(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 May 2009 (07.05.2009)

PCT

(10) International Publication Number
WO 2009/059101 A1

(51) International Patent Classification:
C12N 9/78 (2006.01) *C12P 7/42* (2006.01)
C12N 11/00 (2006.01)

(21) International Application Number:
PCT/US2008/081948

(22) International Filing Date: 31 October 2008 (31.10.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
11/930,550 31 October 2007 (31.10.2007) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(54) **Title:** IMPROVEMENT IN IMMOBILIZED MICROBIAL NITRILASE FOR PRODUCTION OF GLYCOLIC ACID

(57) **Abstract:** The present invention provides a process for preparing an enzyme catalyst having nitrilase activity for hydrolysis of glycolonitrile to glycolic acid with improved retention of recovered catalyst activity in consecutive batch reactions with catalyst recycle, said process comprising pretreating the enzyme catalyst with glutaraldehyde. The glutaraldehyde-pretreated enzyme catalyst has improved specific activity when compared to non-glutaraldehyde-pretreated enzyme catalysts, and thereby, has improved overall catalyst activity and productivity.

WO 2009/059101 A1

TITLE

IMPROVEMENT IN IMMOBILIZED MICROBIAL NITRILASE FOR PRODUCTION OF GLYCOLIC ACID

FIELD OF THE INVENTION

This invention relates to the field of organic acid synthesis and microbiology. More specifically, the present invention provides a process for preparing an enzyme catalyst having improved retention of initial nitrilase activity during hydrolysis of glycolonitrile to glycolic acid, said process comprising pretreating the enzyme catalyst with glutaraldehyde prior to immobilization and cross-linking. The glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst has improved specific activity, as stated above, when compared to non-glutaraldehyde-pretreated immobilized and cross-linked enzyme catalysts, and thereby, has improved overall catalyst activity, catalyst productivity and volumetric productivity for the conversion of glycolonitrile to glycolic acid.

BACKGROUND OF THE INVENTION

Glycolic acid ($HOCH_2COOH$; CAS Registry Number is 79-14-1) is the simplest member of the α-hydroxy acid family of carboxylic acids. Its properties make it ideal for a broad spectrum of consumer and industrial applications, including use in water well rehabilitation, the leather industry, the oil and gas industry, the laundry and textile industry, as a monomer in the preparation of polyglycolic acid (PGA), and as a component in personal care products. Glycolic acid also is a principle ingredient for cleaners in a variety of industries (dairy and food processing equipment cleaners, household and institutional cleaners, industrial cleaners [for transportation equipment, masonry, printed circuit boards, stainless steel boiler and process equipment, cooling tower/heat exchangers], and metals processing [for metal pickling, copper brightening, etching, electroplating, electropolishing]). It has also been reported that polyglycolic acid is useful as a gas barrier material (*i.e.*, exhibits high oxygen barrier characteristics) for packing foods and carbonated drinks (WO 2005/106005 A1). However, traditional chemical synthesis of glycolic acid produces a significant amount of impurities that must be removed prior to use.. New technology to commercially produce glycolic acid, especially one that produces glycolic acid in high purity and at low cost, would be eagerly received by industry.

Microbial enzyme catalysts can hydrolyze a nitrile (*e.g.*, glycolonitrile) directly to the corresponding carboxylic acids (*e.g.*, glycolic acid) using a nitrilase (EC 3.5.5.7), where there is no intermediate production of the corresponding amide (Equation 1), or by a combination of nitrile hydratase (EC 4.2.1.84) and amidase (EC 3.5.1.4) enzymes, where a nitrile hydratase (NHase) initially converts a nitrile to an amide, and then the amide is subsequently converted by the amidase to the corresponding carboxylic acid (Equation 2):

(1) $$HOCH_2CN \xrightarrow[2\ H_2O]{\text{nitrilase}} HOCH_2CO_2H + NH_3$$

(2) $$HOCH_2CN \xrightarrow[H_2O]{\text{NHase}} HOCH_2CONH_2 \xrightarrow[H_2O]{\text{amidase}} HOCH_2CO_2H + NH_3$$

It has been demonstrated that the enzyme catalyst specific activity measured as micromoles glycolic acid produced per minute per g dry cell weight of catalyst, decreases by from 35% to 50% of initial activity after a single use in consecutive batch reactions with biocatalyst recycle. This represents a significant loss in specific activity of the enzyme catalyst in batch reactions, and, in turn, overall enzyme activity and productivity. For a commercially feasible enzymatic process for producing glycolic acid, this loss in enzyme activity needs to be addressed.

One aspect of the loss of enzyme activity may be attributable to impurities and other components present when reacting the enzyme catalyst with glycolonitrile for glycolic acid production. Methods to synthesize glycolonitrile by reacting aqueous solutions of formaldehyde and hydrogen cyanide have previously been reported (US 2,175,805; US 2,890,238; and US 5,187,301; Equation 3).

(3) $$HCN + HCHO \rightleftharpoons HOCH_2CN$$

However, these methods typically result in an aqueous glycolonitrile reaction product that requires significant purification (*e.g.*, distillative purification) as many of the impurities and/or byproducts of the reaction (including excess reactive formaldehyde) may interfere with the enzymatic conversion of glycolonitrile to glycolic acid, including suppression of catalyst activity (*i.e.*, decreased specific activity). In particular, it is well known that formaldehyde can create undesirable modifications in proteins by reacting with amino groups from N-terminal amino acid residues and the side chains of arginine, cysteine, histidine, and lysine residues (Metz *et al.*, *J. Biol. Chem.*, 279 (8): 6235-6243 (2004)). Suppression of catalyst activity decreases the overall productivity of the catalyst (*i*.e., total grams of glycolic acid formed per gram of catalyst), adding a significant cost to the overall process that may make enzymatic production economically non-viable when compared to chemical synthesis. As such, reaction conditions are needed that can help to protect the enzymatic activity against undesirable impurities that decrease the activity of the catalyst.

A method of producing high purity glycolonitrile has been reported by subjecting the formaldehyde to a heat treatment prior to the glycolonitrile synthesis reaction (US 11/3143865 and US 11/314905; Equation 3). However, glycolonitrile can reversibly disassociate into formaldehyde and hydrogen cyanide. As such, there remains a need to protect nitrilase activity against the undesirable effects of both formaldehyde and hydrogen cyanide produced by dissociation of glycolonitrile.

US 5,508,181 also describes similar difficulties related to rapid enzyme catalyst inactivation when converting nitrile compounds to α-hydroxy acids. Specifically, U.S. 5,508,181 provides that α-hydroxy nitrile compounds partially disassociate into the corresponding aldehydes, according to the disassociation equilibrium. These aldehydes were reported to inactivate the enzyme within a short period of time by binding to the protein, thus making it difficult to obtain α-hydroxy acid or α-hydroxy amide in a high concentration with high productivity from α-hydroxy nitriles (col. 2, lines 16-29). As a solution to prevent enzyme inactivation due to accumulation of aldehydes, phosphate or hypophosphite ions were added to the reaction mixture. Similarly, US 5,326,702 describes the use of sulfite, disulfite, or dithionite ions to sequester aldehyde and prevent enzyme inactivation, but concludes that the concentration of α-hydroxy

acid produced and accumulated even by using such additives is not sufficient for most commercial purposes.

Moreover, U.S. 6,037,155 teaches that low accumulation of α-hydroxy acid product is related to enzyme inactivation within a short time due to the disassociated-aldehyde accumulation. These inventors suggest that enzymatic activity is inhibited in the presence of hydrogen cyanide (Asano *et al.*, *Agricultural Biological Chemistry*, Vol. 46, pages 1165-1174 (1982)) generated in the partial disassociation of the α-hydroxy nitrile in water together with the corresponding aldehyde or ketone (Mowry, David T., *Chemical Reviews*, Vol. 42, pages 189-283 (1948)). The inventors address the problem of aldehyde-induced enzyme inactivation by using microorganisms whose enzyme activity could be improved by adding a cyanide substance to the reaction mixture. The addition of a cyanide substance limited the disassociation of α-hydroxy nitrile to aldehyde and hydrogen cyanide. While this tactic provides a benefit to the system, it only addresses one aspect associated with enzyme inactivation in conversion of glycolonitrile to glycolic acid, in that, as stated above, glycolonitrile is known to reversibly disassociate to hydrogen cyanide and formaldehyde, and both are known to negatively effect enzyme catalyst activity.

A separate process has been developed to protect the specific activity of an enzyme catalyst having nitrilase activity when converting glycolonitrile to glycolic acid in the presence of formaldehyde (see copending U.S. Application No. XX/XXXXXX(CL3584) incorporated herein by reference), where significant improvements in catalyst activity and stability were achieved by adding an amine protectant to the reaction mixture, or by immobilization of the nitrilase catalyst in or on a matrix that is comprised of an amine protectant, e.g. PEI, polyallylamine, PVOH/polyvinylamine, etc. In that system, the specific activity of the catalyst in the presence of formaldehyde is improved, but does not address, altogether, issues related to the loss in specific activity of recovered catalyst activity in consecutive batch reactions with catalyst recycle.

U.S. 4,288,552 discloses (column 1, lines 46-49, and column 2, lines 50 – 55) that glutaraldehyde-sensitive enzymes (such as thiol-enzymes (e.g,, nitrilase) and others with an SH group in or very near the active site of the enzyme molecule) are inactivated by thiol-reactive agents such as glutaraldehyde. Therefore, use of glutaraldehyde to improve the

retention of initial catalyst activity during hydrolysis of glycolonitrile to glycolic acid was heretofore unpredictable. Said unpredictable benefit is demonstrated herein.

Therefore there is a need for a process that provides improved retention of initial nitrilase activity during hydrolysis of glycolonitrile to glycolic acid, thereby improving overall catalyst activity, catalyst productivity, and volumetric productivity for the conversion of glycolonitrile to glycolic acid.

SUMMARY OF THE INVENTION

The present invention resolves the need described above by providing a process for preparing an enzyme catalyst having nitrilase activity with improved retention of initial catalyst activity for hydrolysis of glycolonitrile to glycolic acid, said process comprising pretreating the enzyme catalyst with glutaraldehyde prior to immobilization and cross-linking. The glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst has improved specific activity during the conversion of glycolonitrile to glycolic acid when compared to immobilized and cross-linked enzyme catalysts prepared without glutaraldehyde-pretreatment, and thereby, has improved overall catalyst activity, catalyst productivity and volumetric productivity for the conversion of glycolonitrile to glycolic acid.

In one aspect, the present invention provides a process for producing an enzyme catalyst having nitrilase activity with improved retention of the initial specific activity during the conversion of glycolonitrile to glycolic acid, said process comprising:

(a) producing an enzyme catalyst having nitrilase activity by fermentation;

(b) pretreating said enzyme catalyst with glutaraldehyde;

(c) optionally inactivating unreacted glutaraldehyde with bisulfite following glutaraldehyde pretreatment;

(d) recovering the enzyme catalyst from (b) or (c), and immobilizing said enzyme catalyst in carrageenan; and

(e) cross-linking the resulting carrageenan-immobilized enzyme catalyst of (d) with glutaraldehyde and polyethylenimine, whereby a glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst is produced and wherein said glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst has improved retention of initial specific activity during

conversion of glycolonitrile to glycolic acid as compared to non-glutaraldehyde-pretreated immobilized and cross-linked enzyme catalysts under the same reaction conditions.

A further aspect of the invention comprises contacting the glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst of (e) above with glycolontrile in an aqueous solution, under suitable reaction conditions whereby glycolic acid is produced. And further, recovering said glycolic acid.

Another aspect of the invention is directed to the glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst that is produced by the process of steps a) through e) above. Further, said glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst, having nitrilase activity, retains a significantly-greater percentage of its initial specific activity (μmoles of glycolonitrile hydrolyzed per minute per gram of catalyst) when used for the conversion of glycolonitrile to glycolic acid (as the ammonium salt) as compared to a comparable enzyme catalyst without glutaraldehyde pretreatment.

Another aspect of the invention is directed to a glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst having nitrilase activity that is produced by the process of steps a) through e) above, wherein said enzyme catalyst retains at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96 %, or at least about 97% of its initial specific activity after at least four consecutive batch recycles.

Another aspect of the invention is directed to a glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst having nitrilase activity that is produced by the process of steps a) through e) above, wherein said enzyme catalyst retains at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96 %, or at least about 97% of its initial specific activity after the production of at least 40 g of glycolic acid per gram dry cell weight of glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst in a continuous reaction for the production of glycolic acid from glycolonitrile, for example, when running the reaction in a continuously stirred tank reactor (CSTR), or in a fixed-bed plug flow reactor, or in a fluidized-bed or semi-fluidized bed reactor.

A further aspect of the invention is directed to an improved process for hydrolyzing glycolonitrile to glycolic acid comprising improving the

retention of the initial specific activity of an immobilized enzyme catalyst having nitrilase activity during conversion of glycolonitrile to glycolic acid, said process comprising:

(a) producing an enzyme catalyst having nitrilase activity by fermentation;

(b) pretreating said enzyme catalyst with glutaraldehyde;

(c) optionally inactivating unreacted glutaraldehyde with bisulfite following glutaraldehyde pretreatment;

(d) recovering the enzyme catalyst from (b) or (c) and immobilizing said enzyme catalyst in carrageenan;

(e) cross-linking the resulting carrageenan-immobilized enzyme catalyst of (d) with glutaraldehyde and polyethylenimine, whereby a cross-linked immobilized enzyme catalyst is produced; and

(f) contacting the cross-linked immobilized enzyme catalyst of (e) with glycolontrile in an aqueous solution under suitable reaction conditions whereby glycolic acid is produced,

wherein step (f) occurs in at least two consecutive batch recycles.

Further, the process may include recovering the glycolic acid produced by said improved process.

