

**METHODS FOR THE REMOVAL OF UNWANTED MONOMER
AMIDE COMPOUNDS FROM POLYAMIDE PREPARATIONS**

1. FIELD OF THE INVENTION

5 The present invention is related to methods for the
removal of an unwanted monomer amide compound from a
polymerized amide or polyamide preparation, e.g., removal of
unwanted acrylamide monomer from a polyacrylamide preparation
at a pH of about 2 to less than 6, using a pure culture of an
induced microorganism strain capable of removing the monomer
10 compound from the polymer preparation.

2. BACKGROUND OF THE INVENTION

Nitriles are exceedingly versatile compounds that
can be used in the synthesis of a wide variety of compounds,
15 including amines, amides, amidines, carboxylic acids, esters,
aldehydes, ketones, imines, and heterocyclics. One of the
most important commercially important nitriles is
acetonitrile which is a common solvent. Other nitrile
compounds are used as herbicides or in the synthesis of
20 detergents or antiseptics. Another of the most commercially
important nitriles is acrylonitrile, which is used to make
acrylamide, acrylic acid, acrylic fibers, copolymer resins
and nitrile rubbers.

One method of production of acrylonitrile is by use
of the SOHIO/BP process, which entails the direct
25 ammoxidation of propene (a/k/a propylene) by ammonia vapors
in air in the presence of a catalyst (see generally,
Acrylonitrile, 1979, Process Economics Program Report,
Stanford Research International, Menlo Park, CA; Weissermel
and Arpe, 1978, Industrial Organic Chemistry, Verlag Chemie -
30 Weinheim New York, pp. 266-270). The waste stream from this
process contains a complex mixture of nitriles, including
dinitriles, amides and acids at high concentrations. More

particularly, the wastestream generally contains nitriles such as acetonitrile, acrylonitrile, succinonitrile and fumaronitrile as well as acrylamide. In addition, cyanide(s) at variable and/or high concentrations is/are often present.

5 The wastestream generally contains high and/or variable concentrations of ammonium sulfate. This hazardous waste effluent cannot be released into the environment due to its toxicity and in the United States is usually disposed of by deep well injection into sub-surface formations. Such disposal cannot be considered to be a "treatment" of the
10 wastestream, but rather is analogous to the process of landfilling.

Outside the United States, it has been common practice to "treat" the wastestream from the production of acrylonitrile by diluting the wastestream to a low total
15 nitrile concentration of about 250 ppm or less and treating by conventional aerated biological wastestream systems, i.e., activated sewage sludge, after wet/air oxidation, which removes volatiles and partially oxidizes many of the organic constituents. Such method of "treatment" is not suitable to
20 efficient disposal of a nitrile production facility wastestream for the following reasons: (1) wet/air oxidation causes volatile compounds to be stripped, creating an air emission problem; (2) dilution of the wastestream necessitates large wastewater treatment facilities to handle the flow and long residence time required to obtain adequate
25 "treatment"; and (3) combination of wet/air oxidation with biological treatment by activated sludge results in high treatment costs. There remains a long, deep felt need in the nitrile production industry for an efficient, cost effective, environmentally sound method to dispose of the effluent of
30 nitrile production plants.

It has long been known that certain microorganisms are useful to convert a nitrile compound to its corresponding

amide or acid compound biologically. Both the scientific and patent literature contain numerous references describing the use of nitrile converting microorganisms for the production of specialty chemicals, for example, acrylamide and acrylic acid or acrylate from acrylonitrile. See generally, Kobayshi
5 et al., 1992, Trends Biotechnol. 10:402-408. The nitrile converting microorganisms have been shown to have activities including, nitrilase, which converts a nitrile compound to its corresponding acid compound; nitrile hydratase, which converts a nitrile compound to its corresponding amide
10 compound; and amidase, which converts an amide compound to its corresponding acid compound.

To the knowledge of the present inventor, however, in all these specialty chemical productions using nitrile degrading microorganisms, only a single compound has been
15 employed to induce the relevant activity and only a single nitrile compound has been converted to produce a single desired specialty compound.

2.1. MICROORGANISMS WHICH CAN UTILIZE A NITRILE COMPOUND

20 The literature contains certain references which disclose a number of microorganisms which can utilize a nitrile or an amide compound as the sole source of carbon and/or nitrogen.

For example, Asano et al., 1982, Agric. Biol. Chem. 46:1165-1174, describe an isolated strain of *Arthrobacter*
25 which is able to grow using acetonitrile as a sole source of carbon and nitrogen.

Nawaz et al., 1989, 43rd Purdue Ind. Waste Conf. Proc., pp. 251-256 (Nawaz, 1989), describe the isolation of a *Pseudomonas aeruginosa* strain which is able to utilize
30 various nitrile compounds, including acetonitrile, as a sole source of carbon and energy. However, this strain is unable

to utilize other nitrile compounds such as acrylonitrile, acrylamide, benzonitrile and malononitrile.

Nawaz et al., 1992, Appl. Environ. Microbiol. 58:27-31 (Nawaz), describe a *Klebsiella pneumonia* NCTR1 strain which, after acclimation using benzonitrile, could degrade a mixture of benzonitrile and one other nitrile selected from butyronitrile, acetonitrile, glutaronitrile, propionitrile, succinonitrile and methacrylonitrile. In complete contrast to the present method for induction which does not require the presence of an aromatic nitrile, Nawaz's microorganism required benzonitrile in order to induce the ability to degrade the mixtures of benzonitrile and one other nitrile. Moreover, and most importantly, in order to achieve degradation of any of the mixtures of nitriles, benzonitrile had to be present. Since the nitrile wastestream of a nitrile production facility does not contain benzonitrile, this organism and the method disclosed by Nawaz would be completely impractical and, in fact, inoperative for treating such wastestream.

Chapatwala et al., 1993, App. Biochem. Biotech. 39/40:655-666, describe the isolation of a *Pseudomonas putida* strain which is capable of utilizing acetonitrile as a sole source of carbon and nitrogen. However, there is no disclosure of utilization of any other nitrile-containing compound by the strain.

Narayanasamy et al., 1990, Indian J. Exp. Biol. 28:968-971, disclose the utilization of acrylonitrile, acetonitrile, acrylamide and acetamide by an *Arthrobacter* sp. individually. There is no indication that the bacterial strain is able to degrade dinitriles or a mixture of nitriles.

O'Grady and Pembroke, 1994, Biotech. Letters 16:47-50, describe the isolation of an *Agrobacterium* sp. and the ability of the isolated strain to utilize or break down a

number of different nitrile compounds individually. There is no indication that the isolated strain would be able to utilize or break down a mixture of the nitrile compounds.

Martinkova et al., 1992, Folia Microbiol. 37:373-
5 376, disclose the isolation of several bacterial strains, including *Corynebacterium* sp. strain 3 B and *Agrobacterium radiobacter* strain 8/4/1, which are able to utilize acetonitrile as a sole source of carbon and nitrogen. There is no disclosure that the strains are able to utilize any
10 other nitrile compound or a mixture of nitrile compounds.

Nawaz et al., 1994, Appl. Environ. Microbiol. 60:3343-3348, describe the isolation of a bacterium, tentatively identified as a *Rhodococcus* sp., from soil contaminated with the herbicide alachlor. This bacterium was shown to be able to use acrylonitrile as a sole source of
15 carbon and nitrogen.

Nawaz et al., 1993, Can. J. Microbiol. 39:207-212, describe the isolation of a *Pseudomonas* sp. and *Xanthomonas maltophilia*, which each can utilize acrylamide as a sole source of carbon and nitrogen.

Armitage et al., International Patent Publication WO 97/06248, published February 20, 1997, discloses methods for producing an amidase by culturing a suitable
20 microorganism in the presence of an amide or an amide precursor, such as a nitrile, or a mixture thereof, under continuous culture, carbon-limiting conditions in which the
25 amide or amide precursor forms at least 20% mol and preferably substantially all of the carbon. Suitable microorganisms include *Pseudomonas*, *Rhodococcus*, etc. Also disclosed are methods for producing a nitrilase by culturing a microorganism in the presence of a nitrile or a nitrile
30 precursor, or a mixture thereof, under continuous culture carbon-limiting culture conditions. Suitable microorganisms include *Nocardia*, *Rhodococcus* spp., including *Rhodococcus*

ATCC 39484, etc. The induced enzyme is then used, *inter alia*, to convert a nitrile or amide to its corresponding acid.

Although microorganisms which utilize a nitrile
5 compound might possibly be useful to remove a single nitrile
compound from a nitrile containing composition, the use of
such organisms to aid in the disposal of the wastestream of a
nitrile production facility is not to be expected because
nitrile utilization is dependent upon the expression of a
10 nitrilase or nitrile hydratase specific to the single nitrile
compound utilized. Expression of a specific nitrilase or
nitrile hydratase does not assure that the microorganism will
have the ability to convert the mixture of nitrile compounds
or the mixture of nitrile and amide compounds present in the
high concentrations found in the wastestream of a nitrile
15 production facility.

2.2. TREATMENT OF NITRILE WASTES INCLUDING A WASTESTREAM OF A NITRILE PRODUCTION PLANT

A number of references describe attempts to provide
20 a microbiological method to remove a nitrile from a nitrile
waste, including the wastestream of a nitrile production
plant.

U.S. Patent No. 3,940,332 to Kato et al. (Kato)
describes the use of an isolated bacterial strain, *Nocardia*
rubropertincta, ATCC Accession No. 21930, in combination with
25 activated sludge from a sewage treatment plant to degrade a
wastestream containing nitriles and inorganic cyanides. Kato
also indicates that the bacterial strain is able to degrade
nitriles, including, acetonitrile, acrylonitrile,
propionitrile, butyronitrile, crotononitrile, fumaronitrile,
30 valeronitrile, glutaronitrile, and benzonitrile, although no
indication is given of the amount of each of such nitriles or
conditions under which the nitriles are degraded or whether

the nitriles can be degraded together. There is no indication that the strain disclosed by Kato can degrade or detoxify a mixture of nitrile compounds. Further, the nitrile waste treated by Kato was a low strength waste, 50-
5 250 ppm total nitrile concentration. Moreover, the present inventor has tested the strain disclosed by Kato and found that the strain does not remove acetonitrile from a mixture of nitriles with the same efficiency as can be accomplished using the methods of the present invention.

10 Sunarko and Meyer, 1989, DECHMA Biotech. Conf. 3:859-862, disclose that lyophilized cells of *Mycobacterium* UBT5, *Bacillus* UBT2, *Corynebacterium* UBT9, and *Flexibacter* UBT4, which had been induced by growing the cells in the presence of 2-pentenitrile, were able to degrade small quantities of acetonitrile found in laboratory HPLC column
15 effluent.

Brown et al., 1980, Water Res. 14:775-778, disclose that acrylamide spiked at concentrations of 0.5 ppm to 5 ppm into natural and polluted waters of the environment resulted in the degradation of the acrylamide.

20 Kincannon et al., 1983, Journal WPCF 55:157-163, disclose that a mixture of microorganisms isolated from a municipal activated sludge water treatment plant was able to degrade acrylonitrile after a one month acclimation period. Further, the authors also showed that acrolein could be
25 similarly degraded by the mixture of microorganisms.

