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Use of a Nylon Manufacturing Waste as an Industrial Fermentation Substrate

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Nonvolatile residue (NVR), a waste stream from the manufacture of nylon 6'6', contains mainly small carboxylic acids and alcohols, making it a potential fermentation substrate. Above a concentration of 1.3% (wt/vol), NVR inhibited the growth of all microorganisms tested. The most inhibitory of the major NVR components were the monocarboxylic acids (C_4 to C_6) and ε-caprolactone. The inhibitory effects of NVR could be avoided by using a carbon-limited chemostat. Microorganisms were found that could use all of the major NVR components as carbon and energy sources. One such organism, *Pseudomonas cepacia*, was grown in a carbon-limited chemostat with a medium feed concentration of 20.5 g of NVR liter^{-1}. At a dilution rate of 0.14 h^{-1} the yield of biomass ($Y_{x/s}$, where x is biomass produced and s is substrate used) from NVR was 18% (neglecting the water content of NVR). It was concluded that NVR would be a suitable carbon source for certain industrial fermentation processes such as the production of poly-β-hydroxybutyric acid.

Cyclohexane is the only feedstock used in the production of nylon 6'6'. When it is oxidized to form adipic acid, various byproducts are also produced. Adipic acid is separated from the mixture by a distillation process which leaves a waste known as the nonvolatile residue (NVR). Large quantities of NVR are produced, but at present there is no better use for NVR than burning it for its heating value. Its composition suggested that an alternate use was as a feedstock for the fermentation industry. NVR is a dark brown liquid that is slightly denser than and only partially soluble in water at 25°C. It is a variable mixture of monobasic acids, dibasic acids, aldehydes, esters, and other organic compounds. The composition of the NVR used in the present study is shown in Table 1. Since it is an industrial waste, it would be difficult to use NVR for the production of food or health-related products. It may be of more value in the production of biosurfactants, biopolymers, and enzymes used for industrial purposes.

There have been many studies published concerning microbial growth on mixed substrates (2, 3, 5, 7, 12). However, although some success has been achieved (1, 4), very little work has been done to develop practical axenic culture systems for the conversion of complex toxic substrates into products of value. NVR is a particularly suitable carbon source with which to study microbial growth on mixed substrates, since no component accounts for more than 15% of the total. In this paper we examine the potential use of NVR as an industrial fermentation substrate.

MATERIALS AND METHODS

Organisms. The organisms used in the study are listed in Table 2.

NVR. NVR was supplied by the Du Pont Canada, Inc., research center, Kingston, Ontario.

Continuous culture. A Multigen F-2000 2-liter fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) was used as a chemostat with a 1.35-liter working volume. Temperature, aeration, agitation, and pH were controlled. NVR (or caproic acid) and mineral salts medium were added with separate peristaltic pumps since NVR is only slightly soluble in water.

Continuous enrichment culture. As an inoculum an aqueous soil extract, some refinery waste and sludge from the activated sludge pond of a nylon manufacturing plant (Du Pont Canada, Maitland, Ontario) were diluted in 0.05 M K_2HPO_4 buffer adjusted to pH 7.0 with KOH. Several milliliters of this was added as an inoculum to 1.35 liters of nutrient broth. After approximately 24 h, dilute carbon source and mineral salts medium as well as the inoculum were slowly but continuously fed into the reactor. Over a period of days the dilution rate and carbon source concentration were gradually increased, and eventually no more inoculum was added. The fermentation conditions were then kept constant for several days. This resulted in a stable continuous culture. When the carbon source was NVR, a complex mixed culture resulted, whereas when caproic acid alone was used, only one or two strains of microorganisms predominated in the fermentor.

Screening organisms for their ability to use major NVR components as the sole source of carbon and energy for growth. The cultures were maintained on agar (Difco Laboratories, Detroit, Mich.) containing 0.4% nutrient broth

TABLE 1. Composition of NVR used in this study[a]

Component	Concn (wt%)
Butyric acid	3.9
Valeric acid	13.3
Caproic acid	3.4
5-Hydroxyvaleric acid[b]	2.3
6-Hydroxycaproic acid[b]	13.9
Succinic acid	0.4
Glutaric acid	1.7
Adipic acid	8.4
Water	21.7
Others[c]	31.0

[a] Analysis was supplied by Du Pont Canada Inc. NVR also contained trace metals at 125 ppm.

[b] Analyzed by gas chromatography as hydroxymonocarboxylic acids, but probably ocur as lactones in NVR.