BRIEF DESCRIPTION OF THE FIGURE, SEQUENCE LISTING, AND THE BIOLOGICAL DEPOSITS

The invention can be more fully understood from the Figure, sequence listing, biological deposits, and detailed description, that together form this application.

FIGURE

Figure 1, panels A-G, is a CLUSTALW alignment (version 1.83 using default parameters) of various nitrilase sequences. The conserved catalyst signature sequence surrounding the catalyst cysteine residue is highlighted in gray shading. The amino acids representing the catalytic triad (Glu_{48}, Lys_{130}, and Cys_{164}; numbering based on the amino acid sequence SEQ ID NO: 4) are underlined.

SEQUENCE LISTING

The following sequence descriptions and sequences listings attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825. The Sequence Descriptions contain the one letter code for nucleotide sequence characters and the three letter codes for amino acids

as defined in conformity with the IUPAC-IYUB standards described in *Nucleic Acids Research* 13:3021-3030 (1985) and in the *Biochemical Journal* 219 (No. 2):345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

SEQ ID NO: 1 is the amino acid sequence of the catalytic signature motif encompassing the essential cysteine residue of nitrilase enzymes (Formula 1).

SEQ ID NO: 2 is the amino acid sequence of a preferred catalyst signature motif encompassing the essential cysteine residue of nitrilase enzymes (Formula 2).

SEQ ID NO: 3 is the nucleotide sequence of the *Acidovorax facilis* 72W nitrilase coding sequence comprising a change in the start codon from TTG to ATG to facilitate recombinant expression in *E. coli*.

SEQ ID NO: 4 is the deduced amino acid sequence of the *Acidovorax facilis* 72W nitrilase (ATCC 55746).

SEQ ID NO: 5 is the amino acid sequence of the *Alcaligenes faecalis* JM3 nitrilase (GENBANK® BAA02684.1).

SEQ ID NO: 6 is the amino acid sequence of the *Rhodococcus rhodochrous* J1 nitrilase (GENBANK® Q03217).

SEQ ID NO: 7 is the amino acid sequence of the *Rhodococcus rhodochrous* K22 nitrilase (GENBANK® Q02068).

SEQ ID NO: 8 is the amino acid sequence of the *Nocardia sp.* C-14-1 nitrilase (GENBANK® AAX18182.1).

SEQ ID NO: 9 is the amino acid sequence of the *Bordetella bronchiseptica* RB50 nitrilase (GENBANK® NP_887662.1).

SEQ ID NO: 10 is the amino acid sequence of the *Arabidopsis thaliana* nitrilase (GENBANK® AAB60275.1 and AAA19627.1).

SEQ ID NO: 11 is the amino acid sequence of the *Synechococcus elongatus* PCC 7942 nitrilase (GENBANK® YP_399857.1).

SEQ ID NO: 12 is the amino acid sequence of the *Synechococcus elongatus* PCC 6301 nitrilase (GENBANK® YP_171411.1).

SEQ ID NO: 13 is the amino acid sequence of the *Synechocystis sp.* PCC 6803 nitrilase (GENBANK® NP_442646.1).

SEQ ID NO: 14 is the amino acid sequence of the *Pseudomonas entomophila* L48 nitrilase (GENBANK® YP_609048l.1).

SEQ ID NO: 15 is the amino acid sequence of the *Zymomonas moblis* nitrilase (GENBANK® YP_162942.1).

SEQ ID NO: 16 is the amino acid sequence of the *Bacillus sp.* OxB-1 nitrilase (GENBANK® BAA90460.1).

SEQ ID NO: 17 is the amino acid sequence of the *Comamonas testosteroni* nitrilase (GENBANK® AAA82085.1).

SEQ ID NO: 18 is the amino acid sequence of the *Synechococcus sp.* CC9605 nitrilase (GENBANK® YP_381420.1).

SEQ ID NO: 19 is the amino acid sequence of the *Pseudomonas fluorescens* Pf-5 nitrilase (GENBANK® YP_260015.1).

SEQ ID NO: 20 is the amino acid sequence of the *Nocardia farcinica* IFM 10152 nitrilase (GENBANK® YP_119480.1).

SEQ ID NO: 21 is the amino acid sequence of the *Alcaligenes faecalis* 1650 nitrilase (GENBANK® AAY06506.1).

SEQ ID NO: 22 is the amino acid sequence of the *Pseudomonas syringae* pv. syringae B728a nitrilase (GENBANK® AAY35081.1).

SEQ ID NO: 23 is the amino acid sequence of the *Bradyrhizobium sp.* BTAil nitrilase (GENBANK® ZP_00859948.1).

SEQ ID NO: 24 is the amino acid sequence of the *Rhodococcus rhodochrous*
NCIMB 11216 nitrilase (GENBANK® CAC88237).

SEQ ID NO: 25 is the amino acid sequence of *Rhodococcus rhodochrous* ATCC™ 39484

SEQ ID NO: 26 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 201 (L201Q; Leu ➔ Gln).

SEQ ID NO: 27 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 26) comprising a single amino acid substitution at residue position 201 (Leu201 ➔ Gln) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 28 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 201 (L201A; Leu ➔ Ala).

SEQ ID NO: 29 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 28) comprising a single amino acid substitution at residue position 201 (Leu201 ➔ Ala) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 30 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 201 (L201C; Leu ➔ Cys).

SEQ ID NO: 31 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 30) comprising a single amino acid substitution at residue position 201 (Leu201 ➔ Cys) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 32 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 201 (L201T; Leu ➔ Thr).

SEQ ID NO: 33 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 32) comprising a single amino acid substitution at residue position 201 (Leu201 ➔ Thr) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 34 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 201 (L201G; Leu ➔ Gly).

SEQ ID NO: 35 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 34) comprising a single amino acid substitution at residue position 201 (Leu201 ➔ Gly) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 36 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 201 (L201H; Leu ➔ His).

SEQ ID NO: 37 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 36) comprising a single amino acid substitution at residue position 201 (Leu201 ➔ His) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 38 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 201 (L201K; Leu ➔ Lys).

SEQ ID NO: 39 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 38) comprising a single amino acid substitution at residue position 201 (Leu201 ➔ Lys) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 40 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 201 (L201N; Leu ➔ Asn).

SEQ ID NO: 41 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 40) comprising a single amino acid substitution at residue position 201 (Leu201 ➔ Asn) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 42 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 201 (L201S; Leu ➔ Ser).

SEQ ID NO: 43 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 42) comprising a single amino acid substitution at residue position 201 (Leu201 ➔ Ser) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 44 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 168 (F168K; Phe ➔ Lys).

SEQ ID NO: 45 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 44) comprising a single amino acid substitution at residue position 168 (Phe168 ➔ Lys) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 46 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 168 (F168M; Phe ➔ Met).

SEQ ID NO: 47 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 46) comprising a single amino acid substitution at residue position 168 (Phe168 ➔ Met) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 48 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 168 (F168T; Phe ➔ Thr).

SEQ ID NO: 49 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 48) comprising a single amino acid substitution at residue position 168 (Phe168 ➔ Thr) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 50 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 168 (F168V; Phe ➔ Val).

SEQ ID NO: 51 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO:50) comprising a single amino acid substitution at residue position 168 (Phe168 ➔ Val) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 52 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 168 (T210A; Thr ➔ Ala).

SEQ ID NO: 53 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 52) comprising a single amino acid substitution at residue position 210 (Thr210 ➔ Ala) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 54 is the nucleotide sequence of an *A. facilis* 72W nitrilase mutant comprising a codon change which resulted in a single amino acid substitution at residue position 168 (T210C; Thr ➔ Cys).

SEQ ID NO: 55 is the deduced amino acid sequence of the mutant nitrilase (SEQ ID NO: 54) comprising a single amino acid substitution at residue position 210 (Thr210 ➔ Cys) of the *A. facilis* 72W nitrilase.

SEQ ID NO: 56 is the nucleotide sequence of the *A. facilis* 72W nitrilase expressed in *E. coli* strain SS1001 (ATCC PTA-1177).

SEQ ID NO: 57 is the deduced amino acid sequence of the mutant A. facilis 72W nitrilase expressed in *E. coli* SS1001 (ATCC PTA-1177).

BIOLOGICAL DEPOSITS

The following biological deposits have been made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure:

Depositor Identification Reference	Int'l. Depository Designation	Date of Deposit
Acidovorax facilis 72W	ATCC 55746	8 March 1996
E. coli SS1001	ATCC PTA-1177	11 January 2000

As used herein, "ATCC" refers to the American Type Culture Collection International Depository Authority located at ATCC, 10801 University Blvd., Manassas, VA 20110-2209, USA. The "International Depository Designation" is the accession number to the culture on deposit with ATCC.

The listed deposits will be maintained in the indicated international depository for at least thirty (30) years and will be made available to the public upon the grant of a patent disclosing it. The availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by government action.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a process for preparing an immobilized and cross-linked enzyme catalyst having nitrilase activity for hydrolysis of glycolonitrile to glycolic acid with improved retention of initial enzyme catalyst activity during conversion of glycolonitrile to glycolic acid, said process comprising pretreating the enzyme catalyst with glutaraldehyde prior to immobilization. The glutaraldehyde-pretreated immobilized enzyme catalyst has improved retention of initial specific activity, as stated above, when compared to the retention of initial specific activity of non-glutaraldehyde-pretreated immobilized and cross-linked enzyme catalysts during the conversion of glycolonitrile to glycolic acid, and thereby, has improved overall catalyst activity, catalyst productivity and volumetric productivity.

Definitions:

In this disclosure, a number of terms and abbreviations are used. The following definitions apply unless specifically stated otherwise.

As used herein, the term “comprising” means the presence of the stated features, integers, steps, or components as referred to in the claims, but that it does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

As used herein, the term "about" modifying the quantity of an ingredient or reactant of the invention employed refers to variation in the numerical quantity that can occur, for example, through typical measuring and liquid handling procedures used for making concentrates or use solutions in the real world; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of the ingredients employed to make the compositions or carry out the methods; and the like. The term “about” also encompasses amounts that differ due to different equilibrium conditions for a composition resulting from a particular initial mixture. Whether or not modified by the term "about", the claims include equivalents to the quantities. In one embodiment, the term “about” means within 10% of the reported numerical value, preferably within 5% of the reported numerical value.

As used herein, the term “glycolonitrile” is abbreviated as “GLN” and is synonymous with hydroxyacetonitrile, 2-hydroxyacetonitrile, hydroxymethylnitrile, and all other synonyms of CAS Registry Number 107-16-4.

As used herein, the term “glycolic acid” is abbreviated as “GLA” and is synonymous with hydroxyacetic acid, hydroxyethanoic acid, and all other synonyms of CAS Registry Number 79-14-1. The glycolic acid produced by the present processes may in the form of the protonated carboxylic acid and/or the corresponding ammonium salt.

As used herein, the term “ammonium glycolate” is abbreviated “NH_4GLA”.

As used herein, the term “glycolamide” is the amide derived from the reaction of ammonia with glycolic acid and refers to all other synonyms of compounds having CAS Registry Number 598-42-5.

As used herein, the term “glycolide” refers to the compound of CAS Registry Number 502-97-6.

As used herein, the term "formaldehyde" is abbreviated as "FA" and is synonymous with formic aldehyde, methyl aldehyde, oxomethane, and all other synonyms of CAS Registry Number 50-00-0. Commercially available formaldehyde is typically comprised of a mixture of monomeric formaldehyde ("free formaldehyde") and various oligomers of formaldehyde along with some methanol (typically about 1 wt% to about 15 wt %).

As used herein, the term "hydrogen cyanide" is synonymous with prussic acid, hydrocyanic acid, and all other synonyms of CAS Registry Number 200-821-6.

As used herein, the term "glutaraldehyde" is abbreviated "GA" and is synonymous with pentanedial, 1,5-pentanedial, 1,5-pentanedione, diglutaric aldehyde, glutaral, glutardialdehyde, glutaric acid dialdehyde, glutaric dialdehyde, and all other synonyms of CAS Registry Number 111-30-8.

As used herein, the term "bisulfite" or "sodium bisulfite" is synonymous with sulfurous acid sodium salt, sulfurous acid monosodium salt, hydrogen sodium sulfite, hydrogen sulfite sodium, monosodium sulfite, sodium acid sulfite, sodium bisulfite, sodium bisulphate, sodium hydrogen sulfite, sodium sulfite (NaHSO3), and all other synonyms of CAS Registry Number 7631-90-5.

As used herein, the term "recovering" means isolating, purifying, or transferring the product formed by the present process. Methods to isolate and purify the product(s) from the reaction mixture are well known in the art may include, but are not limited to selective precipitation, crystallization, filtration, reactive solvent extraction, ion exchange, electrodialysis, polymerization, distillation, thermal decomposition, alcoholysis, column chromatography, and combinations thereof. In one embodiment, the term "recovering" may also include transferring the product mixture (typically after filtering out the enzyme catalyst) to another reaction to create one or more additional products. In a preferred embodiment, ion exchange is used to recover the glycolic acid.

As used herein, the terms "enzyme catalyst", "nitrilase catalyst" or "microbial cell catalyst" refers to a catalyst that is characterized by a nitrilase activity (*i.e.*, comprises at least one polypeptide having nitrilase activity) for converting glycolonitrile to glycolic acid and ammonia. A nitrilase enzyme directly converts a nitrile (preferably, an aliphatic nitrile) to the corresponding carboxylic acid, without forming the corresponding

amide as intermediate (see Equation 1). Nitrilases share several conserved signature domains known in the art including a signature domain herein referred to as the "catalytic signature sequence" or "signature sequence". This region comprises an essential cysteine residue (*e.g.*, Cys_{164} of SEQ ID NO: 4). As such, polypeptides having nitrilase activity can be identified by the existence of the catalytic domain signature sequence (SEQ ID NO: 1). In a preferred embodiment, the signature sequence is SEQ ID NO: 2. The enzyme catalyst may be in the form of whole microbial cells or permeabilized microbial cells. As used herein, "recycled enzyme catalyst" refers to an enzyme catalyst that is reused as an enzyme catalyst in batch or continuous reactions. Depending on the step in the process of producing or using the enzyme catalyst as described herein, the enzyme catalyst may be glutaraldehyde pretreated, immobilized, and cross-linked.

