enrichment culture that grew on maleimide (10 mM) as the sole source of carbon and nitrogen. However, growth was slow, with a doubling time of 47 h. The maleimide was mineralised to carbon dioxide, ammonia (8 mM) and biomass. The culture supernatant was examined during growth and it was noted that maleimide was transformed to another compound during the lag phase of growth. This compound could be maleamic acid, the hydration product of ring cleavage, which would then be mineralised by the isolate. Maleamic acid was also found to be utilised by several of the isolates obtained for their ability to degrade nitriles and amides as growth substrates, being mineralised to carbon dioxide, ammonia and biomass (data not shown).

Transformation of maleimide

The slow growth rate of the maleimide utilizing isolate could be a problem in a mixed culture system, so additional microorganisms were sought that were capable of transforming maleimide to a product such as maleamic acid that could be readily utilized. Two organisms were obtained by enrichment culture that could rapidly degrade the structural analogue of maleimide, succinimide, as sole source of carbon and nitrogen. These isolates were unable to utilize maleimide but were tested for their ability to hydrolyse the imide ring of maleimide to maleamic acid (Fig. 3). Both isolates were able to hydrolyse maleimide, the rates of conversion being between 0.25 and 0.35 μmoles min^{-1} mg^{-1} dry wt bacteria. The hydrolysis product was tentatively identified by HPLC as maleamic acid.

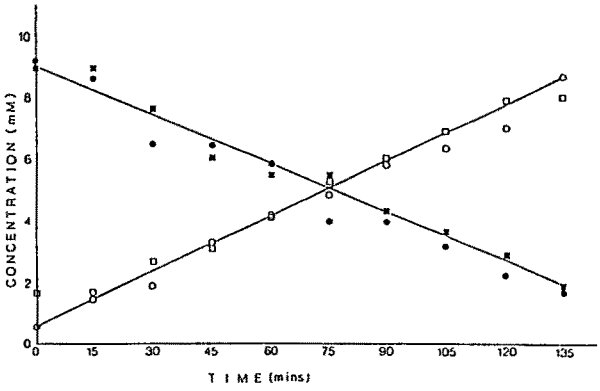

Fig. 3. Biotransformation of maleimide to maleamic acid. The transformation of maleimide to maleamic acid by two organisms (SM-B1 and SM-A2) isolated and grown on succinimide. The figure shows the disappearance of maleimide and appearance of maleamic acid. SM-B1 – ■ maleimide; □ maleamic acid. SM-A2 – • maleimide; ○ maleamic acid. Each organism was grown on succinimide (20 mM), harvested in mid to late exponential phase, resuspended in phosphate buffer (60 mM pH 7.2) to a concentration of 1.5 to 3 mg ml^{-1} dry wt. Maleimide (10 mM) was added to the suspension in a sealed shake flask and incubated with shaking at 25° C. Samples were removed over a time course, the cells were removed by centrifugation (5 min in a microcentrifuge), and the concentration of maleimide and maleamic acid in the supernatant was determined by HPLC. Spontaneous hydrolysis of maleimide in the control flask to which no microorganisms were added was negligible.

Taxonomy

The microbial isolates obtained by enrichment on the major individual compounds of the acrylonitrile waste effluents were tentatively identified (Table 6). Two organisms, CYP-A and MAL-B, were identified by the NCIMB (National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland). The bacterium CYP-A isolated on 3-cyanopyridine as sole carbon and nitrogen source was identified as a *Pseudomonas* sp. probably from rRNA group III. It closely resembled *Pseudomonas saccharophila* (Palleroni 1980) but was atypical for arabinose and malonate utilization tests. The organism, MAL-B, capable of growth on maleimide as

sole source of carbon and nitrogen was identified as a *Bacillus* sp., most probably *Bacillus megaterium.*