[c] Including cyclohexanediols, which account for approximately 4 to 5% of total NVR.

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TABLE 2. Microorganisms used in this study

Microorganism	Source[a]
Alcaligenes eutrophus ATCC 17697	ATCC
Alcaligenes faecalis subsp. *myxogenes* ATCC 14434	ATCC
Arthrobacter viscosus ATCC 19584	MMCC
Aurobasidium pullulans PpKM149	UWOBE
Azotobacter indicus subsp. *myxogenes* ATCC 21423	ATCC
Bacillus subtilis	UWOBE
Corynebacterium equi subsp. *mucilaginosus* ATCC 21521	ATCC
Corynebacterium fascians ICPB CF15	ICPB
Corynebacterium insidiosum ICPB CIBA	ICPB
Corynebacterium lepus	UWOBE
Escherichia coli	MMCC
Isolates I1, P2, P4, and B	NVRI
Isolates SS1 and SS2 (both identified as *P. aeruginosa*)	CAPI
Isolate C12	CAPI
Lactobacillus brevis ATCC 14434	ATCC
Leuconostoc mesenteroides ATCC 10830	ATCC
Lysobacter gummosus ATCC 29489	ATCC
Mycobacterium rhodochrous ATCC 19067	ATCC
Pseudomonas acidovorans ATCC 17476	ATCC
Pseudomonas aeruginosa NRC 2786	NRC
Pseudomonas cepacia ATCC 17759	ATCC
Rhodococcus rhodochrous NRC 43002	NRC
Saccharomyces cerevesiae	MMCC
Sclerotium rolfsii ATCC 15202	ATCC
Staphylococcus epidermidis	RVCC
Torulopsis bombicola ATCC 22214	ATCC
Torulopsis petrophilum ATCC 20225	ATCC
Xanthomonas campestris ATCC 13951	ATCC

[a] ATCC, American Type Culture Collection; ICPB, International Collection of Phytopathogenic Bacteria; NRC, from R. Latta, National Research Council Canada; MMCC, McGill University Department of Microbiology Culture Collection; UWOBE, University of Western Ontario Biochemical Engineering Culture Collection; RVCC, Culture Collection of the Royal Victoria Hospital, Montreal; NVRI, isolated from continuous enrichment cultures with NVR as the sole source of carbon; CAPI, isolated from continuous enrichment cultures using caproic acid as the sole source of carbon.

(Difco) and 0.55% yeast malt extract (Difco). They were transferred to agar plates containing mineral salts medium (but lacking yeast extract) and 0.05% (wt/vol) of the carbon source to be tested. After approximately 72 h at 24°C the cultures were again transferred to agar containing mineral salts and 0.05% of the desired carbon source. Plates lacking a carbon source were used as controls. The plates were observed every 24 h for 4 days and were evaluated as follows: no detectable difference from the control (−); noticeable growth (+); good growth (++).

Determination of the growth effect, I_1, and I_2. The growth effect, I_1 and I_2 were determined to summarize the effects of NVR on growth of microorganisms in shake flasks. Different amounts of NVR were added to 500-ml shake flasks containing 50 ml of a medium in which the organism to be tested could grow well. A 5% (vol/vol) inoculum was added, and flasks were shaken at 27°C. Flasks were sampled after a period of time sufficient to allow measurable growth but before the maximum biomass concentration was attained. The I_1 was the lowest NVR concentration at which less biomass was produced than the control. The I_2 was the lowest NVR concentration at which no growth occurred. NVR was said to have a positive growth effect if some flasks containing NVR had significantly (greater than 5%) more biomass than controls lacking NVR. A negative growth effect was assumed if at 50% of the I_2 concentration there was significantly (greater than 5%) less biomass than the control.

Media. The mineral salts medium consisted of 0.4% (wt/vol) $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.1% $Na_2HPO_4 \cdot 7H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 20 mg of $CaCl_2$ liter^{-1}, 1.6 mg of $(CH_3COO)_2Zn \cdot 2H_2O$ liter^{-1}, 0.3 mg of $FeSO_4 \cdot 7H_2O$ liter^{-1}, 0.6 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ liter^{-1}, 0.6 mg of H_3BO_3 liter^{-1}, 0.01% yeast extract, and 13 μl of HCl liter^{-1}. GM medium contained 2% glucose in mineral salts medium. MSC contained 2.0% sucrose and 0.2% citric acid in mineral salts medium. NB medium was 0.8% nutrient broth, and YM was 2.5% yeast malt broth. All shake flask cultures were grown at an initial pH of 6.0 in 50 ml of medium in 500-ml Erlenmeyer flasks on a rotary shaker (G10 Gyrotory shaker; New Brunswick Scientific) at 180 rpm.

