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(54) Title: ENZYMATIC SYSTEMS AND METHODS FOR SYNTHESIZING NICOTINAMIDE MONONUCLEOTIDE AND NICOTINIC ACID MONONUCLEOTIDE

(57) Abstract: Enzyme-based systems and methods for synthesizing the NAD precursors NMN and NaMN are disclosed. Such methods and systems utilize a mutated form of phosphoribosylpyrophosphate synthetase (PRS) that is superactive and/or other enzyme or enzyme combinations that are immobilized onto a solid surface. The methods and systems substantially increase the efficiency and yield of NAD precursor synthesis.

ENZYMATIC SYSTEMS AND METHODS FOR SYNTHESIZING NICOTINAMIDE MONONUCLEOTIDE AND NICOTINIC ACID MONONUCLEOTIDE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/174,412, filed June 11, 2015, which is incorporated herein by reference as if set forth in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Not Applicable

BACKGROUND

[0003] Nicotinamide Adenine Dinucleotide (NAD^+) is an essential metabolic cofactor. Recent research has indicated that NAD^+ levels decline with age and in certain mammalian disease states, and that therapeutically increasing NAD^+ levels has health benefits. However, NAD^+ is an intracellular metabolite, and does not readily lend itself to external supplementation. It has been suggested that utilizing precursors to the natural synthesis of NAD^+ may be an effective way to increase NAD^+.

[0004] Two exemplary precursors that could be administered to increase NAD^+ are nicotinamide monomucleotide (NMN), which is directly synthesized into NAD^+, and nicotinamide riboside (NR), which is recycled from the utilization of NAD^+, into NMN. There are no known dietary or environmental sources of NMN or NR. Accordingly, in order to use these precursors as drugs or supplements, they must be manufactured.

[0005] U.S. Patent No. 8,106,184, issued to Sauve et. al., describes methods for the efficient manufacture of NR through synthetic chemistry. No such method exists for the production of NMN. U.S. Patent No. 4,411,995, issued to Whitesides and Walt, describes an enzymatic process for producing NMN, but such a method, while efficient in its yield, requires carefully controlled conditions and the addition of costly enzymes.

[0006] Therefore a need exists in the art for an improved method to manufacture NAD precursors at high yield, high purity, and lower cost. Such methods are described herein.

BRIEF SUMMARY

[0007] This disclosure relates to new methods and systems for the enzymatic synthesis of the NAD precursors, such as nicotinamide mononucleotide (NMN) and nicotinic acid mononucleotide (NaMN). Various enzyme-based methods for the production of NMN have been described by, for example, Whitesides (1985), Berghauser (1981), Dietrich (1966), and Preiss (1957). Here we disclose enzyme-based systems and methods for producing NMN or NaMN utilizing one or more improvements. Specifically, the disclosed systems and methods utilize (1) a mutated form of phosphoribosylpyrophosphate synthetase (PRS) that is rendered insensitive to its own reaction product, thus increasing its activity; and/or (2) one or more other enzymes or enzyme combinations (optionally including the mutated form of PRS) that are bound to a solid surface. The one or more enzymes used may be produced by recombinant means in one or more cells, including, without limitation, in yeast, bacteria, baculovirus, or mammalian cell lines. Alternatively, the one or more enzymes used may be produced in cell-containing or cell-free *in vitro* translation systems, such as in reticulocyte lysate.

[0008] Accordingly, in a first aspect, the disclosure encompasses a system for synthesizing an nicotinamide adenine dinucleotide (NAD) precursor. The system includes a superactive phosphoribosylpyrophosphate synthetase (PRS) mutant, wherein the PRS mutant is less sensitive to the product of the reaction that it catalyzes than a wild type PRS.

[0009] In some embodiments, the superactive PRS mutant includes a polypeptide that differs from wild type PRS by one or more amino acid substitutions. In some such embodiments, the one or more amino acid substitutions are Asp51His of human PRS, Asn113Ser of human PRS, Leu128Ile of human PRPP, Asp182His of human PRS, Ala189Val of human PRS, His192Gln of human PRS, any of the equivalent substitutions in a non-human PRS, or any combination of these.

[0010] In some embodiments, the superactive PRS mutant includes one or more affinity tags. In some such embodiments, the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

[0011] In some embodiments, the superactive PRS mutant is recombinantly produced, isolated, or purified from cells.

[0012] In some embodiments, the superactive PRS mutant is immobilized onto a surface. In some such embodiments, the superactive PRS mutant is immobilized onto the surface by

adsorption, affinity binding, ionic bonding, or covalent bonding. In some embodiments, the surface is the surface of a bead or comprises a resin.