Mixed cultures

The general approach to biodegradation of industrial waste effluents containing mixtures of compounds has so far generally been to try to adapt already functional activated sludge systems using the versatility of the microorganisms already present to remove the major contaminants (Hall & Melcer 1983). The approach described below differs in that the waste effluent has been examined to determine the chemical nature of the major components of the acrylonitrile effluent streams, followed by the isolation of bacteria or potential bacterial systems to individually degrade each of the compounds. This is because the toxicity of the nitrile and amide compounds has been found to severely limit the adaptation of a conventional activated sludge system to biodegrade these wastes. In order to determine whether mixtures of components of the AN waste streams could be degraded, mixed cultures of the isolates have been used in continuous culture. Initially degradation of a mixture of the four most readily utilised compounds present in the waste effluents was studied. These were 3-cyanopyridine, acrylamide, acrylic acid and succinonitrile, all at 5 mM. The inoculum was a defined mixture of the isolates obtained by enrichment on these compounds. Microbial activity in batch culture was monitored by measurement of biomass, ammonia release, and the decrease in substrate concentration. When no further overall growth was observed, the culture was switched to continuous fermentation. The rate of addition of the mixture of substrates was 50 ml h^{-1} giving a dilution rate (D) of 0.07 h^{-1}. The parameters controlled were pH, dissolved oxygen concentration (DO) and foaming. Analysis of the culture demonstrated that the four substrates (5 mM each) were not detectable in the effluent (< 1 μM), being degraded to carbon dioxide, ammonia and biomass. The system was operated in continuous mode for 65 h. The microbial population was stable throughout the period of operation (four culture turnovers).

The experiment was repeated, using a defined inoculum consisting of all the isolates used in the previous experiment plus bacteria obtained by enrichment on acrylonitrile (organism AN-2), maleimide (organism MAL-B), fumaronitrile (organisms F1-A), 3-cyanopropenoic acid (organisms 3CP-A and 3CP-B), succinimide (organisms SM-B1 and SM-A2) and allyl alcohol (organisms AYA-A, AYA-B and AYA-C). The system was again grown in batch culture and then switched to continuous culture. The initial substrate mixture contained 3-cyanopyridine, acrylamide, acrylic acid and succinonitrile, all at 5 mM. In continuous culture these compounds were again completely degraded, none being detectable in the effluent (< 1 μM). A stable system resulted (Fig. 4) with a dilution rate of D = 0.07 h^{-1}. After 40 h in continuous mode, acrylonitrile (1 mM) was added to the medium inflow. The addition of this compound did not affect the stability of the culture and the acrylonitrile was also completely degraded. Fumaronitrile (the most toxic and recalcitrant compound of the AN waste stream) was added to the medium inflow after 96 h of continuous operation at a concentration of 1 mM. For approximately 48 h after addition the biomass remained constant and none of the substrate compounds were detected in the effluent. However, the biomass content then decreased by approximately 50% before stabilising. At this stage trace amounts of the substrates were detectable in the effluent. To try to overcome this problem the dilution rate was reduced, thereby decreasing the specific growth rate from D = 0.07 h^{-1} to D = 0.056 h^{-1}. A transient slight increase in biomass was observed. The dilution rate was again lowered from D = 0.056 h^{-1} to D = 0.053 h^{-1}. This resulted in a stable increase in biomass to the original level, with no remaining substrate detectable in the effluent. Over a 400 h period of continuous operation at the new dilution rate there was complete degradation of 3-cyanopyridine, acrylamide, acrylic acid and succinonitrile (all at 5 mM) and acrylonitrile and fumaronitrile (both at 1 mM).

The mixed population of microorganisms was further adapted by gradually increasing the capacity of the culture to biodegrade the more toxic and recalcitrant compounds of the AN waste effluents, fumaronitrile and maleimide, in a long term continuous culture experiment. The culture was established as before with the four more readily degraded substrates, 3-cyanopyridine, acrylamide, acrylic acid and succinonitrile, all at 5 mM. The bacteria were initially grown in batch culture, the inocula being all the isolates used in the previous experiment including those capable of growth or degradation of maleimide. The system was switched into continuous culture at a dilution rate of 0.06 h^{-1}. After 140 hours acrylonitrile (1 mM) was added to the medium with no detrimental effect. Fumaronitrile (1 mM) was added at 300 h, but the culture began to washout, not stabilising as it had previously. At 450 h, when the biomass had