Donberg et al., 1992, Environ. Toxicol. Chem. 11:1583-1594, disclose that a mixture of microorganisms found in soil was able to degrade acrylonitrile under aerobic conditions. Some mixtures were able to degrade 10-100 ppm acrylonitrile on the order of 2 days. However, at higher
30 concentrations of acrylonitrile (1000 ppm), degradation was inhibited. The authors speculated that the inhibition was

due to inhibitory effects of the parent acrylonitrile compound.

Knowles and Wyatt, European Patent No. 274 856 B1 and Wyatt and Knowles, 1995, Biodegradation 6:93-107, describe the degradation of a mixture of nitrile and amide compounds from the wastestreams of a nitrile production plant (using the BP/SOHIO process of acrylonitrile production) by a mixture of microorganisms. The use of a mixture of microorganisms rather than a pure culture, is a serious drawback of the method of Knowles and Wyatt. It is difficult to maintain a mixed culture, for as the conditions of the reaction change, certain strains within the mixed culture will be favored at different times for growth such that the efficacy of degradation can be decreased.

There are a number of disadvantages associated with the above references. For example, many of the individual microbial strains described above have only a limited range of nitrile or amide compounds which they can degrade. The time required for degradation/utilization of nitrile and amide compounds is on the order of days or weeks. Further, disposal by traditional activated sludge treatment has its own drawbacks, such as the large amount of biomass produced, which must eventually be disposed. Moreover, and most importantly, the use of a mixture of microorganisms rather than pure cultures, makes it very difficult because it is difficult to maintain the mixed culture. As conditions of the reaction change, certain strains within the mixed culture will be favored at different times for growth, such that over time the characteristics of the mixed culture will change and the efficacy of degradation can decrease. The mixed culture is not easily reproduced or maintained.

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2.3. TREATMENT OF POLYACRYLAMIDE PREPARATIONS TO REMOVE THE ACRYLAMIDE MONOMER

A number of references describe attempts to provide a microbiological method to remove an unwanted acrylamide monomer from a polyacrylamide preparation.

5 Polyacrylamide polymers are widely used in several industries, including sewage treatment, paper manufacturing, mining, and in biological and chemical research. However, their utility is restricted and they cannot be used in connection with foodstuffs because they are generally
10 contaminated with unreacted acrylamide monomer which is a cumulative neurotoxin and a carcinogen. One method of reducing the amount of unreacted monomer is the "heat treatment" method. However, this treatment increases the cost of manufacture and decreases the efficiency of polymer
15 production due to undesired branching of the polymers.

Another method is the use of amidase obtained from a microorganism. For example, Carver et al., European Patent Publications EP 272,025 A2 and EP 2072,026 A2, describe the decomposition of acrylamide in polyacrylamide preparations using an amidase obtained from an organism such as
20 *Methylophilis* sp. which has been heated to a temperature in the range of 40°C to 80°C. However, the methods disclosed by Carver et al. do not allow for the removal of acrylamide monomer from a cationic polyacrylamide preparation at its native pH value, i.e., a native pH of about 4. See Figure 6
25 in EP 272,025 A2, which clearly shows that in order for the acrylamide monomer to be removed from the cationic polymer, the pH must be raised from a pH of 4 to a pH of 6.

U.S. Patent Nos. 4,687,807 and 4,742,114 to Westgrove et al. describe the production of water-in-oil
30 emulsions of amidase for use in removing unwanted acrylamide monomer from a preformed acrylamide polymer.

Farrar et al., European Patent Publication EP 329,325 A2, describes the removal of acrylamide monomer from polyacrylamide using an aqueous gel of amidase obtained from an amidase-expressing microorganism. Farrar, International Patent Publication WO 92/05205, describes reduction of residual (meth)acrylamide monomer in a polymerized (meth)acrylamide by incorporating amidase into a polymerizable mixture for exothermic polymerization of polymeric (meth)acrylamide.

Armitage et al., International Patent Publication WO 97/06248, published February 20, 1997, also discloses methods for the conversion of meth(acrylamide) to ammonium meth(acrylate) in or after the polymerization of the acrylamide using an amidase which has been induced in a suitable microorganism obtained by culturing the microorganism in the presence of an amide or an amide precursor, such as a nitrile, or a mixture thereof, under continuous culture, carbon-limiting conditions in which the amide or amide precursor forms at least 20% mol and preferably substantially all of the carbon. Suitable microorganisms include *Pseudomonas*, *Rhodococcus*, etc. However, no working examples are provided in which the induced enzyme actually converts the acrylamide monomer in a polyacrylamide preparation, and further, there is no disclosure regarding the pH range in which the conversion reaction could take place.

Citation or identification of any reference in Section 2 or any section of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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3. SUMMARY OF THE INVENTION

The present invention provides methods for removing an unwanted amide monomer from polyamide or polymerized amide preparations, for example, removing acrylamide monomer from a polyacrylamide preparation. An illustrative method entails contacting a polyacrylamide preparation containing acrylamide monomer, said polyacrylamide preparation having a pH of about 2 to less than about 6, with a pure culture or a crude extract of a microorganism strain which has been multiply induced for a period of time sufficient to reduce the amount of acrylamide monomer by converting the acrylamide to the corresponding acrylic acid. Preferably, the polyacrylamide preparation has a pH of about 2 to about 5, more preferably a pH of about 3 to about 4. Also, preferably, the amount of acrylamide monomer in the polyacrylamide preparation is reduced to less than 100 ppm.

A microorganism strain useful to remove an unwanted amide monomer from polyamide preparation can be multiply induced by culturing a pure culture of a microorganism strain on nutritionally complete medium containing a mixture of nitrile compounds or a mixture of nitrile and amide compounds. Alternatively, a microorganism strain useful to remove an unwanted amide monomer from a polyamide preparation can be multiply induced by culturing a pure culture of a microorganism strain on minimal medium containing a mixture of nitrile compounds or a mixture of nitrile and amide compounds as the sole source of carbon and energy and/or nitrogen. The methods for multiple induction do not require the presence of an aromatic nitrile.

The pure cultures of the microorganisms can be stored for extended periods of time after induction, e.g., at least 4 months under normal refrigeration temperatures, i.e., about 4°C, longer (years) under normal freezer temperatures, i.e., -20°C or lower or when freeze-drying or

cryopreservation is employed, without the loss of amide removal activity. Certain microorganisms are capable of utilizing at least one of the nitrile compounds as a sole source of carbon and energy. Certain microorganisms are capable of utilizing at least one of the nitrile or amide compounds as a sole source of carbon and energy and nitrogen.

According to the present invention, once induced, the pure microorganism culture does not have to be actively dividing or even alive for the removal to occur. This decoupling of growth from removal allows for rapid removal of unwanted amide monomer compounds under conditions inhibitory for growth; for example, under very high concentration(s) of nitrile compounds, highly alkaline or acid pH, high temperature, e.g., 55°C, etc. In addition, decoupling: (1) means that since cells need not be growing to remove the amide monomer, then cells (biomass) are not produced or their production is minimized, where cells are growing, it becomes necessary to deal with the waste biomass which is created; (2) because the cells are not growing, compounds which may not serve as growth substrates can be fortuitously removed; and (3) the process results in the production of less toxic or non-toxic intermediates which can easily be degraded in nature (reduction in recalcitrance as well as toxicity).

3.1. DEFINITIONS

As used in this application, a "nitrile" compound is intended to encompass an organic compound containing one or more nitrile moieties, i.e., C≡N, and at least one carbon atom in addition to the C≡N moiety. As presently used, the term "nitrile" includes compounds like acrolein cyanohydrin. It is noted that acrolein in the presence of reactive cyanide exists in the form of acrolein cyanohydrin. The nitrile detoxifying microorganisms are capable of converting a nitrile which is a cyanohydrin to a corresponding acid. For

example, acrolein cyanohydrin is converted to acrylic acid. As presently used, the term "nitrile" includes, but is not limited to, acetonitrile, acrylonitrile, fumaronitrile, succinonitrile, crotononitrile, adiponitrile, benzonitrile, butyronitrile, β -propionisulfononitrile, isovaleronitrile, valeronitrile, phenylnitrile, acrolein cyanohydrin, etc.

As used in this application, an "anionic" polyamide preparation is a polyamide preparation that contains an anionic main chain and/or side chain group(s) and has a net negative charge.

As used in this application, a "cationic" polyamide preparation is a polyamide preparation that contains a cationic main chain and/or side chain group(s) and has a net positive charge.

As used in this application, a "non-ionic" polyamide preparation is a polyamide preparation that either does not contain anionic and/or cationic main chain and/or side chain group(s), or if one or more such groups is present has a net neutral charge.

3.2 OBJECTS OF THE INVENTION

It is an object of the present invention to provide methods for removal of an unwanted amide monomer from a polyamide preparation at a pH of about 2 to less than 6 by converting the amide monomer to the corresponding acid compound.

Other objects and/or advantages of the invention will be apparent to those skilled in the art.

4. DETAILED DESCRIPTION OF THE INVENTION

The present invention encompasses methods for the removal of an unwanted amide monomer from a polyamide or polymerized amide preparation at a pH of about 2 to less than 6 by conversion of the amide monomer to the corresponding

acid compound using a pure culture of a single microorganism strain induced to be capable of converting the monomer amide moieties to acid moieties.

Removal of a monomer amide from the preparation can
5 be monitored by assessing the disappearance of the monomer amide compound and/or the concurrent appearance of the corresponding acid compound by any method known to those of skill in the art, for example, using gas-liquid chromatography with a flame ionization detector (GLC-FID) to
10 detect the amide and high pressure liquid chromatography (HPLC) to detect the corresponding acid compound. Conversion to the corresponding acid results in stoichiometric production of ammonia for each amide group originally present. The extent of conversion of amide compounds can be monitored by measuring the release of ammonia using the
15 technique of Fawcett and Scott, 1960, J. Clin. Pathol. 13:156-159. If ammonia release cannot be measured due to the presence of ammonia or an ammonium salt, the concentration of the amides present can be monitored by methods known to those of skill in the art, including but not limited to, GLC-FID,
20 etc. Alternatively, the disappearance of amide compounds can be measured by assessing the production of the corresponding acid compounds which can be monitored by derivatizing the acid compound and detecting the derivatized product using GLC-FID. Amides and acids can be derivatized for analysis by
25 GLC-FID if first alkylated, esterified or silylated (see generally, Supelco Chromatography Products catalog, 1997, at pages 653-656 (Supelco Inc., Bellefonte PA)).

The acid and other non-polyamide compounds can then, if desired, be further degraded to CO₂, H₂O and biomass.

For clarity of disclosure, and not by way of
30 limitation, the detailed description of the invention is divided into the following sub-sections:

- (1) Methods for induction and identification of microorganism strains having amide removal ability;
- (2) Characterization of the isolated microorganism strains; and
- 5 (3) Applications or methods of use of the induced microorganism strains for removal.