[0013] In some embodiments, the system further includes nicotinamide phosphoribosyltransferase (NAMPT) or nicotinate phosphoribosyltransferase (NAPRT). In some such embodiments, the NAMPT or NAPRT includes one or more affinity tags. In some such embodiments, the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

[0014] In some embodiments, the NAMPT or NAPRT is recombinantly produced, isolated, or purified from cells.

[0015] In some embodiments, the NAMPT or NAPRT is immobilized onto a surface. In some such embodiments, the NAMPT or NAPRT is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding. In some embodiments, the surface is the surface of a bead or comprises a resin.

[0016] In some embodiments where the NAMPT or NAPRT is immobilized onto a surface, the PRS mutant is also immobilized onto a surface. In some such embodiments, the PRS mutant and the NAMPT or NAPRT are immobilized onto different surfaces. In other such embodiments, the PRS mutant and the NAMPT or NAPRT are immobilized onto the same surface.

[0017] In some embodiments, the system further includes adenosine triphosphate (ATP).

[0018] In some embodiments, the system further includes ribose-5-phosphate.

[0019] In some embodiments, the system further includes nicotinamide or nicotinic acid.

[0020] In some embodiments, the system further includes phosphoribosyl pyrophosphate (PRPP).

[0021] In some embodiments, the system further includes nicotinamide mononucleotide (NMN) or nicotinic acid mononucleotide (NaMN).

[0022] In a second aspect, this disclosure encompasses a method for synthesizing an nicotinamide adenine dinucleotide (NAD) precursor. The method includes the step of contacting ribose-5-phosphate with a superactive phosphoribosylpyrophosphate synthetase (PRS) mutant in the presence of adenosine triphosphate (ATP), wherein the PRS mutant is less sensitive to the product of the reaction that it catalyzes than a wild type PRS, and whereby phosphoribosyl pyrophosphate (PRPP) is produced.

[0023] In some embodiments, the superactive PRS mutant comprises a polypeptide that differs from wild type PRS by one or more amino acid substitutions. In some such embodiments, the

one or more amino acid substitutions can be Asp51His of human PRS, Asn113Ser of human PRS, Leu128Ile of human PRPP, Asp182His of human PRS, Ala189Val of human PRS, His192Gln of human PRS, any of the equivalent substitutions in a non-human PRS, and any combination of these.

[0024] In some embodiments, the superactive PRS mutant includes one or more affinity tags. In some such embodiments, the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

[0025] In some embodiments, the superactive PRS mutant is recombinantly produced, isolated, or purified from cells.

[0026] In some embodiments, the superactive PRS mutant is immobilized onto a surface. In some such embodiments, the superactive PRS mutant is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding.

[0027] In some embodiments, the surface is the surface of a bead or comprises a resin.

[0028] In some embodiments, the method further includes the steps of (a)contacting the resulting PRPP with nicotinamide phosphoribosyltransferase (NAMPT) in the presence of nicotinamide, whereby nicotinamide mononucleotide (NMN) is produced; or (b) contacting the resulting PRPP with nicotinate phosphoribosyltransferase (NAPRT) in the presence of nicotinic acid, whereby nicotinic acid mononucleotide (NaMN) is produced.

[0029] In some embodiments, the NAMPT or NAPRT include one or more affinity tags. In some such embodiments, the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

[0030] In some embodiments, the NAMPT or NAPRT is recombinantly produced, isolated, or purified from cells.

[0031] In some embodiments, the NAMPT or NAPRT is immobilized onto a surface. In some such embodiments, the NAMPT or NAPRT is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding. In some such embodiments, the surface is the surface of a bead or comprises a resin.

[0032] In some embodiments, the PRS mutant is also immobilized onto a surface. In some such embodiments, the PRS mutant and the NAMPT or NAPRT are immobilized onto different surfaces. In other such embodiments, the PRS mutant and the NAMPT or NAPRT are immobilized onto the same surface.

[0033] In some embodiments, the method further includes the step of purifying or concentrating the NMN or NaMN produced.

[0034] In a third aspect, this disclosure encompasses a system for synthesizing nicotinamide mononucleotide (NMN). The system includes nicotinamide riboside kinase (NRK) enzyme immobilized onto a surface.

[0035] In some embodiments the NRK includes one or more affinity tags. In some such embodiments, the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

[0036] In some embodiments, the NRK is recombinantly produced, isolated, or purified from a cell.

[0037] In some embodiments, the NRK is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding.

[0038] In some embodiments, the surface is the surface of a bead or comprises a resin.

[0039] In some embodiments, the NRK is purified from cells or produced through recombinant means.

[0040] In some embodiments, the system further includes adenosine triphosphate (ATP).

[0041] In some embodiments, the system further includes nicotinamide riboside.