As used herein, the terms *"Acidovorax facilis"* and *"A. facilis"* are used interchangeably and refer to *Acidovorax facilis* 72W deposited to the American Type Culture Collection (an international depository authority) having accession number 55746 ("ATCC 55746"). The mutant nitrilases derived from *A. facilis* 72W characterized by improved nitrilase activity when converting glycolonitrile to glycolic acid have been previously reported (see co-owned U.S. patent 7,198,927). Examples of these *A. facilis* 72W-derived mutant nitrilases are provided by SEQ ID NOs: 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, and 55.

As used herein, the terms "*Escherichia coli*" and "*E. coli*" are used interchangeably. Several strains of *E. coli* suitable for recombinant expression are described herein including, but not limited to *E. coli* MG1655 having international depository number ATCC 47076, *E. coli* FM5 having international depository number ATCC 53911, *E. coli* W3110 having international depository number ATCC 27325, *E. coli* MC4100 having international depository number ATCC 35695, and *E. coli* W1485 having international depository number ATCC 12435. In one embodiment, suitable *Escherichia coli* strains include *E. coli* FM5 (ATCC 53911) and *E. coli* MG1655 (ATCC 47076).

As used herein, the terms "*E. coli* SS1001" or "SS1001" refer to a transformed *E. coli* strain expressing the *Acidovorax facilis* 72W nitrilase having ATCC Accession No. PTA-1177 (see U.S. patent 6,870,038; herein incorporated in its entirety by reference). The recombinantly expressed *E. coli* SS1001 nitrilase (SEQ ID NO: 57) contains 2 minor sequence

changes in comparison to the wild-type 72W nitrilase sequence (SEQ ID NO: 4). The start codon was changed from GTG to ATG to facilitate recombinant expression and an artifact was introduced during cloning that resulted in a single amino acid change near the C-terminal (Pro367 [CCA] ➔ Ser [TCA]).

As used herein, the terms "suitable aqueous glycolonitrile reaction mixture" and "suitable aqueous reaction mixture" refer to the materials (including at least one amine protectant) and water in which the glycolonitrile and enzyme catalyst come into contact. The components of the suitable aqueous reaction mixture are provided herein and those skilled in the art appreciate the range of component variations suitable for this process.

As used herein, the terms "aqueous ammonium glycolate solution", "aqueous solution comprising ammonium glycolate", and "aqueous solution of ammonium glycolate" will be used to describe an aqueous solution comprising ammonium glycolate produced by the enzymatic hydrolysis of glycolonitrile under typical enzymatic reaction conditions (*i.e.*, a pH range of about 6 to about 8). The aqueous solution of ammonium glycolate comprises ammonium glycolate at a concentration of at least about 0.1 weight percent (wt %) to about 99 wt % ammonium glycolate. In another embodiment, the aqueous solution of ammonium glycolate is comprised of at least about 10 wt % to about 75 wt % ammonium glycolate. In a further embodiment, the aqueous solution of ammonium glycolate is comprised of at least about 20 wt % to about 50 wt % ammonium glycolate. The pH of the aqueous solution of ammonium glycolate can be about 2 to about 12, preferably 5 to about 10, more preferably 6 to about 8. The pH may be adjusted as needed prior to initiating process steps related to recovering glycolic acid (in the form of the acid or salt) from the aqueous ammonium glycolate solution.

As used herein, the terms "catalyst productivity" and "enzyme catalyst productivity" refer to the total amount of product produced per gram of enzyme catalyst dry cell weight. In the present invention, the enzyme catalyst comprises a nitrilase enzyme (EC 3.5.5.7) and the product formed is glycolic acid and/or ammonium glycolate (depending upon the pH of the reaction). In general, the processes produced pursuant to producing glycolic acid are conducted under essentially pH neutral conditions so that the glycolic acid produced is predominantly in the form of the corresponding salt of glycolic acid (i.e. ammonium

glycolate). Generally, in batch reactions with catalyst recycle, the catalyst activity decreases with each recycle reaction (enzyme inactivation).

As used herein, the term "volumetric productivity" refers to the volumetric production of glycolic acid in the reaction, expressed as grams of glycolic acid produced per volume of reaction mixture per unit of time. Typically, volumetric productivity is expressed as grams glycolic acid/L/h.

The term "nitrilase activity" or "specific activity" refers to the enzyme activity per unit mass (for example, milligram) of protein, dry cell weight, or bead weight (immobilized catalyst) when converting glycolonitrile to glycolic acid (or the corresponding ammonium glycolate). Comparisons in nitrilase activity were measured proportional to the dry cell weight or bead weight.

As used herein, the term "one unit of enzyme activity" or "one unit of nitrilase activity" or "U" is defined as the amount of enzyme activity required for the production of 1 μmol of glycolic acid product per minute (GLA U/g dry cell weight or bead weight) at a specified temperature (*e.g.* 25 °C).

As used herein, the terms "relative nitrilase activity", "improved nitrilase activity", and "relative improvement in nitrilase activity" refers to the nitrilase activity expressed as a multiple (or fraction) of a reference (control) nitrilase activity. The nitrilases described herein exhibit a significant improvement in nitrilase activity relative to the nitrilase activity observed with native *Acidovorax facilis* 72W nitrilase. A "significant improvement" in relative nitrilase activity is an improvement of at least 1.5-fold higher nitrilase activity in comparison to the nitrilase activity of a control under identical reaction conditions. In another embodiment, the improvement is at least 2-fold higher nitrilase activity in comparison to the nitrilase activity of the control under identical reaction conditions. In a further embodiment, the improvement is at least 4-fold higher nitrilase activity in comparison to the nitrilase activity of the control under identical reaction conditions.

As used herein, the term "initial reaction rate" is a measurement of the rate of conversion of glycolonitrile to glycolic acid under the stated reaction conditions, where the measurement of reaction rate begins upon the initial addition of glycolonitrile to the reaction mixture, and where the reaction rate is measured over a period of time where the concentration of

glycolonitrile remains above ca. 50 millimolar (mM) during the course of the reaction. The reaction rate is measured as the change in concentration of glycolic acid produced per unit time (e.g., mole glycolic acid/L/min or mM glycolic acid/hour).

As used herein, the term "improved retention of initial specific activity" refers to a comparison of a glutaraldehyde pretreated, immobilized and cross-linked enzyme catalyst with a non-glutaraldehyde pretreated, immobilized and cross-linked enzyme catalyst, both having nitrilase activity, during conversion of glycolonitrile to glycolic acid under the stated reaction conditions, measured as micromoles of glycolic acid produced per minute per g dry cell weight of enzyme catalyst, or micromoles glycolic acid produced per minute per g immobilized and crosslinked enzyme catalyst, wherein the specific activity as measured in a first or "initial" reaction is retained to a greater extent for the glutaraldehyde pretreated immobilized and cross-linked enzyme catalyst than for the non-glutaraldehyde pretreated, immobilized and cross-linked enzyme catalyst, for one or more subsequent reactions. The most notable improvement, as described herein, is for the amount of activity retained for the reaction immediately following an initial batch reaction, measured in one or more subsequent batch reactions with catalyst recycle. A second notable improvement, as described herein, is for the amount of activity retained during the course of running the reaction in a continuously stirred tank reactor (CSTR), or in a fixed-bed plug flow reactor, or in a fluidized-bed or semi-fluidized bed reactor.

As used herein, the terms "recombinant organism", "transformed host", "transformant", "transgenic organism", and "transformed microbial host" refer to a host organism having been transformed with heterologous or foreign DNA. The recombinant organisms of the present invention express foreign coding sequences or genes that encode active nitrilase enzyme. "Transformation" refers to the transfer of a DNA fragment into the host organism. The transferred DNA fragment can be chromosomally or extrachromosomally incorporated (*i.e.,* via a vector) into the host organism. As used herein, the term "transformation cassette" refers to a specific fragment of DNA containing a set of genetic elements

conveniently arranged for insertion into a host cell, usually as part of a plasmid. As used herein, the term "expression cassette" refers to a specific fragment of DNA containing a set of genetic elements conveniently arranged for insertion into a host cell, usually as part of a plasmid that also allows for enhanced gene expression in the host.

As used herein, the terms "nucleic acid fragment" and "nucleic acid molecule" refer to DNA molecule that may encode an entire gene, coding sequence, and/or regulatory sequences preceding (5', upstream) or following (3', downstream) the coding sequence. In one aspect, the present nucleic acid molecules encode for polypeptides having nitrilase activity.

As used herein, the term "gene" refers to a nucleic acid molecule that expresses a specific protein. As used herein, it may or may not including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

As used herein, the term "coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. As used herein, "suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites, and stem-loop structures.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. Promoters that cause a gene to be expressed in most cell types at most times or under most environmental conditions are commonly referred to as "constitutive promoters". Promoters that cause a gene to be expressed only in the presence of a particular compound or environmental condition are commonly referred to as "inducible promoters". Since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

As used herein, the term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid molecule so that the function of one sequence is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (*i.e.,* that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

As used herein, the term "3' non-coding sequences" refers to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences (normally limited to eukaryotes) and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal (normally limited to eukaryotes) is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in using nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its codon usage reflects the preferred codon bias of the host cell. A survey of genes derived from the host cell where sequence information is available can determine its codon bias. Codon-optimization is well known in the art and has been described for various systems including, but not limited to yeast (Outchkourov *et al.*, *Protein Expr Purif*, 24(1):18-24 (2002)) and *E. coli* (Feng *et al.*, *Biochemistry*, 39(50):15399-15409 (2000)).

Enzyme Catalysts Having Nitrilase Activity

All nitrilases (EC 3.5.5.7) share a conserved catalytic triad (Glu, Lys, and Cys) (Chauhan *et al.*, *Appl. Microbiol. Biotechnol.* 61:118-122 (2003); Pace, H. and Brenner, C., *Genome Biol.* [online computer file] 2(1):reviews0001.1-0001.9 (2001)). All known nitrilases have a nucleophilic cysteine in the enzyme active site (Cowan *et al.*, *Extremophiles,* 2:207-216 (1998); Pace, H. and Brenner, C., *supra;* and Chauhan *et al.*, *supra*) and all are susceptible to inactivation by thiol reagents (1.0 mM concentrations of copper chloride, silver nitrate, mercuric acetate, or ferric chloride each produced major decreases in *A. facilis* 72W nitrilase enzyme activity). Cysteine residues are also capable of being irreversibly oxidized to sulfinic acids, resulting in a loss of enzyme activity. Despite the sensitivity of nitrilase enzymes to various inactivating mechanisms, immobilized *A. facilis* 72W cells are robust, capable of retaining much of their nitrilase activity after numerous recycle reactions (US 6,870,038; U.S. 7,148,051; U.S. 7,198,927; and Chauhan *et al.*, *supra*). Nitrilase catalysts derived from the *A. facilis* 72W nitrilase also been shown to catalyze the conversion of α-hydroxynitriles (*i.e.,* glycolonitrile) to α-hydroxycarboxylic acids (*i.e.,* glycolic acid) (see US 6,383,786; US 6,416,980; and U.S. 7,198,927).

Sequence comparisons of the *A. facilis* 72W nitrilase to other bacterial nitrilases have been reported (US 6,870,038; Chauhan *et al.*, *supra*). The 72W nitrilase has several conserved signature domains including a 16-amino acid region near the amino terminus (amino acid residues 40-55 of SEQ ID NO: 4) and a 12 amino acid catalytic region (amino acid residues 160-171 of SEQ ID NO: 4) containing the essential cysteine residue. This essential cysteine residue (Cys_{164} of SEQ ID NO: 4), along with conserved glutamic acid (Glu_{48} of SEQ ID NO:4) and lysine residues (Lys_{130} of SEQ ID NO:4), form the catalytic triad motif found in all nitrilases (Pace, H., and Brenner, C., *supra*).

The regions surrounding each of the catalytic triad residues are highly conserved, especially the region surrounding the catalytic cysteine residue. The essential catalytic cysteine residue is located with a highly conserved region referred to as the "catalytic signature motif" or "signature motif". As such, the present process is useful for protecting the enzymatic activity of any nitrilase comprising the catalytic signature motif defined by Formula 1 (bold indicates strictly conserved amino acid residues, italicized

residues are those that exhibit minimal variability [i.e. minimal variation of 3 or fewer amino acid residues], the catalytic cysteine residue is underlined):

Formula 1 (SEQ ID NO: 1).

Gly-*Xaa*$_1$-*Xaa*$_2$-Xaa$_3$-**Cys**-**Trp**-**Glu**-*Xaa*$_4$-Xaa$_5$-Xaa$_6$-*Xaa*$_7$-*Xaa*$_8$

wherein

Xaa$_1$ = Ala or Gly;

Xaa$_2$ = Leu, Val, or Ala;

Xaa$_3$ = Ala, Asn, Ile, Cys, Val, or Gln;

Xaa$_4$ = His or Asn;

Xaa$_5$ = Leu, Tyr, Phe, Ala, Met, Lys, Val, Thr, or Arg;

Xaa$_6$ = Asn, Gln, Met, Leu, or Ser;

Xaa$_7$ = Pro or Thr; and

Xaa$_8$ = Leu or Val.