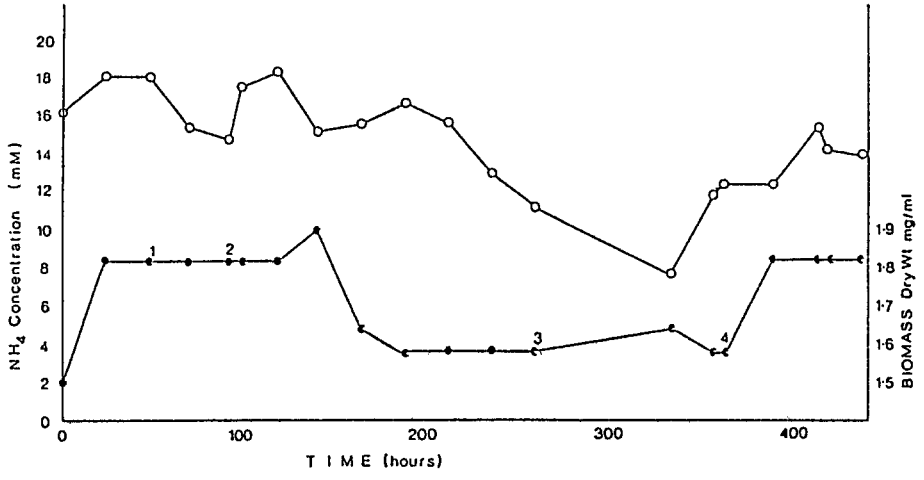

Fig. 4. Variation in biomass and ammonia concentration during continuous fermentation of a mixture of compounds found in acrylonitrile waste streams. At the start of the experiment the medium supply contained 3-cyanopyridine (5 mM), acrylamide (5 mM), acrylic acid (5 mM) and succinonitrile (5 mM). At point (1) acrylonitrile (1 mM) was added to the medium inflow and at point (2) fumaronitrile (1 mM). At point (3) the dilution rate was lowered from 0.07 h^{-1} to 0.056 h^{-1} and at point (4) to 0.053 h^{-1}. The bacteria present initially were as detailed in the text. o – Ammonia concentration (mM). • – Biomass dry wt mg ml^{-1}.

declined to less than half the original value, the fermentation system was switched to batch culture (i.e. the medium feed was stopped) to enable recovery to occur. The biomass level increased during the batch phase and at 500 h the culture was returned to continuous operation. At 600 h the fumaronitrile concentration was increased to 2 mM. There was no detrimental effect on the culture which remained stable for the next 300 h of operation. Maleimide (1 mM) was then added. This had an immediate toxic effect, causing the culture to washout, and 24 h after addition the biomass had dropped from 2 mg/ml to less than 1 mg/ml. The system was therefore switched into batch culture, and the biomass recovered after about 60 h, at which time the system was switched back into continuous culture. A stable culture resulted which was capable of biodegrading maleimide (1 mM) as well as the other compounds present. At 1200 h the concentration of fumaronitrile was increased to 3 mM. This again caused washout to occur but after a brief switch to the batch mode the culture recovered and continuous operation was resumed. At the termination of this experiment the mixed microbial population had been adapted to completely biodegrade the four readily utilisable compounds (each at 5 mM), and acrylonitrile (1 mM) and the two most toxic compounds, fumaronitrile (3 mM) and maleimide (1 mM). The culture was harvested, resuspended in buffer and stored frozen under glycerol at minus 20° C to be used as an inoculum in future experiments. In a subsequent fermentation the same procedure was followed. The concentration of fumaronitrile in the feed was increased to 5 mM without any detrimental effect on the mixed microbial population. The addition of maleimide (2.5 mM) however caused a decline in the biomass and complete degradation of all the components was no longer achieved. By lowering the dilution rate from D = 0.06 h^{-1} to D = 0.03 h^{-1} and switching between batch and continuous culture a recovery was observed. The culture was again monitored for biomass and ammonia production, and the complete degradation of the added substrates was determined by HPLC and GLC. The procedure as previously described was repeated in order to attempt further adaptation. The concentration of maleimide was increased to 5 mM during the experiment and in the later stages other components of the AN waste, acrolein (0.5 mM) and cyanide (1 mM) were also added. The initial dilution rate used was D = 0.06 h^{-1}. This was reduced to D = 0.04 h^{-1} after approximately 250 h of continuous operation and the system was maintained at this lower dilution rate for the remainder of the experiment. The microbial culture proved competent to degrade all of the major components added in the synthetic effluent.