4.1. METHODS FOR INDUCTION AND IDENTIFICATION OF MICROORGANISM STRAINS HAVING AMIDE REMOVAL ABILITY

10 Microorganism strains useful for removing an unwanted amide monomer compound from a polyamide or polymerized amide preparation can be isolated and obtained by growing a microorganism strain in the presence of a mixture of nitrile compounds or a mixture of nitrile and amide
15 compounds. Surprisingly it has been discovered that, in order to induce a broad amide removal ability, i.e., the ability to remove a variety of monomer amides, the culture medium has to be supplemented with more than just one nitrile compound. More particularly, it has been discovered that the ability to remove a broad spectrum of monomer amides can be
20 induced using a mixture of nitrile and amide compounds. In this manner the strains are "multiply induced". After multiple induction, the strains, in pure culture, are able to remove an unwanted amide monomer compound from a polyamide or polymerized amide preparation. The microorganism strains
25 selected to undergo induction are selected from known sources or can be newly isolated microorganisms, and can be thermophiles or other extremophiles, such as halophiles or acidophiles. Advantageously, the multiple induction methods do not require an aromatic nitrile, such as benzonitrile.

30 It has been noted that microorganisms which can be multiply induced to have amide monomer removing activity appear to grow more slowly in media containing a nitrile or amide as the sole source of carbon or as the sole source of

carbon and nitrogen when compared to growth in media containing an easily utilizable carbon source such as a carbohydrate, etc. Preferably, a microorganism strain which can be multiply induced to have amide monomer removal activity is cultured in a medium which contains an easily utilizable source of carbon (such as glucose, maltose, sucrose, acetate, benzoate, etc.). The carbon and a nitrogen source are added incrementally or continuously such that the levels of carbon and nitrogen in the reactor are below 1%. In this manner large amounts of cells are produced quickly and cheaply. Once the desired cell mass has been achieved, then the microorganism is multiply induced according to the methods described in Section 4.1.1 or 4.1.2 below, for example, for the last 20 hours of a production cycle.

4.1.1. INDUCTION USING NITRILES OR NITRILE AND AMIDE COMPOUNDS AND NUTRITIONALLY COMPLETE MEDIUM

One method for the induction of amide monomer removal activity comprises using nutritionally complete culture medium supplemented with a mixture of nitrile compounds or a mixture of nitrile and amide compounds. More particularly, the method comprises culturing a microorganism in a nutritionally complete medium supplemented with a mixture of nitrile compounds or a mixture of nitrile and amide compounds and collecting the cultured microorganisms. When cultured on agar plates, the microorganisms are cultured for about 24 to 48 hours in the presence of a mixture of nitrile compounds or a mixture of nitrile and amide compounds. When cultured in a fermentor, the microorganisms are cultured, in nutritionally complete medium, for 1 to 48+ hours, preferably 1 to 20 hours, more preferably 16 to 20 hours, prior to the addition of a mixture of nitrile compounds or mixture of nitrile and amide compounds; then harvested 4 to 5 hours after addition of the mixture of

nitrile compounds or mixture of nitrile and amide compounds. If a larger biomass is desired, the microorganisms can be cultured in the fermentor for longer time periods prior to the addition of the mixture of nitrile compounds or the
5 mixture of nitrile and amide compounds. As is known to those skilled in the art, additional nutrients can be added, as needed, to maintain growth.

In one alternative method, the microorganism is induced in nutritionally complete medium supplemented with a mixture of nitrile compounds. Useful mixtures of nitrile
10 compounds include the following: acetonitrile at about 50 to about 500 ppm; acrylonitrile at about 50 to about 500 ppm; and succinonitrile at about 25 to about 100 ppm. Optionally, about 1-10 ppm KCN or NaCN may be added to the mixture. Although not intending to be limited to any particular
15 mechanism, the inventor believes the presence of KCN or NaCN during induction acclimates the microorganisms to inorganic cyanide. Also optionally, cobalt at a concentration of about 1-25 ppm may be added to the mixture. Also optionally, urea at a concentration of about 1-10 g/l may be added to the
20 mixture.

Preferably, a nutritionally complete medium is supplemented with a mixture of nitrile compounds containing a mixture of at least one of acetonitrile and acrylonitrile at a concentration of about 150 ppm each and succinonitrile and
25 fumaronitrile at a concentration of about 50 ppm each. More preferably, the nitrile mixture comprises acetonitrile and acrylonitrile at about 150 ppm each and succinonitrile at a concentration of about 50 ppm. Optionally, KCN and cobalt at a concentration of about 10 ppm each and urea at a concentration of about 7 g/l is added to the culture medium.
30 Even more preferably, a nutritionally complete medium which is BACTO™ R2A medium (Difco, Detroit, Michigan) or YEMEA medium or a medium containing the ratio of components of

YEMEA without agar is supplemented with acetonitrile and acrylonitrile at about 150 ppm each and succinonitrile at a concentration of about 50 ppm, KCN and cobalt at about 10 ppm each, and urea at about 7 g/l.

5 In another alternative method, the microorganism is induced in nutritionally complete medium supplemented with a mixture of nitrile and amide compounds. Useful mixtures of nitrile and amide compounds include the following: (1) at least one of succinonitrile at about 25 to 100 ppm, acetonitrile at about 50 to 150 ppm and acrylonitrile at
10 about 50 to 150 ppm; and (2) acetamide at about 50 to 500 ppm and acrylamide at about 50 to 500 ppm. Optionally, KCN or NaCN can be added at about 1 to 10 ppm. Also optionally, cobalt can be added at about 1 to 25 ppm and urea can be added at about 1-10 g/l. Preferably, a nutritionally
15 complete medium is supplemented with 50 ppm succinonitrile and acetamide and acrylamide at 150 ppm each.

Nutritionally complete medium is a growth medium which supplies the microorganism with all necessary nutrients required for its growth, e.g., carbon, and/or nitrogen. For
20 example, and not by way of limitation, BACTO™ R2A medium (Difco, Detroit, Michigan) which contains glucose, peptone, KH_2PO_4 , MgSO_4 , casamino acids, yeast extract, soluble starch and sodium pyruvate is a suitable nutritionally complete medium. Another nutritionally complete medium for use in the present invention is YEMEA medium which contains glucose,
25 malt extract and yeast extract without agar. Another nutritionally complete medium for use in the present invention is a nutritionally complete medium containing glucose, peptone, KH_2PO_4 , MgSO_4 , soluble starch and sodium pyruvate. Any nutritionally complete medium known to those
30 skilled in the art can be used.

The microorganism is cultured under conditions including pH between 3.0 and 11.0, preferably between about

6.0 and 8.0; and temperature between 4°C and 55°C, preferably between about 15°C and 37°C. Further, the dissolved oxygen tension should be between 0.1% and 100%, preferably between about 4% and 80%, more preferably between about 4% and 30%.

5 The dissolved oxygen tension may be monitored and maintained in the desired range by supplying oxygen in the form of air, pure oxygen, peroxide, and/or other peroxy compositions which liberate oxygen.

At the end of the culture period, the cultured microorganisms are collected and concentrated, for example,
10 by scraping, centrifuging, filtering, etc., or by any method known to those skilled in the art.

In one exemplary method, cells are collected at 4°C, prepared as cell concentrates and then rapidly frozen (dry ice and acetone) and then stored at -20°C or lower.
15 Frozen cell concentrate can be used or it can be then immobilized.

The amide monomer removal activity of the collected microorganisms, once multiply induced according to the methods described above, can be stabilized by addition of one
20 or more substrates to the cultured microorganism. Although not intending to be limited to any mechanism, the inventor notes that it is well known to those of skill in the art that amidase enzymes are generally most stable when in the presence of a substrate. Thus, for example, addition of an
25 acid compound, such as isobutyric acid, can stabilize an amidase such that activity is retained for longer time periods.

Stabilization can also be achieved by immobilization of the induced microorganism in polyacrylamide or acrylamide cubes or in alginate which has been cross-
30 linked with polyethylene imide. Preferably, cells are stabilized by immobilization in alginate cross-linked with polyethylene imide.

4.1.2. INDUCTION USING NITRILE OR NITRILE AND AMIDE COMPOUNDS AND MINIMAL MEDIUM

Another method for the induction of amide monomer removal activity comprises using minimal culture medium
5 supplemented with a mixture of nitrile compounds or a mixture of nitrile and amide compounds. More particularly, the method comprises culturing a microorganism in a minimal medium supplemented with a mixture of nitriles or a mixture of nitrile and amide compounds and collecting the cultured
10 microorganisms. When cultured on agar plates, the microorganisms are cultured for about 24 to 48 hours. When cultured in a fermentor, the microorganisms are cultured in a minimal medium supplemented with a mixture of nitrile compounds or a mixture of nitrile and amide compounds for 1
15 to 48+ hours, preferably 1 to 20 hours, more preferably 16 to 23 hours; then harvested 4 to 5 hours after addition of the mixture of nitrile compounds or mixture of nitrile and amide compounds. If a larger biomass is desired the microorganisms can be cultured in the fermentor for longer time periods.

Useful mixtures of nitrile compounds include the
20 following: acetonitrile at about 50 to about 500 ppm; acrylonitrile at about 50 to about 500 ppm; and succinonitrile at about 25 to about 100 ppm. Optionally, 1-10 ppm KCN or NaCN may be added to the mixture. Also optionally, cobalt at a concentration of about 1-25 ppm may
25 be added to the mixture. Also optionally, urea at a concentration of about 1-10 g/l may be added to the mixture.

Preferably, the minimal medium is supplemented with a mixture of nitrile compounds containing at least one of acetonitrile and acrylonitrile at a concentration of about
30 150 ppm each and succinonitrile and fumaronitrile at a concentration of about 50 ppm each. More preferably, the nitrile mixture comprises acetonitrile and acrylonitrile at about 150 ppm each and succinonitrile at a concentration of

about 50 ppm. Optionally, KCN and cobalt at a concentration of about 10 ppm each and urea at a concentration of about 7 g/l is added to the culture medium.

In an alternative method, the microorganism is induced in minimal medium supplemented with a mixture of nitrile and amide compounds. Useful mixtures of nitrile and amide compounds include the following: (1) at least one of succinonitrile at about 25 to 100 ppm, acetonitrile at about 50 to 150 ppm and acrylonitrile at about 50 to 150 ppm; and (2) acetamide at about 50 to 500 ppm and acrylamide at about 50 to 500 ppm. Optionally, KCN or NaCN can be added at about 1 to 10 ppm. Also optionally, cobalt can be added at about 1-25 ppm and urea can be added at about 1-10 g/l.

Preferably, a minimal medium is supplemented with 50 ppm succinonitrile and acetamide and acrylamide at 150 ppm each.

Minimal medium is a nutritionally incomplete medium which does not supply the microorganism with organic carbon for its growth. Rather, the minimal medium must be supplemented with compounds which the microorganisms can use as a source of carbon and/or energy. For example, and not by way of limitation, Stanier's minimal medium (Stanier et al., 1966, J. Gen. Microbiol. 43:159-271) and phosphate buffered saline (PBS) are acceptable minimal media for use in the induction methods.

The microorganism is cultured under conditions including pH between 3.0 and 11.0, preferably between about 6.0 and 8.0; and temperature between 4°C and 55°C, preferably between about 15°C and 37°C. Further, the dissolved oxygen tension should be between 0.1% and 100%, preferably between about 4% and 80%, more preferably between about 4% and 30%. The dissolved oxygen tension may be monitored and maintained in the desired range by supplying oxygen in the form of air, pure oxygen, peroxide, and/or other peroxy compositions which liberate oxygen.