In a preferred embodiment, the nitrilase signature motif of Formula 1 is Xaa$_1$ = Ala or Gly; Xaa$_2$ = Leu; Xaa$_3$ = Ala, Asn, Ile, Cys, Val, or Gln; Xaa$_4$ = His; Xaa$_5$ = Leu, Tyr, Phe, Ala, Met, Lys, Val, Thr or Arg; Xaa$_6$ = Ser, Gln, Asn, or Met; Xaa$_7$ = Pro; and Xaa$_8$ = Leu; resulting in the catalytic signature motif represented by the following:

Gly-Xaa$_1$-Leu-Xaa$_3$-Cys-Trp-Glu-His-Xaa$_5$-Xaa$_6$-Pro-Leu (SEQ ID NO: 2)

Examples of nitrilases, including the sequences and position of the corresponding catalytic signature motif sequence, are provided in Table 1.

Table 1. Conserved Catalytic Cysteine Region – Catalytic Signature Motifs

Nitrilase Source	GenBank® Accession Number	Amino Acid SEQ ID NO.	Sequence of Signature Motif (amino acid residue positions)
Acidovorax Facilis 72W	ABD98457.1	4	GGLNCWEHFQPL (160-171)
Alcaligenes faecalis JM3	BAA02684.1	5	GALCCWEHLSPL (159-170)
Rhodococcus rhodochrous J1	Q03217	6	GALNCWEHFQTL (161-172)
Rhodococcus rhodochrous K22	Q02068	7	GGLNCWEHFQPL (166-177)
Nocardia sp. C-14-1	AAX18182.1	8	GGLNCWEHFQPL (154-165)
Bordetella bronchiseptica RB50	NP_887662.1	9	GAVVCWENYMPL (161-172)
Arabidopsis thaliana	AAB60275.1 AAA19627.1	10	GAAICWENRMPL (175-186)
Synechococcus elongatus PCC 7942	YP_399857.1	11	GALACWEHYNPL (157-168)
Synechococcus elongatus PCC 6301	YP_171411.1	12	GALACWEHYNPL (157-168)
Synechocystis sp. PCC 6803	NP_442646.1	13	GALACWEHYNPL (165-176)
Pseudomonas entomophila L48	YP_609048l.1	14	GAAVCWENYMPL (161-172)
Zymomonas moblis	YP_162942.1	15	GAAICWENYMPV (161-172)
Bacillus sp. OxB-1	BAA90460.1	16	GGLQCWEHFLPL (158-169)
Comamonas testosteroni	AAA82085.1	17	GGLQCWEHALPL (159-170)
Synechococcus sp. CC9605	YP_381420.1	18	GALACWEHYNPL (156-167)
Pseudomonas fluorescens Pf-5	YP_260015.1	19	GAVICWENMMPL (161-172)
Nocardia farcinica IFM 10152	YP_119480.1	20	GALCCWEHLQPL (159-170)
Alcaligenes faecalis 1650	AAY06506.1	21	GALCCWEHLSPL (159-170)
Pseudomonas syringae pv. syringae B728a	AAY35081.1	22	GALCCWEHLQPL (157-168)
Bradyrhizobium sp. BTAil	ZP_00859948.1	23	GALCCWEHLQPL (163-174)
Rhodococcus rhodochrous NCIMB 11216	CAC88237	24	GALNCWEHFQTL (161-172)
Rhodococcus rhodochrous ATCC 39484™	N/A	25	GALNCWEHFQTL (161-172)

In one embodiment, the nitrilase catalyst comprises a polypeptide having nitrilase activity isolated from a genera selected from the group consisting of *Acidovorax*, *Rhodococcus*, *Nocardia*, *Bacillus*, and

Alcaligenes. In one embodiment, the nitrilase catalyst comprises a polypeptide having nitrilase activity isolated from a genera selected from the group consisting of *Acidovorax* and *Rhodococcus*.

In another embodiment, the polypeptide having nitrilase activity is derived from *Acidovorax facilis* 72W (ATCC 55746) or a polypeptide (having nitrilase activity) that is substantially similar to the *Acidovorax facilis* 72W nitrilase (SEQ ID NO: 4) or the *A. facilis* 72W derived enzyme represented by SEQ ID NO: 51.

In one embodiment, the nitrilase catalyst is a microbial host cell transformed to express at least one polypeptide having nitrilase activity. In one embodiment the transformed host cell is selected from the group consisting of *Comamonas sp.*, *Corynebacterium sp.*, *Brevibacterium sp.*, *Rhodococcus sp.*, *Azotobacter sp.*, *Citrobacter sp.*, *Enterobacter sp.*, *Clostridium sp.*, *Klebsiella sp.*, *Salmonella sp.*, *Lactobacillus sp.*, *Aspergillus sp.*, *Saccharomyces sp.*, *Yarrowia sp.*, *Zygosaccharomyces sp.*, *Pichia sp.*, *Kluyveromyces sp.*, *Candida sp.*, *Hansenula sp.*, *Dunaliella sp.*, *Debaryomyces sp.*, *Mucor sp.*, *Torulopsis sp.*, *Methylobacteria sp.*, *Bacillus sp.*, *Escherichia sp.*, *Pseudomonas sp.*, *Rhizobium sp.*, and *Streptomyces sp.* In a preferred embodiment, the microbial host cell is selected from the group consisting of *Bacillus sp.*, *Pseudomonas sp.*, and *Escherichia sp.*. In a preferred embodiment, the catalyst is an *Escherichia coli* host cell recombinantly expressing one or more of the polypeptides having nitrilase activity.

In another embodiment, the nitrilase catalyst comprises a polypeptide having nitrilase activity wherein said polypeptide having nitrilase activity has at least 60% identity to SEQ ID NO: 51, preferably at least 70% identity to SEQ ID NO: 51, even more preferably at least 80% identity to SEQ ID NO: 51, yet even more preferably at least 90% identity to SEQ ID NO: 51, and most preferably at least 95% identity to SEQ ID NO: 51.

Working examples of several catalysts having nitrilase activity derived from various sources are described herein, including a catalyst derived from the *A. facilis* 72W nitrilase. Various mutants derived from the *Acidovorax facilis* 72W nitrilase enzyme have been reported in the art (U.S. Patent 7,148,051 and U.S. 7,198,927).

In one embodiment, the polypeptide having nitrilase activity is selected from the group consisting of SEQ ID NOs: 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, 33, 35,

37, 39, 41, 43, 45, 47, 49, 51, 53, 55, and 57. In another embodiment, the polypeptide having nitrilase activity is selected from the group consisting of 4, 24, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, and 57. In another embodiment, the polypeptide having nitrilase activity is selected from the group consisting of 4, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, and 57. n another embodiment, the polypeptide having nitrilase activity is selected from the group consisting of 4, 24, 25, and 51. In another embodiment, the nitrilase catalyst comprises the polypeptide of SEQ ID NO: 51.

Acidovorax facilis 72W (ATCC 55746) Nitrilase

The *A. facilis* 72W nitrilase (EC 3.5.5.1) is a robust catalyst for producing carboxylic acids from aliphatic or aromatic nitriles (WO 01/75077; US 6,870,038; and Chauhan *et al.*, *supra*). It has also been shown to catalyze the conversion of α-hydroxynitriles (*i.e.,* glycolonitrile) to α-hydroxycarboxylic acids (*i.e.,* glycolic acid) (see US 6,383,786 and US 6,416,980). However, nitrilase catalysts having improved nitrilase activity and/or stability (relative to the *A. facilis* 72W nitrilase) when converting glycolonitrile to glycolic acid would reduce the cost of manufacturing glycolic acid. As such, a method of producing glycolic acid using an improved nitrilase catalyst is useful to reduce the cost of manufacturing glycolic acid, however *A. facilis* 72W nitrilase is an enzyme catalyst for purposes of the processes herein, as well as said improved nitrilases described in detail above.

Industrial Production of the Enzyme Catalyst

Where commercial production of the enzyme catalysts described herein is desired, a variety of culture methodologies may be used. Fermentation runs may be conducted in batch, fed-batch, or continuous mode, methods well-known in the art (Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA, (1989); Deshpande, Mukund V., *Appl. Biochem. Biotechnol.* 36(3): 227-234 (1992)).

A classical batch culturing method is a closed system where the composition of the media is set at the beginning of the culture and not subject to artificial alterations during the culturing process. Thus, at the beginning of the culturing process the media is inoculated with the desired organism or organisms and growth or metabolic activity is permitted to

occur adding nothing to the system. Typically, however, a "batch" culture is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions of the system change constantly up to the time the culture is terminated. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase are often responsible for the bulk of production of end product or intermediate in some systems. Stationary or post-exponential phase production can be obtained in other systems.

A variation on the standard batch system is the Fed-Batch system. Fed-Batch culture processes are also suitable in the present invention and comprise a typical batch system with the exception that the substrate is added in increments as the culture progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the media. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen, and the partial pressure of waste gases such as CO_2. Batch and Fed-Batch culturing methods are common and well known in the art and examples may be found in Brock (*supra*) and Deshpande *(supra*).

Commercial production of the present enzyme catalysts having nitrilase activity may also be accomplished with a continuous culture. Continuous cultures are an open system where a defined culture media is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous cultures generally maintain the cells at a constant high-liquid-phase density where cells are primarily in log phase growth. Alternatively, continuous culture may be practiced with immobilized cells where carbon and nutrients are continuously added and valuable products, by-products or waste products are continuously removed from the cell mass. Cell immobilization may be performed using a wide range of solid supports composed of natural and/or synthetic materials.

Continuous or semi-continuous culture allows for the modulation of one factor or any number of factors that affect cell growth or end cell concentration. For example, one method will maintain a limiting nutrient

such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady-state growth conditions and thus the cell loss due to media being drawn off must be balanced against the cell growth rate in the culture. Methods of modulating nutrients and growth factors for continuous culture processes, as well as techniques for maximizing the rate of cell formation, are well known in the art of industrial microbiology and a variety of methods are detailed by Brock (*supra*).

Fermentation media in the present invention must contain suitable carbon substrates. Suitable substrates may include, but are not limited to monosaccharides such as glucose and fructose, disaccharides such as lactose or sucrose, polysaccharides such as starch or cellulose or mixtures thereof, and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Hence, it is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon-containing substrates and will only be limited by the choice of organism.

Glutaraldehyde Pretreatment of the Enzyme Catalyst Prior to Immobilization

Treatment of an enzyme catalyst fermentation culture with glutaraldehyde can be a convenient way to kill the microbes in the culture, thus avoiding containment and safety issues for handling, storage and transportation associated with live recombinant cultures. It has now been discovered that pretreatment with glutaraldehyde, or glutaraldehyde pretreatment followed by bisulfite treatment, can preserve nitrilase activity in cells in suspension and in an immobilized form.

Preservation of nitrilase activity with glutaraldehyde pretreatment of an enzyme catalyst is affected by time, temperature, glutaraldehyde concentration, pH and the concentration of inhibitory products like ammonia and other amines (e.g., amino acids and peptides) in the media that interact with glutaraldehyde. A preferred glutaraldehyde pretreatment method treats cells from high-density fermentation (100-150 OD_{550}) with 5 -10 wt % glutaraldehyde in water that is preferably delivered with adequate mixing at 50 mg to 500 mg glutaraldehyde /L-min, more preferably delivered with adequate mixing at 50 mg to 200 mg glutaraldehyde/L-min,

most preferably delivered with adequate mixing at 50 mg to 100 mg glutaraldehyde/L-min, resulting in a final concentration of about 3 g to about 5 g glutaraldehyde /L (about 0.025 g to about 0.042 g glutaraldehyde per OD_{550}), more preferably about 3.6 g to about 5 g glutaraldehyde /L (about 0.030 g to about 0.042 g glutaraldehyde per OD_{550}). The glutaraldehyde pretreated culture may be held in the fermenter for about 1 to 5 hours. A 10 wt % solution of sodium bisulfite in water is then optionally added at 1 g/L to inactivate the residual glutaraldehyde.

The preferred pH for the glutaraldehyde pretreatment of the enzyme catalyst in the fermentation broth or cell suspension is from pH 5.0 to 9.0, more preferably from pH 5.0 to 8.0, even more preferably from pH 5.0 to 7.0, still more preferably pH 5.0 to 6.0, and most preferably pH 5.0 to 5.5. The residual glutaraldehyde concentration after glutaraldehyde pretreatment is typically low, in the range of 10 - 200 ppm, and can be inactivated as stated above, with the addition of sodium bisulfite to a final concentration of about 1 g/L. Glutaraldehyde and bisulfite pretreatment were found to have no significant detrimental effect on the nitrilase activity. The glutaraldehyde or glutaraldehyde/ bisulfite pretreated cell suspension is optionally chilled to 5 – 10 °C, and optionally washed (by concentration and re-dilution of the cell suspension or fermentation broth) with water or an appropriate storage buffer to remove residual bisulfite and unreacted glutaraldehyde.

Immobilization of Glutaraldehyde Pretreated Enzyme Catalyst and Chemical Cross-linking

Methods for the immobilization of enzyme catalysts have been widely reported and are well known to those skilled in the art (Methods in Biotechnology, Vol. 1: Immobilization of Enzymes and Cells; Gordon F. Bickerstaff, Editor; Humana Press, Totowa, NJ, USA; 1997). The immobilization of the *A. facilis* 72W nitrilase catalyst has also been previously reported (US 6,870,038).

Further, a method for immobilization in carrageenan and subsequent glutaraldehyde/polyethylenimine cross-linking of the immobilized enzyme catalyst follows (and as disclosed in US 6,870,038, and as described in detail in US 6,551,804 B, herein incorporated by reference), however, one of ordinary skill in the art would recognize and readily apply variations to accomplish immobilization and cross-linking.