[0042] In some embodiments, the system further includes nicotinamide mononucleotide (NMN).

[0043] In a fourth aspect, this disclosure encompasses a method for synthesizing nicotinamide mononucleotide (NMN). The method includes the steps of contacting nicotinamide riboside kinase (NRK) immobilized onto a surface with nicotinamide riboside in the presence of adenosine triphosphate (ATP), whereby NMN is produced.

[0044] In some embodiments, the NRK includes one or more affinity tags. In some such embodiments, the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

[0045] In some embodiments, the NRK is recombinantly produced, isolated, or purified from a cell.

[0046] In some embodiments, the NRK is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding. In some embodiments, the surface is the surface of a bead or comprises a resin.

[0047] Some embodiments further include the step of purifying or concentrating the NMN produced.

[0048] In a fifth aspect, this disclosure encompasses a system for synthesizing nicotinamide mononucleotide (NMN). The system includes the following enzymes immobilized onto a surface: (a) a superactive phosphoribosylpyrophosphate synthetase (PRS) mutant, wherein the PRS mutant is less sensitive to the product of the reaction that it catalyzes than a wild type PRS; (b) hexokinase; (c) glucose-6phosphate dehydrogenase; (d) gluconolactonase; (e) 6-phospho gluconate dehydrogenase; (f) ribulose-5-phosphate isomerase; and (g) nicotinamide phosphoribosyl transferase.

[0049] In some embodiments, one or more of the immobilized enzymes include one or more affinity tags. In some such embodiments, the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

[0050] In some embodiments, one or more of the immobilized enzymes is recombinantly produced, isolated, or purified from a cell.

[0051] In some embodiments, the NRK is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding.

[0052] In some embodiments, the superactive PRS mutant includes a polypeptide that differs from wild type PRS by one or more amino acid substitutions. In some such embodiments, the one or more amino acid substitutions can be Asp51His of human PRS, Asn113Ser of human PRS, Leu128Ile of human PRPP, Asp182His of human PRS, Ala189Val of human PRS, His192Gln of human PRS, any of the equivalent substitutions in a non-human PRS, or any combination of these.

[0053] In some embodiments, the surface is the surface of a bead or comprises a resin.

[0054] In some embodiments, each enzyme is immobilized onto a different surface. In other embodiments, each enzyme is immobilized onto a different surface. In yet other embodiments, the six immobilized enzymes are immobilized to between two and five different surfaces.

[0055] In some embodiments, the system may also include one or more of glucose, nicotinamide, adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate ($NADP^+$), an oxidizing agent, or mixtures thereof.

[0056] In a sixth apect, this disclosure encompasses a method for synthesizing nicotinamide mononucleotide (NMN). The method includes the step of contacting the system described in any of the previous eight paragraphs with nicotinamide in the presence of glucose, adenosine

triphosphate (ATP), Nicotinamide adenine dinucleotide phosphate ($NADP^+$), and an oxidizing agent, whereby NMN is produced.

[0057] In some embodiments, the method further includes the step of purifying or concentrating the NMN produced.

DETAILED DESCRIPTION

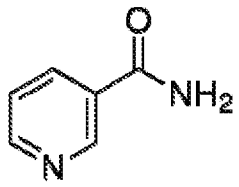
I. Definitions:

[0058] The phrase "a" or "an" entity as used herein refers to one or more of that entity; for example, a compound refers to one or more compounds or at least one compound. As such, the terms "a" (or "an"), "one or more", and "at least one" can be used interchangeably herein.

[0059] The terms "optional" or "optionally" as used herein means that a subsequently described event or circumstance may but need not occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not. For example, "optional bond" means that the bond may or may not be present, and that the description includes single, double, or triple bonds.

[0060] The term "purified," as described herein, refers to the purity of a given compound. For example, a compound is "purified" when the given compound is a major component of the composition, i.e., at least 50% w/w pure. Thus, "purified" embraces at least 50% w/w purity, at least 60% w/w purity, at least 70% purity, at least 80% purity, at least 85% purity, at least 90% purity, at least 92% purity, at least 94% purity, at least 96% purity, at least 97% purity, at least 98% purity, at least 99% purity, at least 99.5% purity, and at least 99.9% purity, wherein "substantially pure" embraces at least 97% purity, at least 98% purity, at least 99% purity, at least 99.5% purity, and at least 99.9% purity.