During the progress of the experiment the biomass changed from a dispersed to a semi-dispersed floccu-

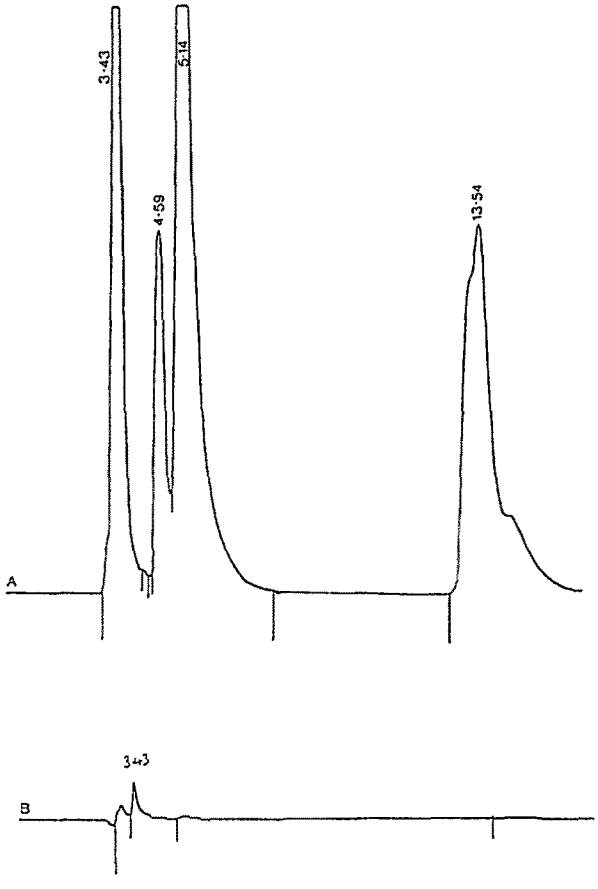

Fig. 5. A comparison (HPLC traces) of the medium entering the fermenter (A) and the run-off from the fermenter (B). These samples were taken after approximately 2400 hours growth of the mixed culture. The analysis of these samples were performed on an LDC isocratic HPLC system using an ODS reverse phase column. The solvent system used was A:B, 2:1 + TMA. A – 5 ml isopropanol, 1 g H_3HP_4 H_2O to 1 litre. B – 5 ml isopropanol, 1 g H_3PO_4, 750 ml methanol, H_2O to 1 litre. TMA – tetramethylammonium hydroxide 0.01 M. Peak time 3.43 min corresponds to acrylic acid. Peak time 4.59 min corresponds to acrylamide. Peak time 5.14 min corresponds to fumaronitrile and maleimide. Peak time 13.54 min corresponds to 3-cyanopyridine.

lar culture. The biomass, therefore, could no longer be determined by measurement of the absorbance at 610 nm. This, of course, is a helpful development if such a microbial culture were to be used in the commercial treatment of the AN wastes. Degradation of the growth substrates was monitored by HPLC and GLC. Figure 5 shows a comparison between the medium entering the fermenter and the subsequent outflow determined by HPLC. These samples were taken in the later stages of the experiment immediately after floccular growth had initiated, demonstrating significant degradation of this synthetic effluent. Overall, these results show that succinonitrile, 3-cyanopyridine, acrylamide, acrylic acid, fumaronitrile and maleimide (all initially at 5 mm), acrylonitrile and cyanide (1 mM) and acrolein (0.5 mm) were all substantially converted to carbon dioxide, ammonia and biomass by microbial activity. The mixed microbial population was examined by plate counts to try to determine the number of microorganisms remaining in the stable degradative population. Of the twenty isolates added initially twelve isolates were detected, although not further identified, being stable parts of the mixed culture. The most prevalent of the bacteria (4 species) were each 10–20% of the population. Several of the bacteria comprised less than 1% of the population, suggesting that, although small in number, they were still a vital component of the population.