At the end of the culture period, the cultured microorganisms are collected and concentrated, for example, by scraping, centrifuging, filtering, etc., or by any method known to those skilled in the art.

5 The amide monomer removal activity of the harvested microorganisms, once multiply induced according to the methods described above, can be stabilized by addition of one or more substrates to the cultured microorganism. Although not intending to be limited to any mechanism, the inventor notes that it is well known to those of skill in the art that
10 amidase enzymes are generally most stable when in the presence of a substrate. Thus, for example, addition of an acid compound, such as isobutyric acid, can stabilize a amidase such that activity is retained for longer time periods.

15 Stabilization can also be achieved by immobilization of the induced microorganism in polyacrylamide or acrylamide cubes or in alginate which has been cross-linked with polyethylene imide. Preferably, cells are stabilized by immobilization in alginate cross-linked with
20 polyethylene imide.

4.1.3. **IDENTIFICATION OF USEFUL MICROORGANISMS**

 According to one embodiment, the present invention provides a method of screening microorganisms to identify and
25 isolate microorganisms useful to remove unwanted amide monomer compounds from polyamide or polymerized amide preparations. The method, in general, entails exposing a microorganism to be tested to the conditions, described above in Sections 4.1.1 and 4.1.2, which are used to multiply
30 induce amide removal ability and then assessing the ability of the putatively "induced" test microorganisms to remove an unwanted amide monomer from a polyamide preparation.

The method to screen for microorganisms useful to remove an amide monomer from a polyamide preparation comprises culturing a test microorganism in a nutritionally complete or minimal medium supplemented with a first mixture of nitrile compounds (see Sections 4.1.1 and 4.1.2, above) for about 24 to 48 hours on agar plates under growth favorable conditions to obtain a putatively induced microorganism; and assessing the ability of the putatively induced microorganism to remove an amide monomer from a polyamide preparation by contacting said microorganism with a polyamide preparation containing an amide monomer, and monitoring the disappearance of the amide monomer, wherein the disappearance of the amide monomer compound in the preparation indicates that the test microorganism has the ability to remove an amide monomer compound from a polyamide preparation. Preferably, the polyamide preparation comprises polyacrylamide and monomer acrylamide and disappearance of all the amide monomer in about 30 minutes indicates that the test microorganism has the ability to remove an amide monomer from a polyamide preparation. Most preferably, disappearance of all the amide monomer in the polyamide preparation in about 10 minutes indicates that the microorganism has the desired ability. If it is desired that the microorganism has the ability to remove an amide monomer in the presence of ammonium sulfate, ammonium sulfate is included in the second mixture at about 1-8% ammonium sulfate.

According to a preferred mode of this embodiment, the nutritionally complete or minimal medium is supplemented with a first mixture of nitrile compounds containing a mixture of at least one of acetonitrile and acrylonitrile at a concentration of about 150 ppm each and succinonitrile and fumaronitrile at a concentration of about 50 ppm each. More preferably, the first mixture of nitriles comprises acetonitrile and acrylonitrile at about 150 ppm each and

succinonitrile at a concentration of about 50 ppm.
Optionally, KCN at a concentration of about 10 ppm is added to the culture medium.

According to another preferred mode of this
5 embodiment, the nutritionally complete or minimal medium is supplemented with a first mixture of nitrile and amide compounds (see Sections 4.1.1 and 4.1.2, above).

Useful nutritionally complete media and minimal media are described above in Sections 4.1.1 and 4.1.2. The test microorganism is cultured under conditions including pH
10 between about 2.0 and 11.0, preferably between about 2.0 and less than 6.0; and temperature between about 4°C and 55°C, preferably between 15°C and 37°C.

The extent of removal of the monomer amide compounds can be monitored by measuring the release of
15 ammonia using the technique of Fawcett and Scott, 1960, J. Clin. Pathol. 13:156-159. If ammonia release cannot be measured due to the presence of ammonia or an ammonium salt, the concentration of the amides present can be monitored by methods known to those of skill in the art, including but not
20 limited to, GLC-FID, etc. Alternatively, the disappearance of amide compounds can be measured by assessing the production of the corresponding acid compounds which can be monitored by derivatizing the acid compound and detecting the derivatized product using GLC-FID. Amides and acids can be
25 derivatized for analysis by GLC-FID if first alkylated, esterified or silylated (see generally, Supelco Chromatography Products catalog, 1997, at pages 653-656 (Supelco Inc., Bellefonte PA)).

4.2. CHARACTERIZATION OF MICROORGANISM STRAINS

30 The microorganism strains, described below, isolated or obtained from known sources have been discovered to have the ability to remove an amide monomer compound from

a polyamide preparation by converting the amide monomer compound to the corresponding acid compound after multiple induction as described above in Section 4.1.

Tables I and II below present certain specific strain characteristics of two *Rhodococcus* strains, DAP 96622 and DAP 96253, derived from two strains obtained from the American Type Culture Collection, Rockville, MD, ATCC Accession No. 33278 and ATCC Accession No. 39484, respectively, and which were discovered to have the ability to remove an amide monomer compound from a polyamide preparation by converting the amide monomer to the corresponding acid compound after multiple induction as described above. In one illustrative example, the two *Rhodococcus* strains were multiply induced using 150 ppm each of acrylonitrile, acetonitrile, and 50 ppm of succinonitrile with or without 50 ppm KCN.

Carbohydrate utilization tests were performed using protocols described in Manual of Methods for General Bacteriology, 1981, Philip Gerhardt, ed., Am. Soc. Microbiol., Washington, D.C. Nitrile utilization tests were performed after the strains were induced by culturing the microorganism on a nutritionally complete medium supplemented with 150 ppm each of acetonitrile and acrylonitrile and 50 ppm succinonitrile. The actual utilization test was performed in a minimal medium supplemented with a particular test compound(s) as a sole source of carbon and/or energy.

TABLE I

Rhodococcus rhodochrous strain DAP 96622:

<u>DIFFERENTIAL CHARACTERISTIC</u>		<u>RESULT</u>
CATALASE/OXIDASE		(+)/(-)
CITRATE UTILIZATION		(+)
TRIPLE SUGAR IRON AGAR		no change
GROWTH AT:	5°C	(-)
	25°C	(+)

		35°C	(+)
		45°C	(+)
	UTILIZATION OF:	GLUCOSE	(+)
		SUCROSE	(+)
		FRUCTOSE	(+)
5		LACTOSE	(+)
		MANNITOL	(+)
		MANNOSE	(+)
		ARABINOSE	(-)
		INOSITOL	(+), No gas
		RHAMNOSE	(+), No gas
10	UREASE		(+)
	NITRATE REDUCTION		(-)
	GELATIN HYDROLYSIS		(-)
	ANTIBIOTIC RESISTANCE:		
		GENTAMICIN	S
		ERYTHROMYCIN	S
15		STREPTOMYCIN	S
		TOBRAMYCIN	S
		RIFAMPIN	S
		PENICILLIN	S
		TETRACYCLINE	S
	UTILIZATION OF CARBON AND NITROGEN:		
20	Stanier's minimal medium with nitrile as the sole carbon and nitrogen source		
	ACRYLONITRILE	(150 ppm/300 ppm)	(+)/(+)
	ACETONITRILE	(150 ppm/300 ppm)	(+)/(+)
	SUCCINONITRILE	(150 ppm/300 ppm)	(+)/(+)
	FUMARONITRILE	(150 ppm/300 ppm)	(+)/(+)
	ACRYLONITRILE/ACETONITRILE/SUCCINONITRILE	(150/300 ppm ea)	(+)/(+)
25	ACRYLONITRILE/ACETONITRILE/FUMARONITRILE	(150/300 ppm ea)	(+)/(-)
	ACRYLONITRILE/ACETONITRILE/SUCCINONITRILE/FUMARONITRILE	(150/300 ppm ea)	(-)/(-)
	UTILIZATION OF CARBON SOURCES		
	Stanier's minimal medium containing 1 g/l ammonium sulfate		
30	P-CRESOL	(150/300 ppm)	(+)/(+)
	TOLUENE	(150/300 ppm)	(NT)/(NT)
	STYRENE	(150/300 ppm)	(NT)/(NT)
	TOLUENE/STYRENE	(150/300 ppm ea.)	(NT)/(NT)

ACETATE	(150/300 ppm)	(+)/(+)
ACRYLATE	(150/300 ppm)	(+)/(+)
SUCCINATE	(150/300 ppm)	(+)/(+)
FUMARATE	(150/300 ppm)	(+)/(+)

5 UTILIZATION OF CARBON SOURCES

Stanier's minimal medium containing 1 g/l ammonium sulfate but with 10 ppm KCN

FUMARONITRILE	(150/300 ppm)	(+)/(+)
SUCCINONITRILE	(150/300 ppm)	(+)/(+)

UTILIZATION OF CARBON SOURCES

10 Stanier's minimal medium without ammonium sulfate but with 10 ppm KCN

FUMARONITRILE	(150/300 ppm)	(+)/(+)
SUCCINONITRILE	(150/300 ppm)	(+)/(+)

TABLE II

15 Rhodococcus sp. strain DAP 96253:

	<u>DIFFERENTIAL CHARACTERISTIC</u>	<u>RESULT</u>
	CATALASE/OXIDASE	(+)/(-)
	CITRATE UTILIZATION	(+)
	TRIPLE SUGAR IRON AGAR	no change
	GROWTH AT: 5°C	(-)
20	25°C	(+)
	35°C	(+)
	45°C	(+)
	UTILIZATION OF: GLUCOSE	(+)
	SUCROSE	(+)
	FRUCTOSE	(+)
25	LACTOSE	(+)
	MANNITOL	(+)
	MANNOSE	(+)
	ARABINOSE	(-)
	INOSITOL	(+), No gas
	RHAMNOSE	(+), No gas
	UREASE	(-)
30	NITRATE REDUCTION	(-)
	GELATIN HYDROLYSIS	(-)
	ANTIBIOTIC RESISTANCE:	

	GENTAMICIN	I*
	ERYTHROMYCIN	S
	STREPTOMYCIN	S
	TOBRAMYCIN	S
	RIFAMPIN	S
5	PENICILLIN	S
	TETRACYCLINE	S

* I means "intermediate" between sensitive and resistant.