[0061] "Nicotinamide," which corresponds to the following structure,

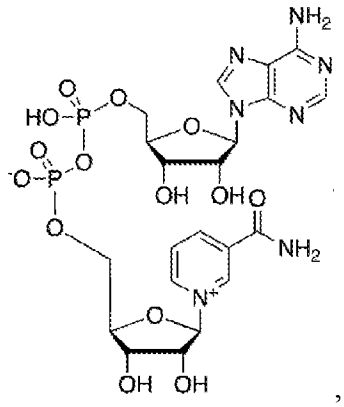


,

is one of the two principal forms of the B-complex vitamin niacin. The other principal form of niacin is nicotinic acid; nicotinamide, rather than nicotinic acid, however, is the major substrate for nicotinamide adenine dinucleotide (NAD) biosynthesis in mammals, as discussed in detail

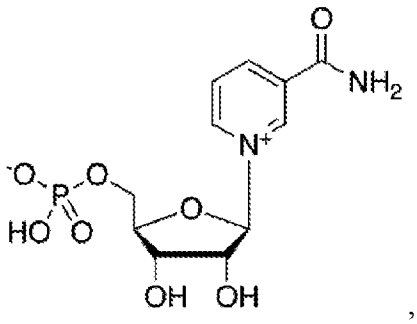
herein. Nicotinamide, in addition to being known as niacinamide, is also known as 3-pyridinecarboxamide, pyridine-3-carboxamide, nicotinic acid amide, vitamin B3, and vitamin PP. Nicotinamide has a molecular formula of $C_6H_6N_2O$ and its molecular weight is 122.13 Daltons. Nicotinamide is commercially available from a variety of sources.

[0062] "Nicotinamide Adenine Dinucleotide" (NAD^+), which corresponds to the following structure,



is produced from the conversion of nicotinamide to NMN, which is catalyzed by Nampt, and the subsequent conversion of NMN to NAD, which is catalyzed by Nmnat. Nicotinamide adenine dinucleotide (NAD) has a molecular formula of $C_{21}H_{27}N_7O_{14}P_2$ and a molecular weight of 663.43. Nicotinamide adenine dinucleotide (NAD) is commercially available from such sources as Sigma-Aldrich (St. Louis, Mo.).

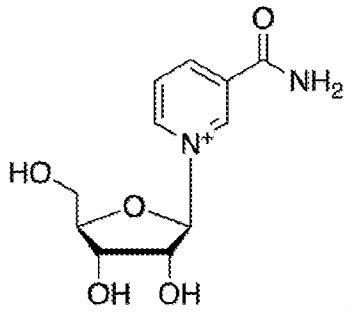
[0063] "Nicotinamide Mononucleotide" (NMN), which corresponds to the following structure,



is produced from nicotinamide in the NAD biosynthesis pathway, a reaction that is catalyzed by Nampt. NMN is further converted to NAD in the NAD biosynthesis pathway, a reaction that is catalyzed by Nmnat. Nicotinamide mononucleotide (NMN) has a molecular formula of

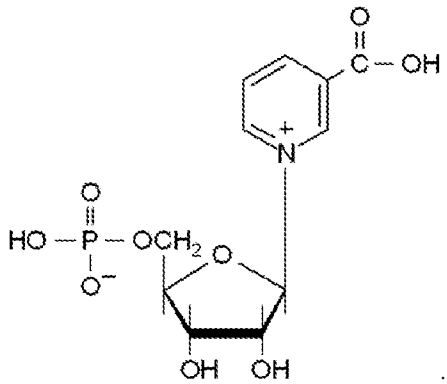
$C_{11}H_{15}N_2O_8P$ and a molecular weight of 334.22. Nicotinamide mononucleotide (NMN) is commercially available from such sources as Sigma-Aldrich (St. Louis, Mo.).

[0064] "Nicotinamide Riboside" (NR), which corresponds to the following structure,

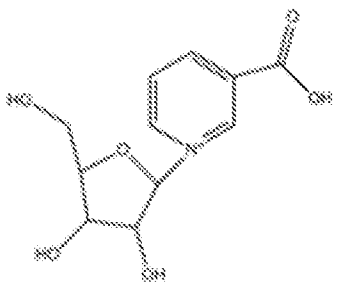


is characterized and a synthesized as described in, for instance, U.S. Patent No. 8,106,184.

[0065] "Nicotinic Acid Mononucleotide" (NaMN) corresponds to the following structure:



[0066] "Nicotinic Acid Riboside" (NaR) corresponds to the following structure:



II. Description of Selected Exemplary Embodiments

[0067] The following exemplary methods and systems are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and the following exemplary systems and methods.

Exemplary Method 1:

[0068] The human enzyme phosphoribosoylpyrophosphate synthetase (PRS) is mutated to increase its activity through rendering it insensitive to the product of its own reaction, phosphoribosyl pyrophosphate (PRPP). Mutations may include, without limitation, Asp51His, Asn113Ser, Leu128Ile, Asp182His, Ala189Val and His192Gln. These mutations are defined relative to the known sequence of human PRS. However, PRS from other species may be used in the disclosed systems and methods, with equivalent mutations in non-human homologs also resulting in the required superactivity.