Conclusions

By using classical enrichment procedures a range of bacteria have been obtained that are capable of utilising six of the eight major toxic components of the acrylonitrile waste effluents (succinonitrile, acrylonitrile, acrylamide, acrylic acid, cyanopyridine and maleimide). Good growth of these isolates occurred on their isolation substrate, with the exception of the maleimide-utilizing bacterium which grew only slowly on maleimide. With the exception of fumaronitrile, acrolein and maleimide, good cross specificity occurred for growth of the isolates on the other major components of the toxic wastes (Table 3). The problems associated with maleimide, fumaronitrile and acrolein were solved by a combination of transformation and degradation. In the case of maleimide, slow growth of the isolate on this compound could cause problems in a mixed culture system growing on the acrylonitrile effluent. This problem has been overcome since several of the isolates previously obtained on other components of the effluent could utilize maleamic acid, the immediate conversion product of maleimide. Other isolates were obtained that grew readily on succinimide, the saturated analogue of maleimide, but not on maleimide itself. However, these bacteria rapidly converted maleimide to maleamic acid, which could then be utilized by the other organisms. Although fumaronitrile is highly biocidal, extended enrichment in continuous culture involving gradual acclimation to fumaronitrile enabled a consortium of bacteria to be developed from which a pure isolate was obtained. However, an alternative approach was also devel-

oped. It was shown that several of the original isolates could grow on succinonitrile, the saturated analogue of fumaronitrile. These bacteria, when grown on succinonitrile, could convert fumaronitrile to 3-cyanopropenoate. Other isolates were then obtained that grew on 3-cyanopropenoate. Isolates able to grow on acrolein were not obtained, probably due to its instability in aqueous culture medium and its volatility. Since this compound is probably an intermediate in the assimilation of allyl alcohol, bacteria were isolated that grew on allyl alcohol. These isolates readily converted acrolein to acrylic acid. We have therefore obtained bacteria or potential bacterial systems that may be capable of degrading all the major components of the acrylonitrile effluent streams.

The microorganisms have been utilised in a complex mixed culture to biodegrade mixtures of the major components of the AN waste effluents as provided in synthetic wastes of similar composition. The mixed microbial population readily biodegraded succinonitrile, acrylamide, acrylic acid, 3-cyanopyridine and acrylonitrile, but the more toxic and recalcitrant compounds maleimide and fumaronitrile were not readily utilized. However, by using a combination of differing dilution rates, switching the system between batch and continuous culture and gradually increasing the concentration of the two recalcitrant chemicals the mixed microbial population has been adapted to give a stable population biodegrades all the major components of the AN waste in a synthetic mixture, mineralising them to carbon dioxide, ammonia and biomass. Since there are many multi-component, difficult to treat industrial effluents, the approach used in this study to develop a novel mixed culture system capable of biodegrading each of the components of a waste effluent may have many applications in the biotechnological treatment of toxic industrial waste effluents. Studies to date of the biodegradation of xenobiotics have generally been of two types. Firstly the degradation of a single compound by pure or mixed cultures of microorganisms obtained either by classical enrichment or by continuous enrichment (Slater & Bull 1982). Secondly mixtures of xenobiotics, usually industrial wastes, have been biodegraded by the gradual adaptation of activated sludge systems to tolerate previously toxic levels of various compounds. An example of the latter is the use of activated sludge systems to treat coke-oven liquors (Catchpole & Stafford 1977). As reported in this paper, using a defined mixed culture of bacteria obtained by enrichment for individual bacteria on each of the major individual components of a waste effluent, followed by combining the isolates to degrade the complex effluent is a novel approach to microbial degradation of toxic waste effluent. Further, the ability of the mixed population to tolerate increased concentrations of the more toxic constituents in continuous culture suggests this approach could be used to develop degradative communities for the detoxification of industrial wastes previously thought to be recalcitrant to microbial degradation.

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