UTILIZATION OF CARBON AND NITROGEN:

10	Stanier's minimal medium with nitrile as the sole carbon and nitrogen source		
	ACRYLONITRILE	(150 ppm/300 ppm)	(+)/(+)
	ACETONITRILE	(150 ppm/300 ppm)	(+)/(+)
	SUCCINONITRILE	(150 ppm/300 ppm)	(+)/(-)
	FUMARONITRILE	(150 ppm/300 ppm)	(+)/(-)
	ACRYLONITRILE/ACETONITRILE/SUCCINONITRILE	(150/300 ppm ea)	(+)/(+)
	ACRYLONITRILE/ACETONITRILE/FUMARONITRILE	(150/300 ppm ea)	(-)/(-)
15	ACRYLONITRILE/ACETONITRILE/SUCCINONITRILE/FUMARONITRILE	(150/300 ppm ea)	(-)/(-)

UTILIZATION OF CARBON SOURCES:

	Stanier's minimal medium containing 1 g/l ammonium sulfate		
	P-CRESOL	(150/300 ppm)	(+)/(+)
20	TOLUENE	(150/300 ppm)	(+)/(+)
	STYRENE	(150/300 ppm)	(+)/(+)
	TOLUENE/STYRENE	(150/300 ppm ea.)	(+)/(+)
	ACETATE	(150/300 ppm)	(+)/(+)
	ACRYLATE	(150/300 ppm)	(+)/(+)
	SUCCINATE	(150/300 ppm)	(+)/(+)
25	FUMARATE	(150/300 ppm)	(+)/(+)

UTILIZATION OF CARBON SOURCES

	Stanier's minimal medium containing 1 g/l ammonium sulfate but with 10 ppm KCN		
	FUMARONITRILE	(150/300 ppm)	(+)/(+)
	SUCCINONITRILE	(150/300 ppm)	(+)/(+)

30

UTILIZATION OF CARBON SOURCES

	Stanier's minimal medium without ammonium sulfate but with 10 ppm KCN		
	FUMARONITRILE	(150/300 ppm)	(+)/(+)

SUCCINONITRILE (150/300 ppm)

(+)/(+)

The following microorganism strains, characterized in the tables below, have been discovered to have the remove
5 an unwanted amide monomer compound from a polyamide
preparation by converting the amide monomer to the
corresponding acid compound after multiple induction as
described above. The isolation of these microorganisms is
described in WO96/18724. Briefly, over 200 separate pure
10 microorganism isolates were cultured from contaminated soil
at the same industrial site. All of these pure isolates were
combined and cultured, aerobically, with a sludge/waste
material containing a mixture of aromatic, nitro-aromatic,
halo-aromatic, aliphatic and halo-aliphatic compounds. A
mixed culture of microorganisms was recovered from the
15 cultured material and has been maintained on BACTO™ R2A
medium (Difco, Detroit, Michigan).

The mixed culture designated DAP-2, which was deposited with the American Type Culture Collection and assigned ATCC Accession No. 55644, aerobically degrades at
20 least the following compounds or mixtures thereof: benzene,
toluene, xylene, ethylbenzene, naphthalene, chlorobenzene,
phenol, cresol, nitrobenzene, aniline, anthracene,
dimethylphenol, styrene, halonaphthalene, 2-, 3- or 4-
chlorotoluene, 2-, 3- or 4-chlorobenzoate, 1,3-
dichlorobenzoate, 1,2-, 1,3- or 1,4-dinitrobenzene, 1-chloro-
25 3-nitrobenzene, 1-chloro-4-nitrobenzene, 1- or 2-
methylnaphthalene, pyrene, acenaphthylene, fluoranthene,
phenanthrene, benzo-(b)-fluoranthene, dibenzofuran, chrysene,
catechol, m-toluic acid, cinnamyl acetate, vanillin, trans-
cinnamaldehyde, mesitylene, salicylate, melamine, cyanuric
30 acid, δ-(-)-limonene, hexadecane, methanol, formaldehyde, and
chloroform.

The following pure cultures were isolated and characterized from the mixed culture designated DAP 2 by isolating single colonies on BACTO™ R2A medium supplemented with 150 ppm each of nitrobenzene, naphthalene, and toluene.

5

Microorganism DAP 623:

DAP 623 is a Gram negative motile rod, generally small single rods, though some pairs are seen. Staining can be uneven and there is some floc formation. The colonies appear white to creamy on BACTO™ R2A medium. In addition, this organism can utilize the following: mesitylene, lactate, succinate, limonene, m-toluic acid, chlorobenzene, salicylate, 2-, 3-, and 4-chlorotoluene, 2-, 3-, and 4-chlorobenzoic acid, and 1,3-dichlorobenzene as a sole source of carbon and energy. DAP 623 was deposited with the American Type Culture Collection and assigned ATCC Accession No. 55722 and is further characterized as shown in Table III.

TABLE III

DAP 623

20	<u>DIFFERENTIAL CHARACTERISTIC</u>		<u>RESULT</u>
	CATALASE/OXIDASE		(+)/(-)
	CITRATE UTILIZATION		(+)
	TRIPLE SUGAR IRON AGAR		acid from glucose
	GROWTH AT:	15°C	(+)
		25°C	(+)
		35°C	(+)
25		41°C	(+)
	UTILIZATION OF:	GLUCOSE	(+)
		FRUCTOSE	(+)
		LACTOSE	(-)
		MANNITOL	(+)
		MANNOSE	(+)
30		2-METHYLNAPHTHALENE	(-)
		α-KETOGLUTARATE	(+)
		GLUTAMATE	(+)

	ETHANOL	(-)
	HEXADECANE	(-)
	NO ₃ - NO ₂	(+)
	ARGININE DECARBOXYLASE	(+)
	LYSINE DECARBOXYLASE	(+)
5	ORNITHINE DECARBOXYLASE	(+)
	GELATIN HYDROLYSIS	(+)
	UREASE	(+)
	ANTIBIOTIC RESISTANCE:	
	HgCl ₂	(-)
	AMPICILLIN	R
10	KANAMYCIN	(-)
	TETRACYCLINE	R
	SPECTINOMYCIN	R
	STREPTOMYCIN	(-)

15 Microorganism DAP 626:

DAP 626 is a Gram variable rod which vary in size and occur singly and in pairs. Growth on flagella plates is seen which indicates flagellar motility. In addition, this organism can utilize the following: mesitylene, lactate, succinate, limonene, cinnamyl acetate, catechol, m-toluic acid, chlorobenzene, 2-, 3-, and 4-chlorotoluene, 2-, 3-, and 4-chlorobenzoic acid, and 1,3-dichlorobenzene as a sole source of carbon and energy. DAP 626 was deposited with the American Type Culture Collection and assigned ATCC Accession No. 55723 and is further characterized as shown in Table IV.

25

TABLE IV

DAP 626

	<u>DIFFERENTIAL CHARACTERISTIC</u>	<u>RESULT</u>
30	CATALASE/OXIDASE	(+)/(+)
	CITRATE UTILIZATION	(-)
	TRIPLE SUGAR IRON AGAR	H ₂ S is produced
	GROWTH AT: 15°C	(+)

	25°C	(+)
	35°C	(+)
	41°C	(+)
5	UTILIZATION OF:	
	GLUCOSE	(-)
	FRUCTOSE	(+)
	LACTOSE	(-)
	MANNITOL	(+)
	MANNOSE	(-)
	2-METHYLNAPHTHALENE	(-)
	α -KETOGLUTARATE	(+)
	GLUTAMATE	(+)
10	ETHANOL	(+)
	HEXADECANE	(+)
	NO ₃ - NO ₂	(-)
	ARGININE DECARBOXYLASE	(-)
	LYSINE DECARBOXYLASE	(-)
	ORNITHINE DECARBOXYLASE	(-)
15	GELATIN HYDROLYSIS	(-)
	UREASE	(+)
	ANTIBIOTIC RESISTANCE:	
	HgCl ₂	(-)
	AMPICILLIN	R
	KANAMYCIN	(-)
	SPECTINOMYCIN	(-)
20	STREPTOMYCIN	R

Microorganism DAP 629:

DAP 629 is a Gram negative small motile rod, almost
 25 cocco-bacillary. Colonies appeared white with a slight
 fluorescence when grown on BACTO™ R2A agar. In addition,
 this organism can utilize the following: fluoranthrene,
 mesitylene, lactate, succinate, limonene, m-toluic acid,
 chlorobenzene, 2-, 3-, and 4-chlorotoluene, 2-, 3-, and 4-
 30 chlorobenzoic acid, and 1,3-dichlorobenzene as a sole source
 of carbon and energy. DAP 629 was deposited with the
 American Type Culture Collection and assigned ATCC Accession
 No. 55726 and is further characterized as shown in Table V.

TABLE V

DAP 629

	<u>DIFFERENTIAL CHARACTERISTIC</u>	<u>RESULT</u>
	CATALASE/OXIDASE	(+)/(+)
5	CITRATE UTILIZATION	(-)
	TRIPLE SUGAR IRON AGAR	no fermentation
	GROWTH AT: 15°C	(+)
	25°C	(+)
	35°C	(+)
	41°C	(-)
10	UTILIZATION OF: GLUCOSE	(+)
	FRUCTOSE	(-)
	LACTOSE	(-)
	MANNITOL	(-)
	MANNOSE	(-)
	2-METHYLNAPHTHALENE	(-)
	α-KETOGLUTARATE	(+)
15	GLUTAMATE	(+)
	ETHANOL	(-)
	HEXADECANE	(-)
	NO ₃ - NO ₂	(+)
	ARGININE DECARBOXYLASE	(-)
	LYSINE DECARBOXYLASE	(+)
20	ORNITHINE DECARBOXYLASE	(-)
	GELATIN HYDROLYSIS	(+)
	UREASE	(-)
	ANTIBIOTIC RESISTANCE:	
	HgCl ₂	(-)
	AMPICILLIN	R
25	KANAMYCIN	(-)
	TETRACYCLINE	(-)
	SPECTINOMYCIN	(-)
	STREPTOMYCIN	(-)

30 Microorganism DAP 632:

DAP 632 is a Gram variable motile slender rod, seen both singly and in pairs. Colonies appeared creamy to yellowish when grown on BACTO™ R2A agar. In addition, this

organism can utilize the following: fluoranthrene, acenaphthalene, mesitylene, lactate, limonene, m-toluic acid, chlorobenzene, 2-, 3-, and 4-chlorotoluene, 2-, 3-, and 4-chlorobenzoic acid, and 1,3-dichlorobenzene as a sole source of carbon and energy. DAP 632 was deposited with the American Type Culture Collection and assigned ATCC Accession No. 55727 and is further characterized as shown in Table VI.

TABLE VI

DAP 632

10	<u>DIFFERENTIAL CHARACTERISTIC</u>	<u>RESULT</u>
	CATALASE/OXIDASE	(+)/(-)
	CITRATE UTILIZATION	(+)
	TRIPLE SUGAR IRON AGAR	no fermentation
	GROWTH AT: 15°C	(+)
	25°C	(+)
15	35°C	(+)
	41°C	(+)
	UTILIZATION OF: GLUCOSE	(-)
	FRUCTOSE	(-)
	LACTOSE	(-)
	MANNITOL	(-)
20	MANNOSE	(-)
	2-METHYLNAPHTHALENE	(-)
	α-KETOGLUTARATE	(-)
	GLUTAMATE	(+)
	ETHANOL	(-)
	HEXADECANE	(-)
25	NO ₃ - NO ₂	(-)
	ARGININE DECARBOXYLASE	(-)
	LYSINE DECARBOXYLASE	(-)
	ORNITHINE DECARBOXYLASE	(-)
	GELATIN HYDROLYSIS	(+)
	UREASE	(+)
30	ANTIBIOTIC RESISTANCE:	
	HgCl ₂	R
	AMPICILLIN	R
	KANAMYCIN	R

TETRACYCLINE	R
SPECTINOMYCIN	R
STREPTOMYCIN	R

5 Microorganism DAP 115:

DAP 115 is a Gram negative motile rod. Growth is observed on flagella plates, indicating motility is flagellar. Colonies appeared white when grown on BACTO™ R2A agar, but appear yellow in nutrient broth. In addition, this
 10 organism can utilize the following: benzo-(b)-fluoranthrene, fluoranthrene, dibenzofuran, acenaphthalene, salicylate, lactate, succinate, glyoxylate, mesitylene, vanillin, limonene, cinnamyl acetate, catechol, m-toluic acid, chlorobenzene, 2-, 3-, and 4-chlorotoluene, 2-, 3-, and 4-
 15 chlorobenzoic acid, and 1,3-dichlorobenzene as a sole source of carbon and energy. DAP 115 was deposited with the American Type Culture Collection and assigned ATCC Accession No. 55724 and is further characterized as shown in Table VII.