[0069] Enzymes may optionally be tagged with affinity tags, such as 6xHis tag or GST tag. Recombinant or purified enzyme mutants as described above may be immobilized on, for example, beads or resin (e.g., agarose beads, sepharose beads) through adsorption, affinity binding (e.g. 6xHis tagged proteins to Ni^{2+} or Co^{2+} beads), ionic binding or covalent bonds. Such methods are well-known in the art.

[0070] Ribose-5-phosphate in the presence of ATP may be passed through beads or resin with immobilized, mutated PRS to yield PRPP. The enzyme nicotinamide phosphoribosyltransferase (NAMPT) may be tagged and immobilized to beads or resin, as described above and known in the art. The product of the previous reaction can be combined with nicotinamide and passed through such a resin to yield nicotinamide mononucleotide (NMN).

[0071] In an alternative method, resin or beads carrying recombinant or isolated NAMPT are placed in a bottom layer of a column, and resin or beads carrying recombinant or isolated PRS mutants are placed in an upper layer of a column. A single mixture containing nicotinamide, ribose-5-phosphate and ATP is then passed through the column to yield NMN as a final product.

[0072] In another alternative method, resin or beads carrying immobilized PRS mutant enzyme and NAMPT enzyme are mixed into a single column, and a mixture containing nicotinamide,

ribose-5-phosphate and ATP is then passed through the column. This latter embodiment will have the advantage of consuming PRPP to further reduce inhibition of PRS enzyme.

[0073] In yet another alternative method, NAMPT is replaced by nicotinate phosphoribosyltransferase and nicotinamide is replaced by nicotinic acid, to yield nicotinic acid mononucleotide (NaMN).

[0074] Exemplary Method 2:

[0075] The enzyme nicotinamide riboside kinase (NRK) may be purified from cells or produced through recombinant means, and then immobilized on a solid support (e.g. resin, beads). Nicotinamide riboside and ATP are then passed over this solid support to yield nicotinamide mononucleotide (NMN).

[0076] Exemplary Method 3:

[0077] Glucose, nicotinamide, ATP, $NADP^+$ and an oxidizing agent are passed over a solid support (e.g. resin, beads) which contain the isolated or recombinant enzymes hexokinase, glucose-6-phosphate dehydrogenase, gluconolactonase, 6-phospho gluconate dehydrogenase, ribulose-5-phosphate isomerase, mutant versions of phosphoriboylpyrophosphatase synthetase, and nicotinamide phosphoribosyl transferase. These enzymes may be immobilized to separate solid supports, and placed in layers in the order listed above from top to bottom. Alternatively, all enzymes may be mixed and immobilized to the same solid support. Glucose and nicotinamide will be converted by these enzymes into NMN, which will consume ATP and require the conversion of $NADP^+$ into NADPH. NADPH will be immediately regenerated back into $NADP^+$ through the addition of an oxidizing agent.

[0078] The midpoint potential of the $NADP^+$/NADPH redox pair is −0.324 volts, meaning that NADPH is comparatively easy to oxidize. Thus, preferred oxidizing agents may include any of a number of very mild oxidizing agents known in the art to be capable of oxidizing NADPH into $NADP^+$.

[0079] The enzymes used in the disclosed systems and methods in all of the above-disclosed exemplary methods may be produced through recombinant means in microbes, such as in yeast, bacteria, baculovirus, or in eukaryotic cells, such as in mammalian cell lines. Methods of

producing recombinant enzymes using such host cells are well-known in the art. Alternatively, the enzymes may be produced through *in vitro* translation methods. A variety of cell-free translation methods are known in the art. A non-limiting example is the use of reticulocyte lysate to facilitate enzyme production.

[0080] Constructs for recombinant expression may be subjected to codon optimization from the parent cDNA to increase protein translation. Again, such techniques are well-known in the art. Although the enzymes described in this disclosure are the human forms, the human form of the enzymes used may be substituted with the orthologous enzymes from other species, depending on the efficiency of their activity.

[0081] Other embodiments and uses will be apparent to those skilled in the art from consideration from the specification and practice of the invention disclosed herein. It is understood that the invention is not confined to the specific reagents, formulations, reaction conditions, etc., herein illustrated and described, but embraces such modified forms thereof as come within the scope of the following claims.

[0082] All references cited herein for any reason, including all journal citations and U.S./foreign patents and patent applications, are specifically and entirely incorporated by reference herein.