Biomass dry weight determination. A 10-ml sample of culture broth was centrifuged for 10 min at 24,000 × *g* at 4°C. The supernatant was discarded, and cells were suspended in 10 ml of distilled water. This was centrifuged as above, and the resulting pellet was washed out of the centrifuge tube into a preweighed aluminum pan and dried at 105°C to a constant mass.

Cellular protein determination. A 10-ml sample of culture broth was centrifuged, washed, and recentrifuged as above. The resulting pellet was analyzed for protein content by the Biuret reaction (11). Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was used as a standard.

Gas analysis. Oxygen in the outlet air stream was measured paramagnetically with a Taylor Servomex oxygen analyzer (Crowborough, Sussex, England). Carbon dioxide was measured with a Lira infrared analyzer model 303 (Mine Safety Appliances, Pittsburgh, Pa.).

RESULTS

Effects of NVR on growth of microorganisms. The results of a growth inhibition study are listed in Table 3, where each line refers to a separate shake flask experiment. The organisms are listed in order of their I_1 value. There was a wide variation in the effect of NVR on growth. Just 0.16% (wt/vol) NVR prevented any growth of *B. subtilis* and *C. lepus*, whereas an NVR concentration of 1.3% (wt/vol) was necessary to totally prevent growth of *P. cepacia*. Growth of *L. gummosus* was measurably inhibited by as little as 0.01% (wt/vol) NVR, whereas growth of *P. cepacia* was not inhibited until an NVR concentration of 1.2% (wt/vol). *P. cepacia* could grow in a higher concentration of NVR than any other

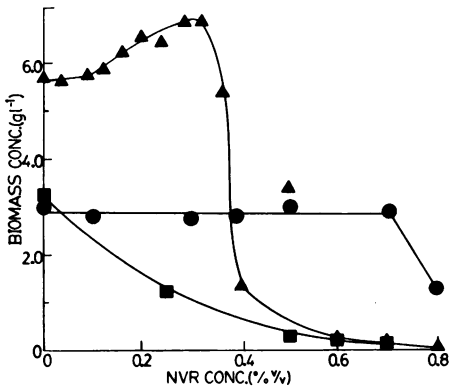

FIG. 1. Effects of NVR on the growth microorganisms in shake flasks. Symbols: ▲, stimulating effect (*T. bombicola*); ●, no effect (*T. petrophilum*); ■, inhibitory effect (isolate G3).

microorganism tested. Attempts were made at batch enrichment culture using 2.0% (vol/vol) NVR. Although a variety of complex inocula were employed, no microorganisms grew at that NVR concentration.

There were three distinct ways in which microorganisms responded to increasing concentrations of NVR (Fig. 1). Some, such as *T. bombicola*, were stimulated in growth by low NVR concentrations (i.e., positive growth effect). Some, such as *T. petrophilum*, were not affected by NVR until the I_2 concentration was approached (i.e., no growth effect). Others, such as isolate G3, were inhibited at NVR concentrations much lower than I_2 (i.e., negative growth effect). The growth effects of NVR on various microorganisms are summarized in Table 3. Generally, organisms that could grow at relatively high NVR concentrations were stimulated by low concentrations of NVR.

Effects of NVR components on microbial growth. Of the major NVR components (listed in Table 1), only the monocarboxylic acids and ε-caprolactone were found to inhibit the growth of microorganisms. This was determined by exposing microorganisms to relatively large concentrations of individual NVR components. The results of one such experiment are listed in Table 4. In this experiment, dicarboxylic acids stimulated growth of isolate 1, whereas 1,2-cyclohexanediol and the trace metals found in NVR had no effect. Monocarboxylic acids and ε-caprolactone greatly inhibited growth of isolate 1. These results were also found with *T. petrophilum*, *B. subtilis*, *C. lepus*, and *X. campestris*, with the exceptions that *T. petrophilum* was not inhibited by 0.6% (wt/vol) ε-caprolactone and growth of *B. subtilis* was not stimulated by dicarboxylic acids in a 0.6% (wt/vol) concentration.

