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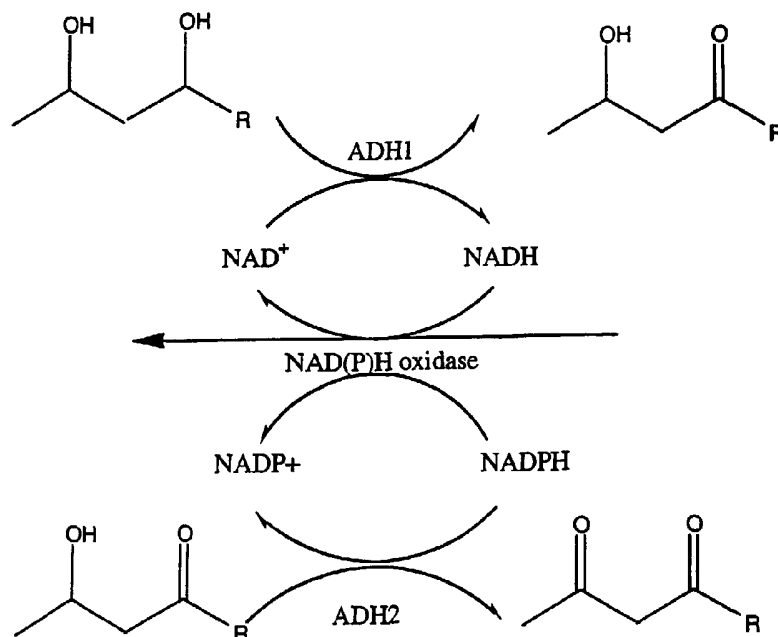
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(54) Title: METHODS AND COMPOSITIONS FOR NAD(P)H OXIDASES



(57) Abstract: The present invention is directed to compositions and methods comprising NAD(P)H oxidases, particularly bacterial oxidases, nucleic acids, recombinant plasmid vectors and recombinant proteins therein encoded, and host cells comprising the oxidases and nucleic acids. The present invention also comprises an isolated bacterial oxidase that oxidizes both NADH and NADPH. Methods for producing the enzymes and enzymatic reactions comprising use of NAD(P)H oxidases and products of such reactions are also disclosed.



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METHODS AND COMPOSITIONS FOR NAD(P)(H) OXIDASES

FIELD OF THE INVENTION

The present invention relates to bacterial NAD(P)H oxidases, their purification, nucleic acids coding for them and vehicles and constructs comprising the nucleic acids or expressed products of the nucleic acids, and products made from enzymatic reactions using the oxidases.

BACKGROUND OF THE INVENTION

Enantiomerically pure compounds (EPCs), especially amino and hydroxy acids as well as alcohols, amines, and lactones are increasingly useful in the pharmaceutical, food, and crop protection industries as building blocks for novel compounds not accessible through fermentation [1-4] as well as for asymmetric synthesis templates.[5-6] One very advantageous route to a wide variety of EPCs is the use of dehydrogenases, to afford either reduction of keto compounds or oxidation of alcohol or amine groups. The repertoire of dehydrogenases useful for synthesis of EPCs encompasses alcohol dehydrogenases (ADHs) [7], D- and L-lactate dehydrogenases (LDHs) [8], D- or L-hydroxyisocaproate dehydrogenases (D- or L-HicDHs) [9,10], or amino acid dehydrogenases such as leucine dehydrogenase (LeuDH) [10], phenylalanine dehydrogenase (PheDH) [11-13] or glutamate dehydrogenase (GluDH).[14] Monooxygenases have been used to synthesize, regio- and enantioselectively, lactones from cyclic ketones useful in the flavor and fragrance industries.[15]

Dehydrogenases and monooxygenases need nicotinamide-based cofactors, such as NAD^+ and NADP^+ or their reduced equivalents, NADH and NADPH, to function. Economic use of dehydrogenases and cofactor necessitates cofactor regeneration.[16] Cofactor costs, for example, \$90 per gram for NAD^+ have to be considered and having cofactors regenerated [17] would cut costs by the turnover number for such cofactors, between 100 and up to 600,000 [18].

Cofactor regeneration with alcohol dehydrogenases can be performed by using the same enzyme for in-situ substrate conversion and cofactor regeneration, usually employing isopropanol as co-substrate, as demonstrated with (S)-ADH from *Thermoanaerobium brockii* for both NADH and NADPH [19] and with (R)-ADH from *L. brevis* [20] for NADPH; this

coupled-substrate approach, however, suffers from equilibrium limitations. The more common coupled-system approach, employing a separate second enzyme for regeneration, has been developed for reducing oxidized cofactors, NAD^+ or NADP^+ , to NADH or NADPH . By far the most successful regeneration enzyme is formate dehydrogenase (FDH) for regeneration to either NADPH [24-25] or NADH , the latter even up to industrial scale [20-23]. Other options include the use of glucose 6-phosphate dehydrogenase [26] (to NADPH only) or of glucose dehydrogenase, GluDH [27-29]. For the opposite direction of regeneration, however, from NAD(P)H to oxidized cofactors NAD^+ or NADP^+ , no universally accepted system exists.

There are some currently known NADH oxidases that are able to oxidize NADH to NAD^+ with simultaneous reduction of O_2 to either H_2O_2 or H_2O [30-34]. Four-electron reduction to benign H_2O is preferred over two-electron reduction to H_2O_2 , which, even in small amounts, can deactivate either enzyme of the production-regeneration cycle. Addition of catalase as a possible remedy, to degrade the H_2O_2 , increases complexity of the system to the point where three enzymes have to be coupled and adjusted as to their activity over time.