CLAIMS

1. A system for synthesizing a nicotinamide adenine dinucleotide (NAD) precursor, the system comprising a superactive phosphoribosylpyrophosphate synthetase (PRS) mutant, wherein the PRS mutant is less sensitive to the product of the reaction that it catalyzes than a wild type PRS.

2. The system of claim 1, wherein the superactive PRS mutant comprises a polypeptide that differs from wild type PRS by one or more amino acid substitutions.

3. The system of claim 1 or claim 2, wherein the one or more amino acid substitutions are selected from the group consisting of Asp51His of human PRS, Asn113Ser of human PRS, Leu128Ile of human PRPP, Asp182His of human PRS, Ala189Val of human PRS, His192Gln of human PRS, any of the equivalent substitutions in a non-human PRS, and any combination thereof.

4. The system of any of claims 1-3, wherein the superactive PRS mutant comprises one or more affinity tags.

5. The system of claim 4, wherein the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

6. The system of any of claims 1-5, wherein the superactive PRS mutant is recombinantly produced, isolated, or purified.

7. The system of any of claims 1-6, wherein the superactive PRS mutant is immobilized onto a surface.

8. The system of claim 7, wherein the superactive PRS mutant is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding.

9. The system of claim 7 or claim 8, wherein the surface is the surface of a bead or comprises a resin.

10. The system of any of claims 1-9, further comprising nicotinamide phosphoribosyltransferase (NAMPT) or nicotinate phosphoribosyltransferase (NAPRT).

11. The system of claim 10, wherein the NAMPT or NAPRT comprises one or more affinity tags.

12. The system of claim 11, wherein the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

13. The system of any of claims 10-13, wherein the NAMPT or NAPRT is recombinantly produced, isolated, or purified.

14. The system of any of claims 10-13, wherein the NAMPT or NAPRT is immobilized onto a surface.

15. The system of claim 14, wherein the NAMPT or NAPRT is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding.

16. The system of claim 14 or claim 15, wherein the surface is the surface of a bead or comprises a resin.

17. The system of any of claims 14-16, wherein, the PRS mutant is also immobilized onto a surface.

18. The system of claim 17, wherein the PRS mutant and the NAMPT or NAPRT are immobilized onto different surfaces.

19. The system of claim 17, wherein the PRS mutant and the NAMPT or NAPRT are immobilized onto the same surface.

20. The system of any of claim 1-19, further comprising adenosine triphosphate (ATP).

21. The system of any of claims 1-20, further comprising ribose-5-phosphate.

22. The system of any of claims 1-21, further comprising nicotinamide or nicotinic acid.

23. The system of any of claims 1-22, further comprising phosphoribosyl pyrophosphate (PRPP).

24. The system of any of claims 1-23, further comprising nicotinamide mononucleotide (NMN) or nicotinic acid mononucleotide (NaMN).

25. A method for synthesizing an nicotinamide adenine dinucleotide (NAD) precursor comprising contacting ribose-5-phosphate with a superactive phosphoribosylpyrophosphate synthetase (PRS) mutant in the presence of adenosine triphosphate (ATP), wherein the PRS mutant is less sensitive to the product of the reaction that it catalyzes than a wild type PRS, and whereby phosphoribosyl pyrophosphate (PRPP) is produced.

26. The method of claim 25, wherein the superactive PRS mutant comprises a polypeptide that differs from wild type PRS by one or more amino acid substitutions.

27. The method of claim 25 or claim 26, wherein the one or more amino acid substitutions are selected from the group consisting of Asp51His of human PRS, Asn113Ser of human PRS, Leu128Ile of human PRPP, Asp182His of human PRS, Ala189Val of human PRS, His192Gln of human PRS, any of the equivalent substitutions in a non-human PRS, and any combination thereof.

28. The method of any of claims 25-27, wherein the superactive PRS mutant comprises one or more affinity tags.

29. The method of claim 28, wherein the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

30. The method of any of claims 25-29, wherein the superactive PRS mutant is recombinantly produced, isolated, or purified.

31. The method of any of claims 25-30, wherein the superactive PRS mutant is immobilized onto a surface.

32. The method of claim 31, wherein the superactive PRS mutant is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding.

33. The method of claim 31 or claim 32, wherein the surface is the surface of a bead or comprises a resin.

34. The method of any of claims 25-33, further comprising:

(a) contacting the resulting PRPP with nicotinamide phosphoribosyltransferase (NAMPT) in the presence of nicotinamide, whereby nicotinamide monocleotide (NMN) is produced; or

(b) contacting the resulting PRPP with nicotinate phosphoribosyltransferase (NAPRT) in the presence of nicotinic acid, whereby nicotinic acid mononucleotide (NaMN) is produced.