The monocarboxylic acids were more inhibitory to microbial growth than was ε-caprolactone. However, although ε-caprolactone did not completely prevent growth of *P. cepacia*, for example, until a concentration of 0.9% (wt/vol), its presence resulted in less growth than in the control at a concentration of only 0.4% (wt/vol) (Fig. 2). It was a general finding that monocarboxylic acids stimulated growth when below their I_2 concentration, whereas ε-caprolactone inhibited growth well below its I_2 for a specific microorganism.

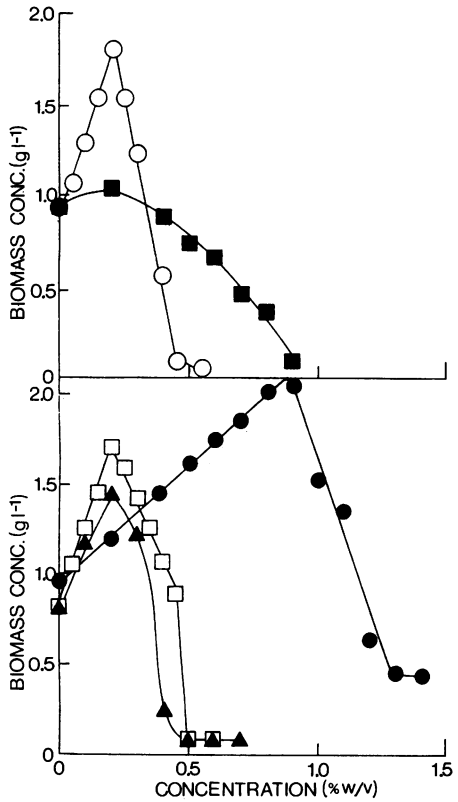

FIG. 2. Effects of NVR and growth inhibitory NVR components on growth of *P. cepacia* in shake flasks. Symbols: ●, NVR; ○, butyric acid; ▲, valeric acid; □, caproic acid; ■, ε-caprolactone.

Use of NVR components as carbon and energy sources by microorganisms. The ability of various microorganisms to use individual NVR components as sole sources of carbon and energy is shown in Table 5. Pseudomonads and corynebacteria were the procaryote genera most capable of using NVR components. No microorganism tested could use 1,2- or 1,4-cyclohexanediol, but bacteria such as *P. cepacia*,

TABLE 3. Effect of NVR on growth of various microorganisms

Organism	Medium[a]	I[b] (%, wt/vol)	I[c] (%, wt/vol)	Growth effect[d]
P. cepacia	NB	1.2	1.3	++
P. acidovorans	NB	0.9	0.9	++
T. peptrophilum	MSC	0.8	0.8	±
E. coli	NB	0.7	1.5	±
Isolate I1	NB	0.6	0.6	++
P. aeruginosa (SS2)	GM	0.5	0.5	++
A. eutrophus	NB	0.4	0.4	++
T. bombicola	MSC	0.34	0.40	+
Isolate B	MSC	0.3	0.3	+
S. epidermidis	NB	0.3	0.3	±
A. viscosus	YM	0.3	0.3	±
Isolate G3	MSC	0.2	0.5	−
B. subtilis	MSC	0.12	0.16	+
C. lepus	MSC	0.12	0.16	±
C. fascians	MSC	0.12	0.16	±
Isolate P2	MSC	0.1	0.5	−
C. insidiosum	MSC	0.1	0.18	−
L. mesenteroides	YM	0.05	0.5	−
X. campestris	YM	0.05	0.3	−
L. gummosus	NB	0.01	0.5	−

[a] NB, Nutrient broth; MSC, mineral salts-citrate broth; GM, glucose-mineral salts broth; YM, yeast malt broth.
[b] Lowest concentration of NVR that was inhibitory to growth.
[c] Lowest concentration of NVR that totally prevented growth.
[d] ++, Great stimulation of growth below the I_2 NVR concentration; +, some stimulation of growth below the I_2 NVR concentration; ±, no effect on growth below the I_2 NVR concentration; −, inhibition of growth at 50% of the I_2 concentration.