20 **TABLE VII**

DAP 115

	<u>DIFFERENTIAL CHARACTERISTIC</u>	<u>RESULT</u>
	CATALASE/OXIDASE	(+)/(+)
	CITRATE UTILIZATION	(+)
	TRIPLE SUGAR IRON AGAR	H ₂ S is produced acid and gas from glucose
25	GROWTH AT:	(+/-)
	15°C	(+)
	25°C	(+)
	35°C	(+)
	41°C	(+)
	UTILIZATION OF:	(+)
	GLUCOSE	(+)
	FRUCTOSE	(+)
30	LACTOSE	(-)
	MANNITOL	(+)
	MANNOSE	(+)

		2-METHYLNAPHTHALENE	(+)
		α -KETOGLUTARATE	(+)
		GLUTAMATE	(+)
		ETHANOL	(-)
		HEXADECANE	(+)
5	NO ₃ - NO ₂		(+)
	ARGININE DECARBOXYLASE		(-)
	LYSINE DECARBOXYLASE		(-)
	ORNITHINE DECARBOXYLASE		(+)
	GELATIN HYDROLYSIS	(+)	
	UREASE		(+)
10	ANTIBIOTIC RESISTANCE:		
	HgCl ₂	R	
	AMPICILLIN	R	
	KANAMYCIN	R	
	TETRACYCLINE	R	
	SPECTINOMYCIN	R	
15	STREPTOMYCIN	R	

Microorganism DAP 120:

DAP 120 is a Gram negative motile rod. Growth is observed on flagella plates, indicating motility is flagellar. In addition, this organism can utilize the following: chrysene, pyrene, lactate, succinate, glyoxylate, salicylate, mesitylene, vanillin, limonene, cinnamyl acetate, catechol, m-toluic acid, chlorobenzene, 2-, 3-, and 4-chlorotoluene, 2-, 3-, and 4-chlorobenzoic acid, and 1,3-dichlorobenzene as a sole source of carbon and energy. DAP 120 was deposited with the American Type Culture Collection and assigned ATCC Accession No. 55725 and is further characterized as shown in Table VIII.

TABLE VIII

DAP 120

	<u>DIFFERENTIAL CHARACTERISTIC</u>	<u>RESULT</u>
	CATALASE/OXIDASE	(+) / (+)
5	CITRATE UTILIZATION	(+)
	TRIPLE SUGAR IRON AGAR	H ₂ S is produced
	GROWTH AT: 15°C	(+)
	25°C	(+)
	35°C	(+)
	41°C	(+)
10	UTILIZATION OF: GLUCOSE	(+)
	FRUCTOSE	(+)
	LACTOSE	(-)
	MANNITOL	(+)
	MANNOSE	(-)
	2-METHYLNAPHTHALENE	(+)
	α-KETOGLUTARATE	(+)
15	GLUTAMATE	(+)
	ETHANOL	(-)
	HEXADECANE	(+)
	NO ₃ → NO ₂	(+)
	ARGININE DECARBOXYLASE	(-)
	LYSINE DECARBOXYLASE	(-)
20	ORNITHINE DECARBOXYLASE	(-)
	GELATIN HYDROLYSIS	(+)
	UREASE	(+)
	ANTIBIOTIC RESISTANCE:	
	HgCl ₂	R
	AMPICILLIN	R
25	KANAMYCIN	R
	TETRACYCLINE	R
	SPECTINOMYCIN	(-)
	STREPTOMYCIN	(-)

30 Table IX below shows that the above-characterized pure cultures, isolated from the mixed culture designated DAP 2, are able to grow on Stanier's minimal medium supplemented solely with 150 ppm each of acetonitrile and acrylonitrile.

The cultures were grown at 25-27°C, colony size determined after 14 days. Values represent mean of five replicate colonies for each determination.

5

TABLE IX

UTILIZATION OF ACETO- AND ACRYLONITRILE

	<u>CULTURE</u>	<u>GROWTH*</u>	<u>COLONY SIZE</u>
	DAP 626	+++	5.3 mm
	DAP 115	+++	6.3 mm
	DAP 632	+++	6.2 mm
	DAP 623	+++	5.0 mm
10	DAP 120	+ / ++ ^a	^a
	DAP 629	+++	5.3 mm

* Growth scored as ++++ luxuriant, +++ good, ++ fair, + modest, +/- scant, - no growth

^a Growth of strain DAP 120 was very thin but rapidly spreading, therefore, precise quantitation was not possible.

15

As demonstrated in Table IX, the strains are able to utilize acetonitrile and acrylonitrile as sole sources of carbon and nitrogen.

20

4.3. APPLICATIONS OR METHODS OF USE OF THE
MICROORGANISMS FOR REMOVAL OF AMIDE MONOMER
COMPOUNDS FROM POLYAMIDE PREPARATIONS

25

According to one embodiment of the invention, a method is provided for removing an amide monomer in a polyamide or polymerized amide preparation, which preparation contains an unwanted or undesired amide monomer, by converting the amide monomer to the corresponding monomeric acid compound, which acid compound can be readily removed from the preparation, for example, by any means of chemical separation known to those skilled in the art. For example, acrylamide can be converted to acrylic acid which is readily removed as the ammonium salt.

30

The method comprises contacting a polyamide or polymerized amide preparation containing an unwanted amide monomer compound with a pure culture of a useful

microorganism strain, multiply induced as described in Section 4.1.1. and 4.1.2, for a period of time sufficient to convert the amide monomer to the corresponding acid. This method is particularly useful to purify a polyamide or
5 polymerized amide preparation, such as a polyacrylamide preparation or a co-polymer containing acrylamide. Any polyamide or polymerized amide preparation containing unwanted monomer can be treated according to this embodiment of the invention, including anionic, cationic, and non-ionic
10 polyamide preparations at a pH of about 2 to less than 6, preferably at a pH of about 3 to about 5, more preferably at a pH of about 3 to about 4. Advantageously, a cationic polyamide or polymerized amide containing an amide monomer can be treated at a pH of less than about 6.0, preferably at a pH of about 2 to 4 and most preferably at a pH of about 3
15 to about 4, such that the cationic side chain groups do not lose their functionality as they might if the pH were adjusted to a pH of about 6 or above before removing the unwanted monomer.

The induced microorganism strain can be growing,
20 i.e., actively dividing or may be resting, i.e., not actively dividing or not alive. When the method entails use of an actively growing culture of microorganisms, conditions for contact with a polyamide or polymerized amide preparation should be such that bacterial growth is supported. Such
25 conditions include, for example, pH between 2.0 and 11.0, preferably between about 2.0 and less than 6.0; temperature between 4°C and 55°C, preferably between about 15°C and 37°C; dissolved oxygen tension between 0.1% and 100%, preferably between about 4% and 80%, more preferably between 4% and 40%
30 of saturation where the oxygen can be supplied by use of an oxygen containing or oxygen liberating composition. The oxygen containing or oxygen liberating composition can be air, pure oxygen, peroxide, or other peroxy chemicals which

liberate oxygen or mixtures thereof. Further, the culture medium may be stirred or may not be stirred, provided with positive dissolved oxygen tension or not.

When the method entails use of a culture of
5 microorganisms which are not actively dividing, conditions for contact with a polyamide preparation containing an unwanted amide monomer should be such that amide removal (enzymatic) activities are supported. For example, temperature is maintained between about 0°C and 65°C,
10 preferably between about 4°C and 55°C, more preferably between about 25°C and 55°C. The pH can be alkaline or acid and is optimally maintained in the range of about pH 2 to less than 6. This particular embodiment is possible because removal of the amide monomer is not growth dependent, i.e., once the amide removal activity is induced, the microorganism
15 no longer needs to grow to remove the amide monomer. This particular mode can be carried out under anaerobic conditions.

The pure culture of a microorganism strain can be encapsulated or immobilized rather than free-swimming to
20 permit collection and reuse. In a preferred embodiment, the microorganism strain is immobilized. The pure culture can be immobilized by sorption, electrostatic bonding, covalent bonding, etc., onto a solid support which aids in the recovery of the microorganisms from the reaction mixture. Suitable solid supports include, but are not limited to
25 granular activated carbon, compost, wood residue products, (e.g., wood chips, nuggets, shredded pallets or trees), alumina, ruthenium, iron oxide, ion exchange resins, (e.g., Amberlite™ IRA-93 or IRA-96 (Rohm & Haas), DOWEX™ (Dow Chemical Co, Inc.), DEAE cellulose, DEAE-SEPHADEX™
30 (Pharmacia, Inc.), ceramic beads, cross-linked polyacrylamide beads or cubes or other gel forms, alginate beads, κ-carrageenan cubes as well as solid particles that can be

recovered from the aqueous solutions due to inherent magnetic ability. The alginate beads can be Ca^{++} alginate beads or hardened alginate beads. The κ -carrageenan cubes can be hardened κ -carrageenan cubes. As an illustrative example, a
5 pure culture of an induced microorganism can be mixed with a sodium alginate solution and calcium chloride to immobilize the microorganisms in alginate beads. In a preferred embodiment, the induced microorganism is immobilized in alginate beads that have been cross-linked with polyethylene imide or immobilized in a polyacrylamide-type polymer.

10 According to another embodiment of the present invention, a method for the removal of an unwanted amide monomer from a polyamide or polymerized amide preparation comprises contacting an extract of a pure culture of a useful microorganism strain, multiply induced according to the
15 present invention, with a polyamide or polymerized amide preparation containing an unwanted amide monomer for a sufficient amount of time to convert the amide monomer to the corresponding acid. Preferably, the extract is a crude extract of an individual microorganism. Extracts of the
20 microorganism are prepared by methods known to those skilled in the art including, but not limited to the following: The cells of a sample of a pure culture of an induced microorganism are disrupted, for example, by sonication, by crushing, employing a French press, etc., to produce a cell lysate. The lysate is filtered or centrifuged to remove
25 cellular debris and unlysed cells to yield a crude extract. The lysate can also be immobilized as described above for whole cells or immobilized using techniques known for use with immobilization of enzymes.

In a particular illustrative embodiment of the
30 application of the invention described above, the removal method is used to convert unwanted acrylamide monomer to acrylic acid in a polyacrylamide preparation. A pure

culture, or an extract, of a useful microorganism strain, multiply induced and immobilized as taught herein, is contacted with a polyacrylamide preparation containing unwanted acrylamide monomer, said preparation having a pH of
5 about 2 to less than 6, for a period of time sufficient to reduce the amount of acrylamide monomer in the polyacrylamide preparation, preferably to less than 100 ppm acrylamide monomer. Alternatively, a pure culture, or an extract, of a useful microorganism strain, multiply induced and immobilized as taught herein, is contacted at a pH of about 2 to less
10 than 6 with an acrylamide monomer preparation simultaneously with a polymerizing agent (an agent which causes the amide monomer to polymerize to polyacrylamide) such that residual acrylamide monomer (after the polymerization reaction) is converted to the corresponding acrylic acid. Such
15 polymerizing agents include, but are not limited to, agents such as a persulfate, a sulfate, a bromate, a chlorate, a peroxide, or a mixture thereof, etc. The concentration of acrylamide monomer in a polyacrylamide preparation may be as high as 100,000 ppm and is generally from about less than
20 10,000 ppm to about 40,000 ppm and using the methods of the present invention, the levels of acrylamide monomer are reduced to less than 1000 ppm, preferably to less than 100 ppm.