Said variations are contemplated herein and are within the scope of the instant process. Further, the amounts or concentrations of components used for immobilization and chemical cross-linking will vary depending on the amount and type of enzyme catalyst and fermentative production of enzyme catalyst. One of ordinary skill in the art would recognize these factors and adjust the immobilization and chemical cross-linking procedures accordingly. With regard to cross-linking with glutaraldehyde and polyethylenimine, US 6,551,804 (supra), describes the processes and procedures for chemically cross-linking alginate immobilized cells. Said description applies here for carrageenan immobilized cells as well.

Hydrolysis of Glycolonitrile to Glycolic Acid Using a Nitrilase Catalyst

The enzymatic conversion of glycolonitrile to glycolic acid (in the form of the acid and/or the corresponding ammonium salt) may be performed by contacting an enzyme catalyst, immobilized enzyme catalyst, or cross-linked immobilized enzyme catalyst having nitrilase activity under suitable reaction conditions as described below (i.e. in an aqueous reaction mixture at certain pH range, temperatures, concentrations, etc.). In one embodiment, whole recombinant microbial cells are immobilized in carrageenan, cross-linked, and the resulting enzyme catalyst used directly for the conversion of glycolonitrile to glycolic acid, or unimmobilized cells can be maintained separately from the bulk reaction mixture using hollow-fiber membrane cartridges or ultrafiltration membranes. In a second embodiment, whole recombinant microbial cells are immobilized in polyacrylamide gel, and the resulting enzyme catalyst used directly for the conversion of glycolonitrile to glycolic acid.

The concentration of enzyme catalyst in an aqueous reaction mixture depends on the specific activity of the enzyme catalyst and is chosen to obtain the desired rate of reaction. The wet cell weight of the microbial cells used as catalyst in hydrolysis reactions typically ranges from 0.001 grams to 0.250 grams of wet cells per mL of total reaction volume, preferably from 0.002 grams to 0.050 grams of wet cells per mL. The indicated wt % of wet cells per volume of total reaction volume may be present in the reaction mixture in the form of an immobilized enzyme catalyst prepared as previously described (supra), where the weight of wet cells as a percentage of the total weight of the immobilized enzyme catalyst is known from the method of preparation of the immobilized enzyme catalyst.

The temperature of the glycolonitrile hydrolysis reaction is chosen to control both the reaction rate and the stability of the enzyme catalyst activity. The temperature of the reaction may range from just above the freezing point of the reaction mixture (approximately 0 ºC) to about 65 ºC, with a preferred range of reaction temperature of from about 5 ºC to about 35 ºC. An enzyme catalyst suspension may be prepared by suspending the immobilized cells in distilled water, or in a aqueous solution of a buffer which will maintain the initial pH of the reaction between about 5.0 and about 10.0, preferably between about 5.5 and about 8.0, more preferably between about 5.5 and about 7.7, and most preferably about 6.0 to about 7.7. As the reaction proceeds, the pH of the reaction mixture may change due to the formation of an ammonium salt of the carboxylic acid from the corresponding nitrile functionality. The reaction can be run to complete conversion of glycolonitrile with no pH control, or a suitable acid or base can be added over the course of the reaction to maintain the desired pH.

Glycolonitrile was found to be completely miscible with water in all proportions at 25 ºC. In cases where reaction conditions are chosen such that the solubility of the substrate (*i.e.*, an α-hydroxynitrile) is also dependent on the temperature of the solution and/or the salt concentration (buffer or product glycolic acid ammonium salt, also known as ammonium glycolate) in the aqueous phase, the reaction mixture may initially be composed of two phases: an aqueous phase containing the enzyme catalyst and dissolved α-hydroxynitrile, and an organic phase (the undissolved α-hydroxynitrile). As the reaction progresses, the α-hydroxynitrile dissolves into the aqueous phase, and eventually a single phase product mixture is obtained. The reaction may also be run by adding the α-hydroxynitrile to the reaction mixture at a rate approximately equal to the enzymatic hydrolysis reaction rate, thereby maintaining a single-phase aqueous reaction mixture, and avoiding the potential problem of substrate inhibition of the enzyme at high starting material concentrations.

Glycolic acid may exist in the product mixture as a mixture of the protonated carboxylic acid and/or its corresponding ammonium salt (dependent on the pH of the product mixture; pKa of glycolic acid is about 3.83), and may additionally be present as a salt of the carboxylic acid with any buffer that may additionally be present in the product mixture. Typically, the glycolic acid produced is primarily in the form of the ammonium salt (pH of the glycolonitrile hydrolysis reaction is typically

between about 5.5 and about 7.7). The glycolic acid product may be isolated from the reaction mixture as the protonated carboxylic acid, or as a salt of the carboxylic acid, as desired.

The final concentration of glycolic acid in the product mixture at complete conversion of glycolonitrile may range from 0.001 M to the solubility limit of the glycolic acid product. In one embodiment, the concentration of glycolic acid will range from about 0.10 M to about 5.0 M. In another embodiment, the concentration of glycolic acid will range from about 0.2 M to about 3.0 M.

Glycolic acid may be recovered in the form of the acid or corresponding salt using a variety of techniques including, but not limited to ion exchange, electrodialysis, reactive solvent extraction, polymerization, thermal decomposition, alcoholysis, and combinations thereof.

Further, when an amount, concentration, or other value or parameter is given either as a range, preferred range, or a list of upper preferable values and lower preferable values, this is to be understood as specifically disclosing all ranges formed from any pair of any upper range limit or preferred value and any lower range limit or preferred value, regardless of whether ranges are separately disclosed. Where a range of numerical values is recited herein, unless otherwise stated, the range is intended to include the endpoints thereof, and all integers and fractions within the range. It is not intended that the scope of the invention be limited to the specific values recited when defining a range.

GENERAL METHODS

The following examples are provided to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus may be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for

General Bacteriology (1994) (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds.), American Society for Microbiology, Washington, DC.) or by Thomas D. Brock, in Biotechnology: A Textbook of Industrial Microbiology, (1989) Second Edition, (Sinauer Associates, Inc., Sunderland, MA). Methods to immobilize enzymatic catalysts can be found in Bickerstaff, G.F., *supra*).

Procedures required for genomic DNA preparation, PCR amplification, DNA modifications by endo- and exo-nucleases for generating desired ends for cloning of DNA, ligations, and bacterial transformation are well known in the art. Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Maniatis, *supra*; and by T. J. Silhavy, M. L. Bennan, and L. W. Enquist, Experiments with Gene Fusions, (1984) Cold Spring Harbor Laboratory Press, Cold Spring, NY; and by Ausubel, F. M. *et al.*, Current Protocols in Molecular Biology, (1994-1998) John Wiley & Sons, Inc., New York.

All reagents and materials were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma/Aldrich Chemical Company (St. Louis, MO) unless otherwise specified.

The abbreviations in the specification correspond to units of measure, techniques, properties, or compounds as follows: “sec” means second(s), “min” means minute(s), “h” or “hr” means hour(s), “d” means density in g/mL, “μL” means microliters, “mL” means milliliters, “L” means liters, “mM” means millimolar, “M” means molar, “mmol” means millimole(s), “wt” means weight, “wt%” means weight percent, “g” means grams, “μg” means micrograms, HPLC” means high performance liquid chromatography, “O.D.” means optical density at the designated wavelength, “dcw” means dry cell weight, “U” means units of nitrilase activity, “EDTA” means ethylenediaminetetraacetic acid, and “DTT” means dithiothreitol. One U of nitrilase activity corresponds to the hydrolysis of 1 μmol glycolonitrile/min.

Analytical Methodology

HPLC Analysis

Unless otherwise noted, the following HPLC method was used. The reaction product mixtures were analyzed by the following HPLC method.

Aliquots (0.01 mL) of the reaction mixture were added to 1.50 mL of water, and analyzed by HPLC (HPX 87H column, 30 cm x 7.8 mm; 0.01 N H_2SO_4 mobile phase; 1.0 mL/min flow at 50 °C; 10 µL injection volume; RI detector, 20 min analysis time). The method was calibrated for glycolonitrile at a series of concentrations using commercially available glycolonitrile purchased from Aldrich.

Quantitative ^{13}C NMR Analysis

Quantitative ^{13}C NMR spectra were obtained using a Varian Unity Inova spectrometer (Varian, Inc., Palo Alto, CA) operating at 400 MHz. Samples were prepared by taking 3.0 mL of the reaction product along with 0.5 mL of D_2O in a 10 mm NMR tube. ^{13}C NMR spectra were typically acquired using a spectral width of 26 KHz with the transmitter located at 100 ppm, 128K points, and a 90-degree pulse (pw90 = 10.7 microseconds at a transmitter power of 56 db). The longest 13C T1 (23 sec) was associated with the GLN nitrile carbon, and the total recycle time was set greater than ten times this value (recycle delay d1 = 240 sec, acquisition time at = 2.52 sec). Signal averaging of 360 scans gave a total experiment time of 26.3 hours. The Nuclear Overhauser Enhancement (NOE) was suppressed by gating on the Waltz-modulated 1H decoupling only during the acquisition time (at).

EXAMPLE 1

Fermentation of *E. coli* MG1655/pSW138-168V

Seed cultures of *E. coli* MG1655/pSW138-168V were grown in 500 mL LB media supplemented with 0.1 mg ampicillin per mL for 6-10 h (OD_{550} = 1-2) at 30 ºC with shaking (300 rpm) prior to inoculation of the fermentor. Growth of *E. coli* MG1655/pSW138-168V nitrilase strain was in 14-L Braun Biostat C fermenters (B. Braun Biotech International Gmbh, Melsungen, Germany) using mineral medium with glucose, ammonia, and salts, and lactose was used for induction. Pre-sterilization fermenter media (7.5 L) is described in Table 2. Post-sterilization additions include filter-sterilized trace elements (Table 3), 0.1 mg ampicillin per mL, 2 g casamino acids (Difco) per L, 4 g glucose per L, and 500 mL seed culture.

Fermentation set points are described in Table 4. NH_4OH (40% w/v) and H_3PO_4 (20% w/v) were used for pH control. The dissolved oxygen concentration was controlled at 25% of air saturation with the agitation set to rise first with increase in oxygen demand, with aeration to follow. The fermentation feed protocol used with lactose induction is given in Table 5.

Glucose feed rates were reduced if glucose accumulated above 5 g/L. After 40-56 h, the fermentation broth was chilled to 5-10 ºC and the cells harvested by centrifugation. Cell paste was frozen and stored at -70 °C. The cell paste was designated as NIT 60 (1910 GLN U/g dcw).

Table 2. Fermentation media, pre-sterilization.

$(NH_4)_2SO_4$	5.0 g/L
K_2HPO_4	4.0 g/L
KH_2PO_4	3.5 g/L
$MgSO_4*7H_2O$	0.6 g/L
$Na_3Citrate*2H_2O$	1.0 g/L
NZ Amine AS (Quest)	2.5 g/L
Antifoam - Biospumex 153K	0.25 ml/L

Table 3. Fermentation trace elements

	Concentration
Citric acid	10 g/L
$CaCl_2*2H_2O$	1.5 g/L
$FeSO_4*7H_2O$	5 g/L
$ZnSO_4*7H_2O$	0.39 g/L
$CuSO_4*5H_2O$	0.38 g/L
$CoCl_2*6H_2O$	0.2 g/L
$MnCl_2*4H_2O$	0.3 g/L

Table 4. Fermentation set points

	Initial Set-Point	Minimum	Maximum
Stirrer (rpm)	400	400	1000
Airflow (slpm)	2	2	10
pH	6.8	6.8	6.8
Pressure (kPa)	0.5	0.5	0.5
DO	25%	25%	25%
Temperature °C	30	30	30

Table 5. Fermentation feed protocol used with lactose induction

EFT (h)	Feed Rate (g/min)	Substrate
0	0	Glucose (batched)
5	0.27	Glucose (50% w/w)
14	1.3	Lactose (25% w/w)

EXAMPLE 2

Immobilization of *E. coli* MG1655/pNM18-168V in GA/PEI-cross-linked Carrageenan Beads

With rapid stirring, 12 g of carrageenan (FMC GP911) was slowly added to 228 g deionized distilled water at 50 °C, the resulting mixture heated to 80 °C until the carrageenan was completely dissolved, and the resulting solution cooled with stirring to 52 °C. In a separate beaker equipped with stir bar, 83.2 g of frozen *E. coli* MG1655/pNM18-168V cells (25.2 % dcw) were added to 84.8 g of 0.35 M Na_2HPO_4 (pH 7.3) at ca. 25 °C and mixed until the cells were suspended, then a deoxyribonuclease I solution (10 μL of 12,500 U/mL DNase (Sigma)/100 mL of cell suspension) was added. The cell suspension was filtered consecutively through a 230 micron and 140 micron Nupro TF strainer element filter, and heated with stirring to 50 °C. With stirring, 160.0 g of *E. coli* MG1655/pNM18-168V cell suspension at 50 °C was added to the carrageenan solution at 52 °C, and the resulting cell/carrageenan suspension was pumped through an electrically-heated 20 gauge needle at 47 ºC and dripped into 0.25 M $KHCO_3$ (pH = 7.3) with stirring at ca. 37-38 ºC); the flow rate through the needle was set at 5 - 8 mL/min. The resulting beads were allowed to harden in this same buffer for 1 h at room temperature with stirring, and were stored in 0.25 M potassium bicarbonate (pH 7.3).