TABLE 4. Effect of the major NVR components on the growth of isolate 1

NVR component	Concn (%, wt/vol)	Biomass dry wt (g liter^{-1})	Final pH	Growth effect
Control	0	1.96	7.6	
1,2-cyclohexanediol (*cis* plus *trans*)	0.6	1.95	7.9	None
Trace metal 1		1.68	7.5	None
Trace metal 2		1.85	7.6	None
ε-Caprolactone	0.6	0.11	6.5	Inhibitory
Butyric acid	0.6	0.10	6.4	Inhibitory
Valeric acid	0.6	0.02	6.4	Inhibitory
Caproic acid	0.6	0.15	6.4	Inhibitory
Succinic acid	0.6	3.56	9.5	Stimulatory
Glutaric acid	0.6	3.81	9.5	Stimulatory
Adipic acid	0.6	5.48	9.2	Stimulatory

TABLE 5. Use of major NVR constituents as sole sources of carbon and energy by various microorganisms[a]

Organism	NVR	ε-Caprolactone	Monocarboxylic acids			Dicarboxylic acids		
			C_4	C_5	C_6	C_4	C_5	C_6
A. eutrophus	+	+	+	−	+	+	+	+
A. indicus	−	−	−	−	−	−	−	−
A. viscosus	−	−	−	−	−	++	+	+
B. subtilis	−	−	−	−	−	+	−	−
C. equi	+	+	+	+	+	+	−	+
C. fascians	−	−	−	−	−	+	−	−
C. lepus	+	+	+	+	−	+	+	+
E. coli	−	−	−	−	−	+	−	−
Isolate B	−	−	−	−	−	−	−	−
Isolate CAP12	+	++	+	+	+	+	+	+
Isolate Il	+	−	+	+	+	+	+	+
Isolate P2	−	−	−	−	−	+	+	+
Isolate P4	−	−	−	−	−	+	−	−
L. brevis	−	−	−	−	−	−	−	−
L. gummosus	−	−	−	−	−	−	−	−
L. mesenteroides	−	−	−	−	−	−	−	−
M. rhodochrous	+	−	+	+	−	+	+	+
P. acidovorans	+	−	+	+	+	+	+	+
P. aeruginosa	+	+	+	+	+	+	+	+
P. aeruginosa (SS1)	+	+	+	+	+	+	+	+
P. aeruginosa (SS2)	+	+	+	+	+	+	−	+
P. cepacia	+	+	++	++	++	+	+	+
R. rhodochrous	+	−	−	−	−	+	+	+
S. cerevesiae	−	−	−	−	−	−	−	−
S. epidermidis	−	−	−	−	−	+	−	−
S. rolfsii	−	−	−	−	−	+	−	−
T. bombicola	−	−	−	−	−	−	−	−
T. petrophilium	+	−	+	+	+	−	−	−
X. campestris	−	−	−	−	−	+	−	−

[a] Results are expressed as no detectable difference from the control (−), noticeable growth (+), or good growth (++) on the indicated substrate.

P. aeruginosa, and CAP 12 could use all of the other major NVR components as sole carbon and energy sources. *A. eutrophus*, *P. acidovorans*, *C. lepus*, and *C. equi* could use most of the major NVR components as sole carbon and energy sources. *A. eutrophus* is not known to use valeric acid (8) and did not when it was supplied in solid agar; however, when it was grown with the same mineral salts medium in a carbon (NVR or fructose)-limited chemostat, a pulse of valeric acid elicited a sharp increase in oxygen consumption. This indicates the ability to use that component as a carbon source (9), especially since the shape of the oxygen uptake response curve was identical to those elicited by butyric and caproic acids, which the organism can certainly use as carbon and energy sources.

Growth of *P. cepacia* on NVR. To prove conclusively that microorganisms can grow on NVR, *P. cepacia* was grown in a carbon-limited chemostat with NVR as its sole source of carbon and energy. The dilution rate was constant at 0.14 h^{-1}. Steady-state growth (monitored by gas analysis) was achieved at feed concentrations of up to 17.8 g of NVR $liter^{-1}$. Above this level, oxygen limitation occurred due to the mass transfer limitations of the fermentor. The yield ($Y_{x/s}$) was 13.6 g of biomass for every 100 g of NVR supplied. (Fig. 3). When the water content of NVR was subtracted, the calculated yield ($Y_{x/s}$, where x is the biomass produced and s is the substrate used) was 18%. Under carbon-limited conditions, the protein content of *P. cepacia* was 60% when growing on NVR using $(NH_4)_2SO_4$ as the nitrogen source. The Q_{O_2} was 9.2 mM g^{-1} h^{-}, and 9.5 mmol of O_2 was consumed for every g of NVR supplied under carbon-limited conditions at this dilution rate.