35. The method of claim 34, wherein the NAMPT or NAPRT comprises one or more affinity tags.

36. The method of claim 35, wherein the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

37. The method of any of claims 34-36, wherein the NAMPT or NAPRT is recombinantly produced, isolated, or purified.

38. The method of any of claims 34-37, wherein the NAMPT or NAPRT is immobilized onto a surface.

39. The method of claim 38, wherein the NAMPT or NAPRT is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding.

40. The method of claim 38 or claim 39, wherein the surface is the surface of a bead or comprises a resin.

41. The method of any of claims 38-40, wherein, the PRS mutant is also immobilized onto a surface.

42. The method of claim 41, wherein the PRS mutant and the NAMPT or NAPRT are immobilized onto different surfaces.

43. The method of claim 17, wherein the PRS mutant and the NAMPT or NAPRT are immobilized onto the same surface.

44. The method of any of claims 34-43, further comprising purifying or concentrating the NMN or NaMN produced.

45. A system for synthesizing nicotinamide mononucleotide (NMN), the system comprising nicotinamide riboside kinase (NRK) immobilized onto a surface.

46. The system of claim 45, wherein the NRK comprises one or more affinity tags.

47. The system of claim 46, wherein the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

48. The system of any of claims 45-47, wherein the NRK is recombinantly produced, isolated, or purified.

49. The system of any of claims 45-48, wherein the NRK is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding.

50. The system of any of claims 45-49, wherein the surface is the surface of a bead or comprises a resin.

51. the system of any of claims 45-50, wherein the NRK is purified from cells or produced through recombinant means.

52. The system of any of claims 45-51, further comprising adenosine triphosphate (ATP).

53. The system of any of claims 45-52, further comprising nicotinamide riboside.

54. The system of any of claims 45-53, further comprising nicotinamide mononucleotide (NMN).

55. A method for synthesizing nicotinamide mononucleotide (NMN), the method comprising contacting nicotinamide riboside kinase (NRK) immobilized onto a surface with nicotinamide riboside in the presence of adenosine triphosphate (ATP), whereby NMN is produced.

56. The method of claim 55, wherein the NRK comprises one or more affinity tags.

57. The method of claim 56, wherein the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

58. The method of any of claims 55-57, wherein the NRK is recombinantly produced, isolated, or purified.

59. The method of any of claims 55-58, wherein the NRK is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding.

60. The method of any of claims 55-59, wherein the surface is the surface of a bead or comprises a resin.

61. The method of any of claims 55-60, further comprising purifying or concentrating the NMN produced.

62. A system for synthesizing nicotinamide mononucleotide (NMN), the system comprising the following enzymes immobilized onto a surface:

(a) a superactive phosphoribosylpyrophosphate synthetase (PRS) mutant, wherein the PRS mutant is less sensitive to the product of the reaction that it catalyzes than a wild type PRS;

(b) hexokinase;

(c) glucose-6phosphate dehydrogenase;

(d) gluconolactonase;

(e) 6-phospho gluconate dehydrogenase;

(f) ribulose-5-phosphate isomerase; and

(g) nicotinamide phosphoribosyl transferase.

63. The system of claim 62, wherein one or more of the immobilized enzymes comprises one or more affinity tags.

64. The system of claim 63, wherein the affinity tag is a 6xHis tag or a glutathione S-transferase (GST) tag.

65. The system of any of claims 62-64, wherein one or more of the immobilized enzymes is recombinantly produced, isolated, or purified from a cell.

66. The system of any of claims 62-65, wherein the NRK is immobilized onto the surface by adsorption, affinity binding, ionic bonding, or covalent bonding.

67. The system of any of claims 62-66, wherein the superactive PRS mutant comprises a polypeptide that differs from wild type PRS by one or more amino acid substitutions.

68. The system of claim 67, wherein the one or more amino acid substitutions are selected from the group consisting of Asp51His of human PRS, Asn113Ser of human PRS, Leu128Ile of human PRPP, Asp182His of human PRS, Ala189Val of human PRS, His192Gln of human PRS, any of the equivalent substitutions in a non-human PRS, and any combination thereof.

69. The system of any of claims 62-68, wherein the surface is the surface of a bead or comprises a resin.

70. The system of any of claims 62-69, wherein each enzyme is immobilized onto a different surface.

71. The system of any of claims 62-69, wherein each enzyme is immobilized onto a different surface.

72. The system of any of claims 62-69, wherein the six immobilized enzymes are immobilized to between two and five different surfaces.

73. The system of any of claims 62-72, further comprising one or more of the group consisting of glucose, nicotinamide, adenosine triphosphate (ATP), Nicotinamide adenine dinucleotide phosphate ($NADP^+$), an oxidizing agent, and mixtures thereof.