Preferably, the extract is a crude extract of an individual microorganism strain. Methods for preparing
25 extracts of the microorganism strain are described above in this application. Preferably, a pure culture or an extract of a microorganism, described in detail above in Section 4.2, induced as taught herein, is employed. Alternatively, a pure culture or an extract of a microorganism capable of removing
30 an amide monomer compound from a polyamide preparation which has been identified using the screening method(s) described above in Section 4.1.3 is employed. The crude extract,

optionally, can be further purified by enzyme purification methods known in the art such as ion exchange column chromatography before contacting.

In a preferred embodiment, the microorganism
5 strain, or an extract thereof, is immobilized. Methods for immobilization of the microorganism strain, or extract thereof, are described above in this application. More preferably, the induced microorganism, or extract thereof, is immobilized in alginate beads that have been cross-linked
10 with polyethylene imide or immobilized in a polyacrylamide-type polymer or on Dowex™ (Dow Chemical Co., Inc.), an ion exchange resin. Alternatively, in another preferred embodiment, the microorganism or extract is immobilized on a planar surface of a material. A polyamide, e.g., polyacrylamide, preparation is passed over the planar surface
15 having the immobilized microorganism or extract thereof. In one embodiment, the polyamide preparation is an aqueous preparation. In an illustrative embodiment, the planar surface is in the form of a sheet or a series of sheets of a material on which an induced microorganism or extract is
20 immobilized. In the preferred embodiment, the immobilized multiply induced microorganism or extract can easily be reused by simply flowing a new preparation over the planar surface and there is no need to recover the multiply induced microorganism or extract from the polyamide product. Immobilization allows for maximum retention and re-use of the
25 microorganism, or extract thereof, to remove more monomer, thus decreasing the amount of microorganism, or extract thereof, utilized, and thus, reducing costs. Furthermore, the need for mixing, i.e., imparting additional energy, and the amount of microorganism, or extract thereof, in the
30 polyamide preparation is minimized. Also alternatively, the microorganism, or extract thereof, is immobilized by contact with an amide monomer preparation together with a

polymerizing agent and a cross-linking agent, such that the microorganism, or extract thereof, becomes immobilized in the resulting polyamide preparation, in which it removes residual amide monomer.

5 Moreover, in the manufacturing of polyacrylamide preparations, unwanted residual monomer nitrile compounds can also be present and the removal method detailed above also allows for the conversion of these nitrile monomer compounds, in addition to the residual amide monomer compounds present, to the corresponding acid compounds, which are readily
10 removed in their salt form.

Any method for contacting the induced microorganism strain with a composition comprising polyamides and monomer amide compounds can be used according to the present invention. Such methods for contacting include, but are not
15 limited to, contacting in a closed vessel or container or with an apparatus containing the induced strains, etc.

The methods of the present invention can further comprise monitoring conversion of the monomer amide compound to the corresponding acid compound by assessing the
20 disappearance of the monomer amide compound and/or the concurrent appearance of the corresponding acid compound by any method known to those of skill in the art, for example, using gas-liquid chromatography with a flame ionization detector (GLC-FID) to detect the amide, and high pressure
25 liquid chromatography (HPLC) to detect the corresponding acid compound. Conversion to the corresponding acid results in stoichiometric production of ammonia for each amide group originally present. The extent of conversion of amide compounds can be monitored by measuring the release of ammonia using the technique of Fawcett and Scott, 1960, J.
30 Clin. Pathol. 13:156-159. If ammonia release cannot be measured due to the presence of ammonia or an ammonium salt, the concentration of the amides present can be monitored by

methods known to those of skill in the art, including but not limited to, GLC-FID, etc. Alternatively, the disappearance of amide compounds can be measured by assessing the production of the corresponding acid compounds which can be monitored by derivatizing the acid compound and detecting the derivatized product using GLC-FID. Amides and acids can be derivatized for analysis by GLC-FID if first alkylated, esterified or silylated (see generally, Supelco Chromatography Products catalog, 1997, at pages 653-656 (Supelco Inc., Bellefonte PA)).

The polyamide or polymerized amide preparations containing an unwanted amide monomer may be in solid, liquid, and/or gaseous form. When a polyamide preparation is in the gaseous and/or liquid form, it may be sorbed onto a material, such as a solid.

Energy can be imparted, for example, by imparting mechanical energy, e.g., by mixing; by imparting acoustic energy; e.g., by setting up a standing acoustic wave in the fluid; or by imparting an electrical or electrostatic field.

At different time points one may remove samples and measure the concentration of the amide monomer or polyamide or polymerized amide compounds by methods known to those skilled in the art, for example, GLC-FID, etc.

4.3.1. MODES OF OPERATION

The methods for removal of an unwanted amide monomer compound from a polyamide preparation can be operated in a variety of modes, including batch mode, sequencing batch mode, continuous or semi-continuous mode, and flow-through using a biofilter.

In all modes of operation, samples of the contents may be removed periodically to monitor removal of the compounds of interest. Additionally, the agitating and/or mixing of the reactor contents may induce foaming. In these

cases, an anti-foaming agent may be added to prevent foaming. Suitable anti-foaming agents include such as silicon containing anti-foam emulsion (e.g., Dow ANTIFOAM-A®; a silicon based anti-foaming agent).

5

4.3.1.1. BATCH MODE OPERATION

Batch mode operation entails placing a fluid containing an unwanted amide monomer compound into a vessel, such as a bioreactor, inoculating with microorganisms induced as described in Section 4.1 and incubating the mixture to
10 culture the microorganisms such that the unwanted amide monomer compound is removed. After a predetermined time period, the incubation is stopped and the contents are removed and the solids, if any, are separated from the liquid by filtration. Samples may then be taken from both the solid
15 and liquid phase and tested, for example, by GLC-FID, to assess the level of the monomer amide compounds and to confirm that the monomer amide compounds have been removed. The reactor solids are subsequently dewatered and may be further processed into, for example, a landfill or may be
20 used as bacterial inoculum for the next batch mode. In batch mode the dewatered solid residue is re-added at about 2%-40% by weight or volume, preferably at about 5%-20%. Air or oxygen may be pumped into the reactor and the contents agitated, mechanically in the bioreactor.

25

4.3.1.2. SEQUENCING BATCH MODE OPERATION

Sequencing batch mode is operated much the same as batch mode except that after the incubation period is over, the reactor is allowed to settle for a time, usually about 15 minutes, and the top 60%-95% of the reactor contents are
30 removed, leaving settled solids, if any, at the bottom as inoculum for the next batch of neutralized fluid. Preferably between 70% and 90% of the contents are drawn off.

Sequencing batch mode is a preferred embodiment for removal of fluids containing an unwanted amide monomer because the lag or acclimation phase is reduced, high levels of biomass are retained in the reactor, variability in the composition of the waste feed is better accommodated, and the residual solids remaining after biotreatment are potentially reduced.

4.3.1.3. SEMI-CONTINUOUS/CONTINUOUS MODE

Semi-continuous/continuous mode is similar to both batch and sequencing batch modes. However, rather than stopping the incubation after a predetermined time, fresh fluid containing an unwanted amide monomer is pumped into the bioreactor in a fixed amount over a given period of time as treated fluid is drawn out of the bioreactor. This provides for a continuous treatment of fluid without having to stop the removal process.

4.3.2. BIOFILTERS

Biofilters can be used for the removal of an amide monomer compound from a polyamide or polymerized amide preparation in effluents such as air, vapors, aerosols, and water or aqueous solutions. For example, if volatile amide monomer compounds are present, the volatiles may be stripped from solid or aqueous solution in which they are found and steps should be carried out in such a way that the volatiles are trapped in a biofilter. Once trapped, the volatiles can be contacted with a pure culture or an extract of a multiply induced microorganism strain to remove the monomer amide compound. A classic-type biofilter can be used, in which air containing a polyamide preparation containing an unwanted amide monomer is passed through the biofilter apparatus. A trickle-bed biofilter can be used, in which air and aqueous polyamide solutions containing an unwanted amide monomer is passed through the biofilter apparatus.

The biofilters can comprise an apparatus having a pure culture of an microorganism induced according to the methods described above, or an extract thereof, immobilized on a solid support. The microorganism can be actively
5 dividing or not actively dividing. The microorganism can also have been lyophilized before combination with the biofilter apparatus. Suitable solid supports include, but are not limited to granular activated carbon, compost, wood residue products, (e.g., wood chips, nuggets, shredded
10 pallets or trees), alumina, ruthenium, iron oxide, ion exchange resins, (e.g., Amberlite™ IRA-93 or IRA-96 (Rohm & Haas), DOWEX™ (Dow Chemical Co, Inc.), DEAE cellulose, DEAE-SEPHADEX™ (Pharmacia, Inc.), ceramic beads, polyacrylamide beads, alginate beads, κ-carrageenan cubes as well as solid particles that can be recovered from the aqueous solutions
15 due to inherent magnetic ability. Preferably, the solid support is alginate beads that have been cross-linked with polyethylene imide. Alternatively, the microorganism or extract thereof can be immobilized on a planar surface of a material. The biofilter apparatus can have influx and efflux
20 orifices, such that the material to be treated can flow through the apparatus. Preferably, the microorganism attached to the solid support is selected from the group consisting of microorganisms having ATCC Accession No. 55899, 55898, 55722, 55723, 55726, 55727, 55724, and 55725, which has been induced as described above.

25 The biofilters can be used in a method which comprises flowing an effluent, containing a polyamide or polymerized amide preparation and an unwanted amide monomer compound, through a biofilter which comprises an apparatus having a pure culture of a microorganism induced as described
30 above, or an extract thereof, immobilized on a solid support. The method may further comprise monitoring the effluent to determine that the amide monomer compound has indeed been

removed. Preferably, the microorganism attached to the solid support is selected from the group consisting of microorganisms having ATCC Accession No. 55899, 55898, 55722, 55723, 55726, 55727, 55724, and 55725, which has been induced
5 as described above.

The following examples are presented for purposes of illustration only and are not intended to limit the scope of the invention in any way.

10 5. **EXAMPLE: IMMOBILIZATION OF WHOLE CELLS IN
POLYACRYLAMIDE**

Whole cells of *Rhodococcus* strain, DAP96253 were immobilized in polyacrylamide to anchor the cells in a stable matrix. In addition, the polyacrylamide provides for mechanical integrity and strength which results in more
15 effective retention of the cells in the reactor vessel and serves to stabilize the amidase activity in the cells.