Chemical cross-linking of the immobilized cell/carrageenan beads was performed by addition of 0.5 g of 25 % glutaraldehyde (GA) in water (Sigma M 752-07) to 20 g beads suspended in 48 mL of 0.25 M potassium bicarbonate (pH 7.3), and stirring for 1 h at room temperature. To the suspension of beads was then added 2.0 g of 12.5 wt % polyethylenimine (PEI, BASF LUPASOL PS) in water, and the bead suspension stirred for an additional 18 h at room temperature. The GA/PEI-cross-linked beads were recovered from the suspension, stirred twice for 15 min in 48 mL of 0.25 M potassium bicarbonate (pH 7.3), then stored in 1.0 M ammonium bicarbonate (pH 7.3) at 5 °C. Prior to use as catalyst for conversion of glycolonitrile to glycolic acid (as the ammonium salt), the beads were washed twice for 15 min with 180 mL of 0.1 M ammonium glycolate (pH 7.3) at room temperature to remove the 1.0 M ammonium bicarbonate (pH 7.3) storage buffer. The resulting immobilized cell catalyst was identified as immobilized NIT 60.

EXAMPLE 3:

Pretreatment of *E. coli* MG1655/pSW138-168V with Glutaraldehyde Prior to Immobilization

A 200-L fermentation was performed to produce a broth containing *E. coli* MG1655/pSW138-168V cells that were subsequently pretreated with glutaraldehyde in-situ prior to immobilization. A pre-seed culture was first prepared by charging a 2-L shake flask with 0.5 L seed medium containing yeast extract (Ambrex 695, 5.0 g/L), K_2HPO_4 (10.0 g/L), KH_2PO_4 (7.0 g/L), sodium citrate dihydrate (1.0 g/L), $(NH_4)_2SO_4$ (4.0 g/L), $MgSO_4$ heptahydrate (1.0 g/L) and ferric ammonium citrate (0.10 g/L). The pH of the medium was adjusted to 6.8 and the medium was sterilized in the flask. Post sterilization additions included glucose (10 mL, 50 wt %) and 1 mL ampicillin (25 mg/mL). The pre-seed medium was inoculated with a 1-mL frozen stock culture of *E. coli* MG1655/pSW138-168V in 20% glycerol, and cultivated at 35 °C and 300 rpm. The seed culture was transferred at ca. 2 OD_{550} to a 14L seed fermentor (Braun) with 8 L of medium containing KH_2PO_4 (3.50 g/L), $FeSO_4$ heptahydrate (0.05 g/L), $MgSO_4$ heptahydrate (2.0 g/L), sodium citrate dihydrate (1.90 g/L), yeast extract (Ambrex 695, 5.0 g/L), Biospumex153K antifoam (0.25 mL/L, Cognis Corporation), NaCl (1.0 g/L), $CaCl_2$ dihydrate (10 g/L), and NIT trace elements solution (10 mL/L). The trace elements solution contained citric acid monohydrate (10 g/L), $MnSO_4$ hydrate (2 g/L), NaCl (2 g/L), $FeSO_4$ heptahydrate (0.5 g/L), $ZnSO_4$ heptahydrate (0.2 g/L), $CuSO_4$ pentahydrate (0.02 g/L) and $NaMoO_4$ dihydrate (0.02 g/L). Post sterilization additions included 120 g glucose solution (50% w/w) and ampicillin 16 mL stock solution (25 mg/mL).

The dissolved oxygen (dO) concentration was controlled at 25% of air saturation. The dO was controlled first by impeller agitation rate (400 to 1400 rpm) and later by aeration rate (2 to 10 slpm). The pH was controlled at 6.8. NH_4OH (29% w/w) and H_2SO_4 (20% w/v) were used for pH control. The temperature was controlled at 35 ^oC and the head pressure was 0.5 bars. At ca 6 OD_{550} the culture was transferred to the 200L Biostat-D Braun fermenter. The medium used was the same as in the seed fermenter, the initial working volume was 140 L and 50% w/w glucose was charged to 8 g/L. The fermentation started as a batch operation, and once the glucose was depleted (<0.5 g/L) a fed batch operation with 50% w/w glucose was initiated with a predetermined rate (Table 6), at ca 25 OD_{550}

the feed was switched to 25% D-lactose solution with a pre-determined rate (Table 7).

The temperature was controlled at 35.0 °C, the head pressure at 0.5 bar, the pH at 1st stage (glucose phase) at 6.8 and at the 2nd stage (lactose phase) at 7.2, NH_4OH (29% w/w) and H_2SO_4 (20% w/v) were used for pH control, the dO controlled at 1st stage at 25% of air saturation and 2nd stage at 10%, the dO was controlled by agitation first (250-450 rpm) and later by aeration (25-35 slpm). Glucose and lactose levels were monitored during the fed operation and if the levels of glucose exceeds 0.1 g/L or lactose above 1 g/L the feed program was either temporarily halted or reduced. The run was ended 40 h after the initiation of lactose feed, and cells were either harvested by centrifugation or microfiltration or kept in the vessel for treatment with glutaraldehyde. The fermentation produced about 8 kg dry cell weight with a nitrilase specific activity of 2819 BZN U/g dcw (1788 GLN U/g dcw).

Table 7: Feed protocol

Feed time intervals (h)	Feed rate g/min	Substrate	Stage
0	6.13	50% w/w glucose	1st
1	7.13	50% w/w glucose	1st
2	8.28	50% w/w glucose	1st
3	9.62	50% w/w glucose	1st
4	11.18	50% w/w glucose	1st
5	11.18	50% w/w glucose	1st
6	11.18	50% w/w glucose	1st
7	11.18	50% w/w glucose	1st
8	11.18	50% w/w glucose	1st
0	11.22	25% w/w lactose	2nd
2	24.42	25% w/w lactose	2nd
20	16.72	25% w/w lactose	2nd
30	18.7	25% w/w lactose	2nd
40	18.7	25% w/w lactose	2nd

At the end of the fermentation, the agitation was reduced to 150 rpm, the aeration stopped and the temperature maintained at 35 °C. Part of the fermentation broth was withdrawn, leaving ca. 180 kg in the fermenter. This remaining broth was titrated to pH 5.2 and maintained at

this pH with 20% H_2SO_4 (20% w/w) and NaOH (50% w/w) while 9.0 L of aqueous glutaraldehyde (GA, 10% w/w) was added with stirring at a rate of ~90 mL/min; this rate of addition was equivalent to 50 mg glutaraldehyde/L fermentation broth/min, and the final concentration of glutaraldehyde was ca. 5 g glutaraldehyde/L (0.035 g glutaraldehyde/ OD_{550}). After 5 h from initiation of glutaraldehyde addition to the broth, the pH was adjusted to 7.0, and 1.8 L of aqueous sodium bisulfite (10% w/w, pH 7) was added (ca. 1 g sodium bisulfite/L final concentration) with stirring, and the broth stirred for an additional 15 min. The temperature of the broth was then decreased to 10 °C, and the agitation decreased to 100 rpm. The broth was concentrated to 40 kg of cell suspension using a Diskstack centrifuge (Alfa Laval), then 50 kg DI water (20 °C) was added to the suspension and the mixture was concentrated by centrifugation to produce 40 kg of washed cell suspension. The suspension (identified as NIT 188A-C2) was stored at 5 °C, and a portion of the cell suspension was used directly for the preparation of an immobilized cell catalyst (Example 6). Nitrilase specific activity during each process step is summarized in Table 8.

Table 8: Nitrilase activity during different stages of GA and bisulfite treatment

fermentation stage	BZN U/g dcw
pre GA treatment	2819
post GA	3300
post NaHSO3	2493

EXAMPLE 4

Immobilization of Glutaraldehyde Pretreated *E. coli* MG1655/pNM18-168V in GA/PEI-cross-linked Carrageenan Beads

The final cell suspension concentrate recovered from the glutaraldehyde and sodium bisulfite-treated fermentation broth of Example 5 was centrifuged at 5 °C. The resulting cell pellet was re-suspended in a 5-fold by weight amount of 0.35 M potassium phosphate buffer (pH 7.2), and centrifugation of the resulting cell suspension at 5 °C produced a wet cell paste that was immobilized and chemically cross-linked with GA and PEI as described in Example 2. The resulting immobilized cell catalyst was identified as immobilized NIT 188A-C2.

EXAMPLE 5

Improvement in Biocatalyst Specific Activity in Consecutive Batch Reactions with Catalyst Recycle using Glutaraldehyde/polyethylenimine Cross-linked Carrageenan-immobilized *E. coli* MG1655/pSW138-F168V Transformant

In a typical procedure, duplicate sets of batch reactions for the conversion of glycolonitrile to glycolic acid were run in 50-mL jacketed reaction vessels equipped with overhead stirring and temperature control. Each reactor was charged with 8 g of GA/PEI-cross-linked *E. coli* MG1655/pSW138-168V/carrageenan beads (prepared using the process as described in Example 1 (no GA pretreatment prior to immobilization) or Example 4 (GA pretreatment prior to immobilization)) containing 5 % (dcw) transformant expressing the *A. facilis* 72W nitrilase mutant F168V (SEQ ID NO: 51). To the vessel was then added 14.78 mL of distilled water and 6.0 mL of aqueous ammonium glycolate (4.0 M, pH 7.0), and the reaction vessel flushed with nitrogen. The mixture was stirred at 25 °C while programmable syringe pumps were used to simultaneously add 1.07 mL of 60 wt % glycolonitrile (GLN) in water (12.0 mmol GLN, 0.084 mmol formaldehyde; Fluka (redistilled, stabilized with 0.5 wt% glycolic acid)) and 0.150 mL of aqueous ammonium hydroxide (1.875 wt%); one equivalent volume of GLN and ammonium hydroxide solutions was added simultaneously every 2 h (for a total of eight equivalent additions each of GLN solution and aqueous ammonium hydroxide) to maintain the concentration of GLN at $\leq$ 400 mM and the pH within a range of 6.5 – 7.5. Four 0.050-mL reaction samples were removed at pre-determined times after the first GLN addition and analyzed by HPLC to determine the initial reaction rate and the catalyst specific activity (μmol glycolic acid/min/g dcw biocatalyst). At completion of the reaction, there was 100% conversion of GLN to produce glycolic acid (as the ammonium salt) in > 99 % yield.

At the end of the first reaction, the aqueous product mixture was decanted from the catalyst (under nitrogen), leaving ca. 10.3 g of a mixture of immobilized cell catalyst (8.0 g) and remaining product mixture (ca. 2.3 g). To the reaction vessel then added 20.78 mL of distilled, deionized water, and a second reaction was performed at 25 ºC by the addition of aliquots of aqueous GLN and ammonium hydroxide as described immediately above. The specific activities of recovered biocatalyst in consecutive batch reactions with catalyst recycle are listed in Table 7.

Table 7. Dependence of recovered biocatalyst specific activity in consecutive batch reactions with catalyst recycle on glutaraldehyde pretreatment of cells prior to immobilization.

		biocatalyst specific activity (GLN U/g dcw) in consecutive batch reactions				
immobilized cell biocatalyst	glutaraldehyde pretreatment of cells	reaction 1	reaction 2	reaction 3	reaction 4	decrease in specific activity, rxn1 to rxn4 (%)
NIT 188C2	yes	1826	1518	1596	1759	4
NIT 188C2	yes	1857	1656	1581	1947	0
NIT 027	no	1312	872	898	816	38
NIT 027	no	1416	856	931	621	56
NIT 49	no	1694	702	718	886	48
NIT 49	no	1665	868	869	946	43
NIT 60-A	no	1319	730	744	563	57
NIT 60-A	no	1410	750	725	699	50
NIT 60-B	no	1222	757	857	802	34
NIT 60-B	no	1335	722	839	808	39

EXAMPLE 6

Storage Stability of Glutaraldehyde/polyethylenimine Cross-linked Carrageenan-immobilized *E. coli* MG1655/pSW138-F168V Prepared using Glutaraldehyde Pretreated Cells.

Freshly-prepared glutaraldehyde/polyethylenimine cross-linked carrageenan-immobilized *E. coli* MG1655/pSW138-F168V prepared using glutaraldehyde pretreated cells (as described in Example 4) were stored for 32 days in 1.0 M ammonium bicarbonate (pH 7.3) at 5 °C. Prior to use, the beads were washed twice for 15 min with 180 mL of 0.1 M ammonium glycolate (pH 7.0) at room temperature. Biocatalyst stored for either 4 days or 32 days at 5 °C were each evaluated in duplicate sets of consecutive batch reactions with biocatalyst recycle using the procedure described in Example 5 (Table 8).

Table 8. Specific activity of biocatalyst prepared using glutaraldehyde pretreatment of cells prior to immobilization in consecutive batch reactions with catalyst recycle, using biocatalyst stored at 5 °C for 4 or 32 days.

		biocatalyst specific activity (GLN U/g dcw) in consecutive batch reactions				
immobilized cell biocatalyst	days stored at 5 °C	reaction 1	reaction 2	reaction 3	reaction 4	decrease in specific activity, rxn1 to rxn4 (%)
NIT 188C2	4	1826	1518	1596	1759	4
NIT 188C2	4	1857	1656	1581	1947	0
NIT 188C2	32	1910	1455	1580	1543	19
NIT 188C2	32	1987	1434	1472	1783	10

CLAIMS

What is claimed is:

1. A process for improving the retention of the initial specific activity of an enzyme catalyst having nitrilase activity during the conversion of glycolonitrile to glycolic acid, said process comprising:

(a) producing an enzyme catalyst having nitrilase activity by fermentation;

(b) pretreating said enzyme catalyst with glutaraldehyde;

(c) optionally inactivating unreacted glutaraldehyde with bisulfite following glutaraldehyde pretreatment;

(d) recovering the enzyme catalyst from (b) or (c) and immobilizing said enzyme catalyst in carrageenan; and

(e) cross-linking the resulting carrageenan-immobilized enzyme catalyst of (d) with glutaraldehyde and polyethylenimine, whereby a glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst is produced and wherein said glutaraldehyde-pretreated immobilized and cross-linked enzyme catalyst has improved retention of initial specific activity during conversion of glycolonitrile to glycolic acid as compared to non-glutaraldehyde-pretreated immobilized and cross-linked enzyme catalysts under the same reaction conditions.