74. A method for synthesizing nicotinamide mononucleotide (NMN), the method comprising contacting the system of any of claims 62-72 with nicotinamide in the presence of glucose, adenosine triphosphate (ATP), Nicotinamide adenine dinucleotide phosphate ($NADP^+$), and an oxidizing agent;

whereby NMN is produced.

75. The method of claim 74, further comprising purifying or concentrating the NMN produced.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2016/000874

A. CLASSIFICATION OF SUBJECT MATTER

C12N 9/12 (2006.01) C07H 19/00 (2006.01) C12P 19/30 (2006.01) C12N 11/18 (2006.01) C12N 11/00 (2006.01)

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)

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 practicable, search terms used)

EPOQUE: EPODOC, WPIAP, TXTE, TXPEA, TXPEB, TXPEC, TXPEE, TXPEF, TXPEI, TXPEP, TXPEPEA, TXPES, TXPUS01A, TXPUSE1A, TXPUSEA, TXPUSEB, TXPWOEA. STN: CAPLUS, MEDLINE, EMBASE, BIOSIS. Searched for keywords: PRPS, PRPP, PHOSPHORIBOSYL PYROPHOSPHATE, SUPER ACTIVE, PRODUCT INSENSITIVE, MUTANT, NICOTINAMIDE MONONUCLEOTIDE, ASP51HIS OR ASN113SER OR LEU128ILE OR ASP182HIS OR ALA189VAL OR HIS192GLN and like terms.

GENOME QUEST: Wild-type human PRS1 protein was searched at >80% sequence identity. Motifs representing each mutation of claims 3, 27 and 68 were searched at 100% identity.

PATENT LENS, GOOGLE SCHOLAR and internal databases provided by IP Australia. Searched for applicant and inventor names.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[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 application or patent 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
4 November 2016	04 November 2016
Name and mailing address of the ISA/AU	**Authorised officer**
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INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/IB2016/000874**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BECKER, M., et al., "The genetic and functional basis of purine nucleotide feedback-resistant phosphoribosylpyrophosphate synthetase superactivity.", Journal of Clinical Investigation, 1995, vol. 96, no. 5, pages 2133-2141. Abstract, Page 2134 column 2 paragraphs 2 and 4, Figures 3 and 4, Tables I and III	1-44 and 62-75
X	ZAKATAEVA, N., et al., "Wild-type and feedback-resistant phosphoribosyl pyrophosphate synthetases from Bacillus amyloliquefaciens: purification, characterization, and application to increase purine nucleoside production.", Applied Microbiology and Biotechnology, 2012, vol. 93, no. 5, pages 2023-2033. Abstract, Figures 2-4, Tables 1 and 3, Page 2026 'Expression and purification of recombinant PRSBas', Page 2026 - 2027 'PRS activity assay and kinetic analysis'	1-44 and 62-75
X	LI, B., et al., "Negative feedback-defective PRPS1 mutants drive thiopurine resistance in relapsed childhood ALL.", Nature Medicine, 2015 (ePub date 11 May 2015), vol. 21, no. 6, pages 563-571. Abstract, Figure 2, Supplementary Figures 3D-E and 15, Supplementary Tables 9 and 10	1-44 and 62-75
X	GROSS, A., et al., "Practical synthesis of 5-phospho-D-ribosyl. alpha.-1-pyrophosphate (PRPP): enzymatic routes from ribose 5-phosphate or ribose.", Journal of the American Chemical Society, 1983, vol. 105 no. 25, pages 7428-7435. Abstract, Schemes I and IV, 'Syntheses and Use of PRPP' page 7430-7431, Page 7431 column 1 paragraph 2, Page 7433 column 1 paragraph 3 - column 2 paragraph 1, Page 7431 column 1 paragraph	1-44 and 62-75
X	WO 2015069860 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 14 May 2015 Abstract, Example 1, Figures 1 and 3, Claim 5, Page 5 lines 1-4, Page 7 line 26 - Page 8 line 4	1-44 and 62-75

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2016/000874

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-44, 62-75

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box

Continuation of: **Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

Invention 1. Claims 1-44, 62-75 are directed to methods and systems for synthesising a nicotinamide adenine dinucleotide precursor. The feature of a superactive phosphoribosylpyrophosphate synthetase mutant, that is less sensitive to the product of the reaction that it catalyses, is specific to this group of claims.

Invention 2. Claims 45-61 are directed to methods and systems for synthesising nicotinamide mononucleotide. The feature of a nicotinamide riboside kinase immobilised onto a surface is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a priori*.

Due to the lack of unity only invention 1 has been searched and examined.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/IB2016/000874**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2015069860 A1	14 May 2015	WO 2015069860 A1	14 May 2015
		US 2016287621 A1	06 Oct 2016

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.