At a lower dilution rate (0.09 h^{-1}), the steady state was achieved at an NVR feed concentration of 20.5 g $liter^{-1}$. This could be attained because the oxygen requirements were less at the lower dilution rate. The biomass yield was the same as at the 0.14 h^{-1} dilution rate.

DISCUSSION

Since shake flasks containing I_1 or I_2 concentrations of NVR by definition contained less biomass at the time of sampling than did the control and since flasks were sampled relatively soon after inoculation, I_1 and I_2 must reflect the effect of NVR on growth rate rather than on yield. Most of the organisms tested were inhibited in growth by NVR concentrations greater than 0.35% (wt/vol). Such low I_1 values make these organisms unsuitable for industrial fermentations lacking rigid control. If during poorly controlled chemostat growth the concentration of NVR reached the I_1 value, growth would be inhibited. If the specific growth rate decreased to less than the dilution rate, washout would occur.

Since the highest I_2 value found was only 1.3% (wt/vol), conventional batch culture technique must be discounted as an economical method of using NVR as a fermentation substrate. Carbon-limited fed-batch and carbon-limited chemostat culture are the likeliest alternatives. These fermentation methods would be successful, provided the microorganism used in the process was capable of utilizing the major growth-inhibiting components of NVR. Under carbon-limited conditions, this would keep the steady-state concentration of these components well below their inhibitory concentration. This is verified by the observation that *P. cepacia* can be grown in a chemostat with a medium feed

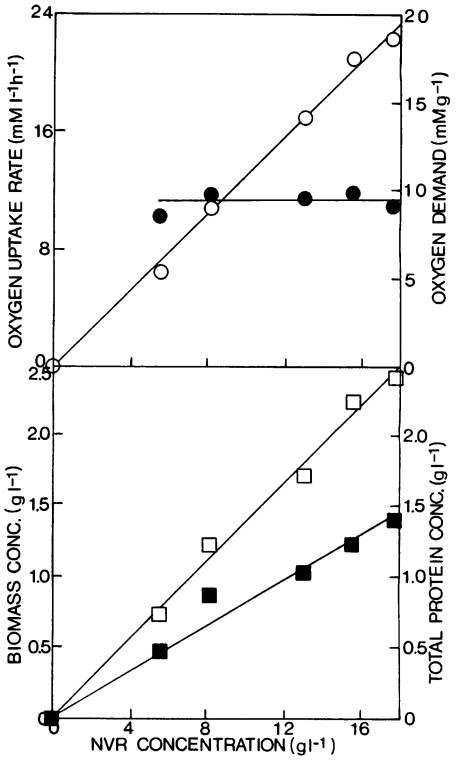

FIG. 3. Growth of *P. cepacia* in an NVR-limited chemostat at a dilution rate of 0.14 h^{-1} at 30°C. Symbols: □, biomass concentration; ■, total protein concentration; ○, oxygen uptake rate; ●, oxygen demand (millimoles of oxygen used per gram of NVR supplied).

concentration of NVR much higher than the I_2 value for that organism. The use of carbon-limited fed-batch or chemostat culture also eliminates the problem of multiple growth phases (i.e., diauxy) associated with batch growth on mixed substrates. Since ε-caprolactone and monocarboxylic acids (C_4 to C_6) are the major toxic NVR components, only microorganisms able to use those components could be considered for an industrial fermentation process with NVR.

It should be noted that temperature, pH, and the degree of mixing greatly influence the apparent I_1 and I_2 values. Any factor affecting the solubility of carboxylic acids in the medium affects the apparent I_1 and I_2 for NVR.

Those microorganisms with the highest I_1 and I_2, with the greatest ability to utilize toxic NVR components, and that were stimulated in growth by low NVR concentration belonged to the genus *Pseudomonas* and to related genera such as *Alcaligenes*. NVR could most easily be used as a fermentation substrate in processes utilizing these organisms. One such process is the manufacture of poly-β-hydroxybutyric acid, which can be used as a thermoplastic (6, 10). *P. cepacia* and *A. eutrophus* both accumulate poly-β-hydroxybutyric acid as their major storage polymer.

It can be concluded that NVR is a potential fermentation substrate for a limited number of industrial processes involving a small number of microbial genera. To be of value in such a process, a microorganism must not be inhibited by low concentrations of NVR, must be able to utilize monocarboxylic acids (C_4 to C_6) and ε-caprolactone, and must produce from NVR some useful product not related to the food or health industries.

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