2. The process of claim 1 wherein the enzyme catalyst of (a) comprises the amino acid sequence signature motif SEQ ID NO: 1 or SEQ ID NO: 2.

3. The process of Claim 2 wherein said enzyme catalyst comprises a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, and 57.

4. The process of claim 3 wherein said enzyme catalyst is *Acidovorax facilis* 72W.

5. The process of claim 1 wherein the pH is maintained between 5.0 and 9.0 during pretreatment with glutaraldehyde.

6. The process of claim 1 wherein the pretreating with glutaraldehyde in step (b) comprises adding glutaraldehyde to a fermentation broth produced by step (a) in an amount in the range of about 3 g/L (0.025 g GA per OD_{550}) and about 5 g/L (0.042 g GA per OD_{550}).

7. The process of claim 1 wherein the pretreating with glutaraldehyde in step (b) comprises adding glutaraldehyde to a fermentation broth produced by step (a) at a rate of 50 mg/L/h to 500 mg/L/h.

8. The glutaraldehyde pretreated, immobilized and cross-linked enzyme catalyst produced by the process of claim 1.

9. The enzyme catalyst of claim 8, wherein said catalyst retains at least about 70% of its initial specific activity after at least four consecutive batch recycle reactions.

10. An improved process for hydrolyzing glycolonitrile to glycolic acid comprising improving the retention of the initial specific activity of an immobilized enzyme catalyst having nitrilase activity during conversion of glycolonitrile to glycolic acid, said process comprising:

(a) producing an enzyme catalyst having nitrilase activity by fermentation;

(b) pretreating said enzyme catalyst with glutaraldehyde;

(c) optionally inactivating unreacted glutaraldehyde with bisulfite following glutaraldehyde pretreatment;

(d) recovering the enzyme catalyst from (b) or (c) and immobilizing said enzyme catalyst in carrageenan;

(e) cross-linking the resulting carrageenan-immobilized enzyme catalyst of (d) with glutaraldehyde and polyethylenimine, whereby a cross-linked immobilized enzyme catalyst is produced; and

(f) contacting the cross-linked immobilized enzyme catalyst of (e) with glycolontrile in an aqueous solution under suitable reaction conditions whereby glycolic acid is produced.

11. The process of claim 10 further comprising step (g) recovering the glycolic acid produced in step (f).

12. The process of claim 10 wherein said cross-linked immobilized enzyme catalyst retains at least about 70% of its initial specific activity after the production of at least 40 g of glycolic acid per gram dry cell weight of immobilized enzyme catalyst, and wherein said process has improved productivity for glycolic acid production as compared to a process for producing glycolic acid with a cross-linked immobilized enzyme catalyst comprising an enzyme catalyst having nitrilase activity not pretreated with glutaraldehyde.

```
CLUSTAL W (1.83) multiple sequence alignment


SEQ ID NO:4      ----------MVSYNSKFLAATVQAEPVWLDADATIDKSIGIIEEAAQKGASLIAFPEVF
SEQ ID NO:5      -----------MQTRKIVRAAAVQAASPNYDLATGVDKTIELARQARDEGCDLIVFGETW
SEQ ID NO:6      ----------MVEYTNTFKVAAVQAQPVWFDAAKTVDKTVSIIAEAARNGCELVAFPEVF
SEQ ID NO:7      -----MSSNPELKYTGKVKVATVQAEPVILDADATIDKAIGFIEEAAKNGAEFLAFPEVW
SEQ ID NO:8      ------------------MKVATVQAEPVILDADATIDKAIGYIEEASKNGAEFIAFPEVW
SEQ ID NO:9      --------------MTTHRIAVIQDGPVPGDAMATAEKMSRLAASAKAQGARLALFPEAF
SEQ ID NO:10     MSTSENTPFNGVASSTIVRATIVQASTVYNDTPATLEKANKFIVEAASKGSELVVFPEAF
SEQ ID NO:11     -------------MADKIIVAAAQIRPVLFSLEGSVARVLAAMAEAAAAGVQLIVFPETF
SEQ ID NO:12     -------------MADKIIVAAAQIRPVLFSLEGSVARVLAAMAEAAAAGVQLIVFPETF
SEQ ID NO:13     -----MLGKIMLNYTKNIRAAAAQISPVLFSQQGTMEKVLDAIANAAKKGVELIVFPETF
SEQ ID NO:14     --------------MPKSIVAALQVGSLPEGKAATLEQILGYEQAIREAGARLVVMPEAL
SEQ ID NO:15     --------------MSCHRVAVIQAGTSLFDTEKTLDRMEALCRQAAEQNVELAVFPEAY
SEQ ID NO:16     -----------MSNYPKYRVAAVQASPVLLDLDATIDKTCRLVDEAAANGAKVIAFPEAF
SEQ ID NO:17     -----------MKNYPTVKVAAVQAAPVFMNLEATVDKTCKLIAEAASMGAKVIGFPEAF
SEQ ID NO:18     ---------------MTTVKVAAAQIRPVLFSLDGSLQKVLDAMAEAAAQGVELIVFPETF
SEQ ID NO:19     --------------MPKSVVAALQIGALPEGKAATLEQILSYEAAIIEAGAQLVVMPEAL
SEQ ID NO:20     -----------MSQRDSFRAAAVQAAPVWLDGAATVDKCVALIEEAADNGAALIAFPETF
SEQ ID NO:21     -----------MQTRKIVRAAAVQAASPNYDLATGVDKTIELARQARDEGCDLIVFGETW
SEQ ID NO:22     -------------MKEPLKVACVQAAPVFLDLDATVDKTITLMEQAAAAGAGLIAFPETW
SEQ ID NO:23     ------------LAHPKYKVAVVQAAPAWLDLDASIKKTIALIEEAADKGAKLIAFPEVF
SEQ ID NO:24     ----------MVEYTNTFKVAAVQAQPVWFDAAKTVDKTVSIIAEAARNGCELVAFPEVF
SEQ ID NO:25     ----------MVEYTNTFKVAAVQAQPVWFDAAKTVDKTVSIIAEAARNGCELVAFPEVF
                                    :  *  .   .      :           .  .  : *.
```

FIGURE 1A

Replacement Figures
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```
SEQ ID NO:4   IPGYP----YWAWLGDVKYSLS--FTSRYHENSLELGDDRMRRLQLAARRNKIALVMGYS
SEQ ID NO:5   LPGYP----FHVWLGAPAWSLK--YSARYYANSLSLDSAEFQRIAQAARTLGIFIALGYS
SEQ ID NO:6   IPGYP----YHIWVDSPLAGMAK-FAVRYHENSLTMDSPHVQRLLDAARDHNIAVVVGIS
SEQ ID NO:7   IPGYP----YWAWIGDVKWAVSD-FIPKYHENSLTLGDDRMRRLQLAARQNNIALVMGYS
SEQ ID NO:8   IPGYP----YWAWIGDVKWAVSE-FIPKYHENSLTLGDDRMRRLQLAARQHNIAMVVGYS
SEQ ID NO:9   VGGYPKGADFHIFLGGRTPQGRA-QYQRYAETAIAVPGPVTERIGQIAAEQDMFIVVGVI
SEQ ID NO:10  IGGYPRGFRFGLGVGVHNEEGRD-EFRKYHASAIKVPGPEVEKLAELAGKNNVYLVMGAI
SEQ ID NO:11  LPYYP----YFSFVEPPVLMGRS--ILKLYEQATTMTGPELQQIARAARQIRLFVLLGVN
SEQ ID NO:12  LPYYP----YFSFVEPPVLMGRS--HLKLYEQAFTMTGPELQQIARAARQHRLFVLLGVN
SEQ ID NO:13  VPYYP----YFSFVEPPVLMGKS--HLKLYQEAVTVPGKVTQAIAQAAKTHGMVVVLGVN
SEQ ID NO:14  LGGYPKGEGFGTQLGYRLPEGRE-AFARYFANAIDVPGSETAALAGLSARTGASLVLGVI
SEQ ID NO:15  IGGYPKGLDFGARMGTRTEAGRE-DFLRYWKAAIDVPGKETARIGSFAAKMKAYLVVGVI
SEQ ID NO:16  IPGYP----WWIWLGNADYGMK--YYIQLYKNSVEIPSLAVQKLSSAG-TNKVYFCVSVT
SEQ ID NO:17  IPGYP----YWIWTSNMDFTGM--MWAVLFKNAIEIPSKEVQQISDAAKKNGVYVCVSVS
SEQ ID NO:18  LPYYP----YFSFVEPPVLMGRS--HLALYEQAVVVPGPVTDAVAAAASQYGMQVLLGVN
SEQ ID NO:19  LGGYPKGEGFGTQLGYRLPEGRE-AFARYFANAIEVPGVETDALAALSARTGANLVLGVI
SEQ ID NO:20  VPGYP----WWLWLDSPAWGMQ--FVARYFDNSLALDGPLFARLREAARRSAITVVTGHS
SEQ ID NO:21  LPGYP----FHVWLGAPAWSLK--YSARYYANSLSLDSAEFQRIAQAARTLGIFIALGYS
SEQ ID NO:22  IPGYP----WFLWLDAPAWNMP--LVQRYHQQSLVLDSVQARRISDAARHLGLYVVLGYS
SEQ ID NO:23  IPGYP----WHIWMDSPAWCIGRGFVQRYFDNSLAYDSPQAEALRAAVRKAQLTAVLGLS
SEQ ID NO:24  IPGYP----YHIWVDSPLAGMAK-FAVRYHENSLTMDSPHVQRLLDAARDHNIAVVVGIS
SEQ ID NO:25  IPGYP----YHIWVDSPLAGMAK-FAVRYHENSLTMDSPHVQRLLDAARDHNIAVVVGIS
              :  **    :                :.  .    :          .
```

FIGURE 1B

Replacement Figures
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```
SEQ ID NO:4   EREAGSRYLSQVFIDERGEIVANRRKLKPTHVERTIYGEGNGTDFLTHDFA-FGRVGGLN
SEQ ID NO:5   ERSGGSLYLGQCLIDDKGQMLWSRRKLKPTHVERTVFGEGYARDLIVSDTE-LGRVGALC
SEQ ID NO:6   ERDGGSLYMTQLVIDADGQLVARRRKLKPTHVERSVYGEGNGSDISVYDMP-FARLGALN
SEQ ID NO:7   EKDGASRYLSQVFIDQNGDIVANRRKLKPTHVERTIYGEGNGTDFLTHDFG-FGRVGGLN
SEQ ID NO:8   EKDGASRYLSQVFIDQNGDIVANRRKLKPTHVERTIYGEGNGTDFLTHDFG-FGRVGGLN
SEQ ID NO:9   ERDGGTLYCTILFSPEGELLGKHRKLMPTALERLLWGYGDGSTFPVYDTP-LGKLGAVV
SEQ ID NO:10  EKDGYTLYCTALFTSPQGFLGKIRKLMPTSLERCIWGQGDGSTIPVYDTP-IGKLGAAI
SEQ ID NO:11  ERDGGSLYNTQLLISDQGDLLLKRRKITPTYHERMVWGQGGGAGLTVVETV-LGKVGALA
SEQ ID NO:12  ERDGGSLYNTQLLISDQGDLLLKRRKITPTYHERMVWGQGGGAGLTVVETV-LGKVGALA
SEQ ID NO:13  EREEGSLYNTQLIFDADGALVLKRRKITPTYHERMVWGQGDGAGLRTVDTT-VGRLGALA
SEQ ID NO:14  ERSGNTLYCTVLFFEPEGGLVAKHRKLMPTGTERLIWGKGDGSTLPVVDGR-AGRIGAAV
SEQ ID NO:15  ERSEATLYCTALFFAPDGTLIGKHRKLMPTATERLVWGQGDGSTIEILDTA-VGKLGAAI
SEQ ID NO:16  EKDGGSLYLTQLWFDPNGDLIGKHRKLKATNAEKTIWGDGDGSMMPVFETE-FGNLGGLQ
SEQ ID NO:17  EKDNASLYLTQLWFDPNGNLIGKHRKFKPTSSERAVWGDGDGSMAPVFKTE-YGNLGGLQ
SEQ ID NO:18  ERDGGTLYNTQLLFNSCGELVLKRRKITPTYHERMVWGQGDGSGLKVVQTP-LARVGALA
SEQ ID NO:19  ERSGSTLYCTALYFDPQQGLSGKHRKLMPTGTERLIWGKGDGSTLPVLDTQ-VGRVGAVI
SEQ ID NO:20  ERDGGSLYMGQAIIGADGEVLAARRKLKPTHVERTVFGESDGSNLTVVDTE-LGRLGALC
SEQ ID NO:21  ERSGGSLYLGQCLIDDKGEMLWSRRKLKPTHVERTVFGEGYARDLIVSDTE-LGRVGALC
SEQ ID NO:22  ERNKASLYIGQWIIDDHGETVGVRRKLKATHVERTMFGEGDGASLRTFETP-VGVLGALC
SEQ ID NO:23  ERDGGSLYIAQWLIGADGETIAKRRKLRPTHAERTVYGEGDGSDLAVHERPDIGRIGALC
SEQ ID NO:24  ERDGGSLYMTQLIIDADGQLVARRRKLKPTHVERSVYGEGNGSDISVYDMP-FARLGALN
SEQ ID NO:25  ERDGGSLYMTQLIIDADGQLVARRRKLKPTHVERSVYGEGNGSDISVYDMP-FARLGALN
              *:.  : *    :          :**: .*  *: ::* . .    .    . :*.
```