10 grams of DAP 96253 multiply induced cells, wet weight, were suspended in 40 ml distilled water. The 40 ml of cells was added to 40 ml distilled water containing 4.5 g acrylamide and 0.5 g N-,N-methylene bisacrylamide. To the 80
20 ml solution, 5 ml α -dimethylaminopropionitrile and 10 ml of a 2.5% potassium persulfate solution were added and the resultant mixture was stirred vigorously and then placed on ice. After the solution was polymerized, the polymerized solution was cut into small cubes which were washed with
25 distilled water to remove any unpolymerized monomer.

6. **EXAMPLE: REMOVAL OF CONTAMINATING ACRYLAMIDE
MONOMER FROM A CATIONIC POLYACRYLAMIDE PREPARATION**

6.1 **USE OF AN IMMOBILIZED EXTRACT**

30 In one series of experiments, a crude cell extract of multiply induced DAP 96253 cells, obtained as described in Section 4.1, above, by culturing the cells on nutritionally complete medium supplemented with 150 ppm acrylonitrile, 150

ppm acetonitrile and 50 ppm succinonitrile, was immobilized on DOWEX™ ion exchange resin (Dow Chemical Co., Inc.). The immobilized extract was added directly to a commercial cationic polyacrylamide preparation having a pH of about 3.5. 5 The reaction was carried out by mixing the preparation with the immobilized extract for 2 hours at 25-27°C.

The concentration of residual acrylamide monomer in the cationic polyacrylamide preparation was reduced by about 50 to 60% after 2 hours.

10 In another series of experiments, a crude cell extract of multiply induced DAP 96253 cells was obtained as follows. The cells were multiply induced as described in Section 4.1, above, on nutritionally complete medium supplemented with 150 ppm acetonitrile, 150 ppm acrylonitrile and 50 ppm succinonitrile. The cells were harvested, washed 15 and resuspended in phosphate buffered saline. The multiply induced cells were lysed and a crude extract of the cells was obtained as described in Section 4.1.

The crude extract was immobilized on DOWEX™ ion exchange resin (Dow Chemical Co., Inc.) as follows. One gram 20 of DOWEX™ WR2 anionic resin was prewashed with 50% ethanol and then contacted with 150 µg of crude extract, allowed to react and washed two times. The immobilized crude extract was tested for amidase activity by assessing the ability of the immobilized extract to convert acrylamide to acrylic acid at pH 7 and at 30°C. The immobilized extract exhibited an 25 amidase activity of 20 units/gram (One unit equals 1 µmole of acrylamide converted per minute at pH 7, 30°C).

Approximately 0.5 grams of the immobilized extract (10.3 units) was added directly to two separate commercial cationic polyacrylamide preparations (Cytac Industries, 30 Stamford, CT), each having a pH of less than 4 and each having a residual concentration of 1000-1100 ppm monomeric acrylamide. The reaction was carried out by mixing the

preparation with the immobilized extract and samples were taken at various time points for determination of acrylamide concentration using gas-liquid chromatography.

The concentration of residual acrylamide monomer in the first cationic polyacrylamide preparation was reduced to less than 1 ppm in 360 minutes. In the second preparation, the concentration of acrylamide monomer was reduced to approximately 400 ppm in 360 minutes.

In yet another series of experiments, when this particular extract was not immobilized, this particular extract showed no monomer amide removing activity at a pH of less than 4.

6.2 USE OF IMMOBILIZED MICROORGANISMS

Multiply induced cells, DAP 96253, are obtained as described in Section 13 above, and are immobilized on DOWEX™ ion exchange resin (Dow Chemical Co., Inc.). The immobilized cells are added directly to a commercial cationic polyacrylamide preparation, which has a pH of about 3.5. The reaction is carried out by mixing the preparation with the immobilized cells for a sufficient time, e.g., 2 hours at about ambient temperature, e.g., 25-27°C, and the concentration of the amide monomer is monitored.

7. DEPOSIT OF MICROORGANISMS

The following microorganisms were deposited on December 10, 1996 with the American Type Culture Collection (ATCC), Rockville, MD, and have been assigned the indicated Accession numbers:

	<u>Microorganism</u>	<u>ATCC Accession No.</u>
	Rhodococcus sp. DAP 96253	55899
30	Rhodococcus rhodochrous DAP 96622.	55898

The following microorganisms were deposited on November 30, 1995 with the American Type Culture Collection

(ATCC), Rockville, MD, and have been assigned the indicated Accession numbers:

	<u>Microorganism</u>	<u>ATCC Accession No.</u>
	DAP 623	55722
5	DAP 626	55723
	DAP 629	55726
	DAP 632	55727
	DAP 115	55724
	DAP 120	55725

10 The invention described and claimed herein is not
to be limited in scope by the specific embodiments herein
disclosed since these embodiments are intended as
illustrations of several aspects of the invention. Any
equivalent embodiments are intended to be within the scope of
this invention. Indeed, various modifications of the
15 invention in addition to those shown and described herein
will become apparent to those skilled in the art from the
foregoing description. Such modifications are also intended
to fall within the scope of the appended claims.

20 A number of references are cited herein, the entire
disclosures of which are incorporated herein, in their
entirety, by reference.

25

30

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 51, lines 26-31 and on page 52, lines 3-8 of the description *

A. IDENTIFICATION OF DEPOSIT *

Further deposits are identified on an additional sheet *

Name of depositary institution *

American Type Culture Collection

Address of depositary institution (including postal code and country) *

10801 University Blvd.
Manassas, VA 20110-2209
US

Date of deposit * November 28, 1995 Accession Number * 55722

B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *** (if the indications are not all designated States)**D. SEPARATE FURNISHING OF INDICATIONS *** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the International application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau *

was

(Authorized Officer)

- 52.2

International Application No: PCT/ /

Form PCT/RO/134 (cont.)

American Type Culture Collection
10801 University Blvd.,
Manassas, VA 20110-2209
US

<u>Accession No.</u>	<u>Date of Deposit</u>
55723	November 28, 1995
55724	November 28, 1995
55725	November 28, 1995
55726	November 28, 1995
55727	November 28, 1995
55898	December 11, 1996
55899	December 11, 1996

WHAT IS CLAIMED IS:

1. A method for removing unwanted amide monomer from a polyamide preparation containing said monomer by converting the amide monomer to the corresponding acid,
5 comprising contacting a pure culture or an extract of a multiply induced microorganism, with a polyamide preparation containing amide monomer, said polyamide preparation having a pH of about 2 to less than 6, for a sufficient time to convert the amide monomer to the corresponding acid.
- 10 2. The method according to claim 1, in which the pure culture or extract is encapsulated or immobilized.
3. The method according to claim 2, in which the pure culture or extract is immobilized onto a solid support
15 selected from the group consisting of granular activated carbon, wood chips, alumina, ruthenium, iron oxide, ceramic beads, alginate beads, k-carrageenan cubes, and ion exchange resin.
- 20 4. The method according to claim 2, in which the culture or extract is immobilized on a planar surface.
5. The method according to claim 1, in which the polyamide preparation is a polyacrylamide preparation.
- 25 6. The method according to claim 1, in which the microorganism is selected from the group consisting of microorganisms having ATCC Accession Nos. 55899, 55898, 55722, 55723, 55726, 55727, 55724 and 55725 and which has been multiply induced.
- 30 7. The method according to claim 1, in which the multiply induced microorganism is obtained by a method



comprising culturing a pure culture of a microorganism in a culture medium supplemented with a mixture of nitrile compounds containing at least one of acetonitrile and acrylonitrile at a concentration of about 150 ppm each and
5 succinonitrile and fumaronitrile at a concentration of about 50 ppm each.

8. The method according to claim 7, in which the culture medium is supplemented with a mixture of nitrile compounds containing acetonitrile and acrylonitrile at about
10 150 ppm each and succinonitrile at about 50 ppm.

9. The method according to claim 7, in which the culture medium is supplemented further with about 1-10 ppm KCN or NaCN.
15

10. The method according to claim 1, in which the multiply induced microorganism is obtained by a method comprising culturing a pure culture of a microorganism in a culture medium supplemented with a mixture of nitrile and
20 amide compounds containing (a) at least one of succinonitrile at about 50 ppm, acetonitrile at about 150 ppm and acrylonitrile at about 150 ppm and (b) acetamide and acrylamide at about 150 ppm each.

11. The method according to claim 10, in which the
25 culture medium is supplemented with a mixture of nitrile and amide compounds containing succinonitrile at about 50 ppm and acetamide at about 150 ppm and acrylamide at about 150 ppm.

12. The method according to claim 10, in which the
30 culture medium is supplemented further with about 1-10 ppm KCN or NaCN.



13. A method for removing unwanted amide monomer from a polyamide preparation containing said monomer by converting the amide monomer to the corresponding acid, comprising contacting, at a pH of about 2 to less than 6, a
5 pure culture or extract of a multiply induced microorganism, with an amide monomer with a polymerizing agent.

14. The method according to claim 13, in which the pure culture or extract is encapsulated or immobilized.

10 15. The method according to claim 14, in which the pure culture or extract is immobilized onto a solid support selected from the group consisting of granular activated carbon, wood chips, alumina, ruthenium, iron oxide, ceramic
15 beads, alginate beads, κ -carrageenan cubes, and ion exchange resin.

16. The method according to claim 14, in which the culture or extract is immobilized on a planar surface.

20 17. The method according to claim 13, in which the amide monomer is acrylamide.

18. The method according to claim 13, in which the microorganism is selected from the group consisting of microorganisms having ATCC Accession Nos. 55899, 55898,
25 55722, 55723, 55726, 55727, 55724 and 55725 and which has been multiply induced.

19. The method according to claim 13, in which the multiply induced microorganism is obtained by a method
30 comprising culturing a pure culture of a microorganism in a culture medium supplemented with a mixture of nitrile compounds containing at least one of acetonitrile and



acrylonitrile at a concentration of about 150 ppm each and succinonitrile and fumaronitrile at a concentration of about 50 ppm each.

5 20. The method according to claim 19, in which the culture medium is supplemented with a mixture of nitrile compounds containing acetonitrile and acrylonitrile at about 150 ppm each and succinonitrile at about 50 ppm.

10 21. The method according to claim 19, in which the culture medium is supplemented further with about 1-10 ppm KCN or NaCN.

 22. The method according to claim 13, in which the multiply induced microorganism is obtained by a method
15 comprising culturing a pure culture of a microorganism in a culture medium supplemented with a mixture of nitrile and amide compounds containing (a) at least one of succinonitrile at about 50 ppm, acetonitrile at about 150 ppm and acrylonitrile at about 150 ppm and (b) acetamide and
20 acrylamide at about 150 ppm each.

 23. The method according to claim 22, in which the culture medium is supplemented with a mixture of nitrile and amide compounds containing succinonitrile at about 50 ppm and acetamide at about 150 ppm and acrylamide at about 150 ppm.
25

 24. The method according to claim 22, in which the culture medium is supplemented further with about 1-10 ppm KCN or NaCN.

30 25. The method according to claim 1, in which the polyacrylamide preparation contains acrylamide monomer at a concentration of about 1,000-40,000 ppm.

26. The method according to claim 1, in which the polyacrylamide preparation also contains an unwanted nitrile monomer and which is removed by the conversion of the monomer to the corresponding acid.

5

27. The method according to claim 13, in which the polyacrylamide preparation also contains an unwanted nitrile monomer and which is removed by the conversion of the monomer to the corresponding acid.

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