FIGURE 1C

Replacement Figures
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```
SEQ ID NO:4   CWEHTQPLSKFMMYSLGEQVHVASWPAMSPLQPDVFQLSIEANATV----------TRSYAIE
SEQ ID NO:5   CWEHLSPLSKYALYSQHEAIHIAAWPSFSLYSEQAHALSAKVNMAA----------SQIYSVE
SEQ ID NO:6   CWEHTQTLTKYAMYSMHEQVHVASWPGMSLYQPEVPAFGVDAQLTA----------TRMYALE
SEQ ID NO:7   CWEHTQPLSKYMMYSLNEQIHVASWPAMFALTPDVHQLSVEANDTV----------TRSYAIE
SEQ ID NO:8   CWEHTQPLSKYMMYSLNEQIHVASWPAMFALTPDVHQLSVEANDTV----------TRSYAIE
SEQ ID NO:9   CWENYMPLLRMAMYGKQIQIYCAPTADDKPTWVSTMQ----------------------IIVALE
SEQ ID NO:10  CWENRMPLYRTALYAKGIELYCAPTADGSKEWQSSML----------------------HIAIE
SEQ ID NO:11  CWEHYNELARFSLMTQGEEIHCAQFPGSLVGPIFSEQTAVT----------LRHHALE
SEQ ID NO:12  CWEHYNELARFSLMTQGEEIHCAQFPGSLVGPIFSEQTAVT----------LRHHALE
SEQ ID NO:13  CWEHYNPLARYALMAQHEQIHCGQFPGSMVGQIFADQMEVT----------MRHHALE
SEQ ID NO:14  CWENYMPLLRTAMYAKGVQLWCAPTVDERELWQVSMR----------HVAAE
SEQ ID NO:15  CWENYMPYLRQVMYAGGVNIWCAPTVDQREIWQVSMR----------HIAYE
SEQ ID NO:16  CWEHTLPLNVAAMASMNEQVHVASWPIGMPQ--EGHLFGPEQCVTA----------TKYYAIS
SEQ ID NO:17  CWEHALPLNIAAMGSLNEQVHVASWPAFVPKGAVSSRVSSSVCASTNAMHQIISQFYAIS
SEQ ID NO:18  CWEHYNPLARYALMAQGEEIHCAQFPGSLVGPIFTEQTAVT----------MRHHALE
SEQ ID NO:19  CWENMMPLLRTAMYAQGIEVWCAPTVDEREMWQVSMR----------HIAHE
SEQ ID NO:20  CWEHLQPLTKYAMYSQHEQIHVAAWPSFSVYRGAAYALGPEVNTGA----------ARQYAVE
SEQ ID NO:21  CWEHLSPLSKYALYSQHEAIHIAAWPSFSLYSEQAHALSAKVNMAA----------SQIYSVE
SEQ ID NO:22  CWEHLQPLSKYAMYAQNEQIHVAAWPSFSLYRNATSALGPEVNTAA----------SRVYAAE
SEQ ID NO:23  CWEHLQPLSKYAMYAQNEQVHVAAWPSFSLYDPFAPALGAEVNNAA----------SRVYAVE
SEQ ID NO:24  CWEHTQTLTKYAMYSMHEQVHVASWPGMSLYQPEVPAFGVDAQLTA----------TRMYALE
SEQ ID NO:25  CWEHTQTLTKYAMYSMHEQVHVASWPGMSLYQPEVPAFGVDAQLTA----------TRMYALE
              ****:  ::  ::    :  .                                        : .
```

FIGURE 1D

Replacement Figures
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```
SEQ ID NO:4   GQTFVLCSTQVIG----PSAIETFCLNDE--QRALLPQGCGWARIYGPDGSELAKPLAED
SEQ ID NO:5   GQCFTIAASSVVT----QETLDMLEVGEH--NASLLKVGGGSSMIFAPDGRTLAPYLPHD
SEQ ID NO:6   GQTFVVCTTQVVT----PEAHEFFCDNDE--QRKLIGRGGGFARIIGPDGRDLATPLAED
SEQ ID NO:7   GQTFVLASTHVIG----KATQDLFAGDDDA-KRALLPLGQGWARIYGPDGKSLAEPLPED
SEQ ID NO:8   GQTFVLAATHVIG----KATQDLFAGDDEA-KRALLPLGQGWARIYGPDGKSLAEPLAEN
SEQ ID NO:9   GRCFVLSACQHLRGKDFPPEFHNALDVQP-----DTVLMRGGSCIVDPMGQLLAGPVY-D
SEQ ID NO:10  GGCFVLSACQFCLRKDFPDHPDYLFTDWYDDKEPDSIVSQGGSVIISPLGQVLAGPNF-E
SEQ ID NO:11  AGCFVLSSTAWLD----PADYDTITPDRS---LHKAFQGGCHTAIISPEGRYLAGPLP-E
SEQ ID NO:12  AGCFVLSSTAWLD----PADYDTITPDRS---LHKAFQGGCHTAIISPEGRYLAGPLP-E
SEQ ID NO:13  SGCFVINATGWLT----AEQKLQITTDEK---MHQALSGGCYTAIISPEGKHLCEPIA-E
SEQ ID NO:14  GRCFVISACQVQ---DSPAALGMEVANWPA----ERPLINGGSLIVGPLGDVLAGPLL-G
SEQ ID NO:15  GRLFVLSACQYMTRADAPADYDCIQGNDP-----ETELIAGGSVIIDPMGNILAGPLY-G
SEQ ID NO:16  NQVFCLLSSQIWT----EEQRDKICETEE--QRNFMKVGHGFSKIIAPNGMEIGNKLAHD
SEQ ID NO:17  NQVYVIMSTNLVG----QDMIDMIGKDEF--SKNFLPLGSGNTAIISNTG-EILASIPQD
SEQ ID NO:18  AGCFVICSTGWLH-----PDDYASITSESG---LHKAFQGGCHTAVISPEGRYLAGPLP-D
SEQ ID NO:19  GRCFVVSACQVQ---ASPEELGLEIANWPA----QRPLIAGGSVIVGPMGDVLAGPLV-G
SEQ ID NO:20  GQCFVLSPCAVID----EAGVELFCDTPA--KRELLLPGGGFAQIYGPDGRELGTALPET
SEQ ID NO:21  GQCFTIAASSVVT----QETLDMLEVGEH--NAPLLKVGGGSSMIFAPDGRTLAPYLPHD
SEQ ID NO:22  GQCFVLAPCAIVS----PEMIEMLCDSDA--KRSLLQAGGGHARIFGPDGSDLATPLGEH
SEQ ID NO:23  GSCFVLAPCATVS----QAMIDELCDRPD--KHALLHAGGGHAAIFGPDGSALAAQLPPD
SEQ ID NO:24  GQTFVVCTTQVVT----PEAHEFFCENEE--QRKLIGRGGGFARIIGPDGRDLATPLAED
SEQ ID NO:25  GQTFVVCTTQVVT----PEAHEFFCENEE--QRMLIGRGGGFARIIGPDGRDLATPLAED
                 : :: .                                  : ::   *  :
```

FIGURE 1E

Replacement Figures
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```
SEQ ID NO:4   AEGILYAEIDLEQILLAKAGADPVGHYSRPDVLSVQFDPRNHTPVHRIGIDGRLDVNTRS
SEQ ID NO:5   AEGLIIADLNMEEIAFAKAINDPVGHYSKPEATRLVLDLGHREPMTRVHSK---SVIQEE
SEQ ID NO:6   EEGILYADIDLSAITLAKQAADPVGHYSRPDVLSLNFNQRHTTPVN----------TAISTI
SEQ ID NO:7   AEGLLYAELDLEQIILAKAAADPAGHYSRPDVLSLKIDTRNHTPVQYITADGRTSLNSNS
SEQ ID NO:8   AEGLLYAELDLEQIIVAKAAADPAGHYSRPDVLSLKVDTRNHTPVQYVTEDGGSSLNSNS
SEQ ID NO:9   EDAILVADIDLDAVTRGKMDFDVVGHYARPDIFSLTVDERPKPPVTTL----------K
SEQ ID NO:10  SEGLITADLDLGDVARAKLYFDSVGHYSRPDVLHLTVNEHPKKPVTFI----------S
SEQ ID NO:11  GEGLAIAELDKSLITKRKRMMDSVGHYSRPDLLSLRINRSPATQVQAIG----------S
SEQ ID NO:12  GEGLAIAELDKSLITKRKRMMDSVGHYSRPDLLSLRINRSPATQVQAIG----------S
SEQ ID NO:13  GEGLAIADLDFSLIAKRKRMMDSVGHYARPDLLQLTLNNQPWSALEAN----------P
SEQ ID NO:14  ARGLVCAEVDTDELVRARYDFDVVGHYARPDVFELSVDERPRPGVR----------F
SEQ ID NO:15  QEGVLVADIDLSDTIKARYDLDVSGHYGRPDIFEIKVDRQSHQVITDQ----------F
SEQ ID NO:16  EEGITYADIDLEQIIPGKFLIDSAGHYSTPGFLSLSFDRTEKKPIKHIG----------E
SEQ ID NO:17  AEGIAVAEIDLNQIIYGKWLLDPAGHYSTPGFLSLTFDQSEHVPVKKIG----------E
SEQ ID NO:18  GEGLAIADLDLALITKRKRMMDSVGHYSRPELLSLQINSSPAVPVQNM----------S
SEQ ID NO:19  RAGLISAQIDTADLVRARYDYDVVGHYARPDVFELTVDQRPRPGVR----------F
SEQ ID NO:20  EEGLVYADLEASAVAVAKSAADPVGHYSRPDVLQLLWDP---RPRSVVR----------Q
SEQ ID NO:21  AEGLIIADLNMEEIAFAKAINDPVGHYSKPEATRLVLDLGHRDPMTRVHSK---SVTREE
SEQ ID NO:22  EEGLLYATLDPAALTLAKVAADPAGHYSRPDVTRLMFNP---NPTPCVV----------D
SEQ ID NO:23  QEGLLIAEIDLGMIGIAKNAADPAGHYSRPDVTRLLLNK---KPLNRVE----------H
SEQ ID NO:24  EEGILYADIDLSAITLAKQAADPVGHYSRPDVLSLNFNQRRTTPVN----------TPLSTI
SEQ ID NO:25  EEGILYADIDLSAITLAKQAADPVGHYSRPDVLSLNFNQRRTTPVN----------TPLSTI
                .:  * ::      :   *  ***. *    :  :
```

FIGURE 1F

Replacement Figures
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```
SEQ ID NO:4   RVENTRLRQAAEQERQASKRLGTKLFEQS----------LLAEEPVPAK--
SEQ ID NO:5   APEPHVQSTAAPVAVSQTQDSDTLLVQEPS----------
SEQ ID NO:6   HATHTLVPQSGALDGVRELNGADEQRALPS----------THSDETDRATASI
SEQ ID NO:7   RVENYRLHQLADIEKYENAEAATLPLDAPAPAP----------APEQKSGRAKAEA
SEQ ID NO:8   RVENYRLRQLADIEKYENADSATVPLDVTTPEKQSGDVNANGNAKVNTNPSAKAKA
SEQ ID NO:9   P----------
SEQ ID NO:10  KVEKAEDDSNK----------
SEQ ID NO:11  AAALPELPNLEAAPAETAEDYLHA----------
SEQ ID NO:12  AAALPELPNLEAAPAETAEDYLHA----------
SEQ ID NO:13  VTPNAIPAVSDPELTETIEALPNNPIFSH----------
SEQ ID NO:14  IG----------
SEQ ID NO:15  SRDQATEKKPVSDSEISQLD----------
SEQ ID NO:16  SAQETVTYEEIQYGNKANVKVHS----------
SEQ ID NO:17  QTNHFISYEDLHEDKMDMLTIPPRRVATA----------
SEQ ID NO:18  TASVPLEPATATDALSSMEALNHV----------
SEQ ID NO:19  T----------
SEQ ID NO:20  VA-LSVASPAESAD----------DAEPAVR----------
SEQ ID NO:21  APEQGVQSKIASVAISHPQDSDTLLVQEPS----------
SEQ ID NO:22  LPDLPISSESIELL----------RPDIALEV----------
SEQ ID NO:23  FS-LPVDSAAAALPGEAAVARPDQSI----------
SEQ ID NO:24  HATHTFVPQFGALDGVRELNGADEQRALPS----------THSDETDRATATL
SEQ ID NO:25  HATHTFVPQFGALDGVRELNGADEQRALPS----------THSDETDRATATL
```

FIGURE 1G

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/081948

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/78 C12N11/00 C12P7/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 233 057 A (MITSUBISHI RAYON CO [JP]) 21 August 2002 (2002-08-21) paragraph [0018]; claim 6	1-12
X	US 4 355 105 A (LANTERO JR ORESTE J) 19 October 1982 (1982-10-19) abstract column 2, lines 12-23	1-12
X	GB 1 553 662 A (UNION CARBIDE CORP) 26 September 1979 (1979-09-26) the whole document	1-12
X	WO 2006/069114 A2 (E.I. DUPONT DE NEMOURS AND COMPANY, USA) 29 June 2006 (2006-06-29) pages 32-34; examples 21,35,36	1-12

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 January 2009	04/02/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Petri, Bernhard

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2008/081948

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PANOVA, ANNA ET AL: "Chemoenzymatic synthesis of glycolic acid" ADVANCED SYNTHESIS & CATALYSIS , 349(8+9), 1462-1474 CODEN: ASCAF7; ISSN: 1615-4150, 2007, XP009110927 page 1464, right-hand column, paragraph 2 - page 1465, left-hand column, paragraph 3	1-12
P,X	BEN-BASSAT, ARIE ET AL: "Optimization of biocatalyst specific activity for glycolic acid production" ADVANCED SYNTHESIS & CATALYSIS , 350(11+12), 1761-1769 CODEN: ASCAF7; ISSN: 1615-4150, 2008, XP002511198 the whole document	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2008/081948

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