For reductive reactions with dehydrogenases or for monooxygenases, NAD(P)H has to be regenerated from NAD(P)^+ . For this problem, the system formate dehydrogenase (FDH)/formate is now used almost universally [35-37]



FDH functions as a universal regeneration enzyme in tandem with dehydrogenases catalyzing extremely enantioselective reduction reactions.[38-39]

For oxidative reactions requiring regeneration of NAD(P)^+ from NAD(P)H , prior to the present invention, no universal cofactor regeneration system was known. Alcohol dehydrogenase (ADH) itself can be utilized to catalyze both the oxidative production reaction as well as the reductive regeneration reaction by adding isopropanol which is oxidized to acetone, but such a scheme tends to be equilibrium-limited and plagued by deactivation of ADH.[40] Both the ADH and the lactate dehydrogenase (LDH) systems [41] cannot take NADPH , in contrast to glutamate dehydrogenase (GluDH), which has been utilized to reduce α -ketoglutarate to L-glutamate.[42,43] NADH oxidases from thermophiles have been employed which regenerate NAD^+ from NADH by reducing O_2 to H_2O_2 .[44]

What is needed are enzymes that regenerate NAD(P)H to oxidized cofactors NAD⁺ and NADP⁺ and synthesis methods that employ such enzymes alone or in coupled reactions. What is also needed are enzymes that perform the oxidation of NADH to NAD⁺ with the concomitant reduction of molecular oxygen to water as a solution to the cofactor regeneration problem from NADH to NAD⁺. Further, what is needed are methods for efficiently isolating the enzymes.

SUMMARY OF THE INVENTION

The present invention comprises methods and compositions comprising NAD(P)H oxidases (NOX). Compositions of the present invention comprise NOX that have activity in NAD⁺ regeneration and that have activity for both NAD⁺ and NADP⁺ regeneration. Additionally, the NOX show concomitant reduction of molecular oxygen to water. NOX is expected to be produced easily and be available in sufficient amounts for large-scale use. Compositions of the present invention also include isolated NOX from *Borrelia burgdorferi* (BNOX) and from *Lactobacillus sanfranciscensis* (SFNOX).

Further compositions comprise nucleic acids that encode the NOX, and recombinant plasmid vectors, and cells comprising the NOX-encoding nucleic acids. Such compositions include recombinant plasmid vectors and cells where the NOX-encoding nucleic acids are found alone or are found in combination with other enzyme-encoding nucleic acids. For example, compositions of the present invention comprise a cell comprising at least one plasmid comprising an enzyme-encoding nucleic acid, wherein in at least one encoding nucleic acid expresses at least one NOX of the present invention. The vectors of the present invention may be separate, under the control of one or more promoters, i.e., functioning like an individual plasmid, or may be intercalated with other vector constructs or genomic sequences.

Compositions of the present invention comprise whole cell catalysts, wherein the cells comprise NOX proteins and/or NOX-encoding nucleic acids and also comprise other enzymes or nucleic acids encoding such enzymes, so that all or part of a coupled enzymatic reaction can occur under the correct conditions. For example, a whole cell catalyst could comprise at least NOX and/or NOX-encoding nucleic acids and a dehydrogenase and/or dehydrogenase-encoding nucleic acids.

Compositions of the present invention further comprise nucleic acids and proteins encoded thereby that are derived by mutation or alteration of the nucleic acids taught herein. Such mutated sequences encode proteins that have NAD(P)H activity and are used in the vectors, plasmids, constructs and whole cell catalysts taught herein. Additionally, such mutated sequences are contemplated in the methods steps taught herein, for example, in isolating or using the NOX sequences or proteins.

Methods of the present invention comprise methods for isolating NOX from cells and methods for producing recombinant NOX from cells. Novel methods for isolation of NOX from cells are provided herein that has utility for large-scale production of such enzymes. Methods for producing a recombinant NOX comprise cultivating a cell containing a construct comprising NOX encoding nucleic acid, and collecting the NOX produced by the cell.

The present invention also comprises methods of use of the NOX described herein in enzymatic reactions, and compositions of the products of such reactions. Some enzymatic reactions contemplated by the present invention comprise methods of producing one or more chiral enantiomer-enriched organic compounds in reactions comprising one or more NOX. Such enzymatic reactions may be performed in in vitro systems or in in vivo, living cell systems.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are schematic drawings of enzyme reactions of NOX.

Figure 2A-2F are sequence comparisons for BNOX (A-C) and SFNOX (D-F).

Figure 3A-3C are graphs of the kinetics of SFNOX and BNOX with NAD(P)H cofactor in air-saturated solution at pH 7 and 30° C.

Figure 4 is a graph showing activity-pH-profile of *L. sanfranciscensis* NADPH oxidase

Figure 5 is a graph of the standard curve for selective ion monitoring of phenylethanol (• mass122) acetophenone (▲ mass 120)

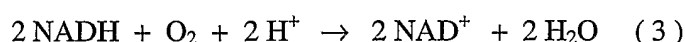
DETAILED DESCRIPTION OF THE INVENTION

The present invention comprises compositions and methods comprising novel NAD(P)H oxidases (NOX) from bacterial sources, and is particularly directed to SFNOX and BNOX. In summary, the compositions comprising NOX of the present invention include isolated enzymes, recombinantly produced enzymes, nucleic acids encoding the NOX, NOX

sequences, proteins and recombinant constructs wherein the altered sequences are derived by mutational methods, vectors and plasmids comprising the NOX nucleic acids, and cells comprising the enzymes or nucleic acids encoding the NOX proteins. Compositions also include products made in enzymatic reactions in which NOX participates and enantiomer-
 5 enriches an unreacted racemate. SFNOX reacts with both NADH and NADPH, whereas BNOX reacts with NADH. Both enzymes reduce oxygen to water. As used herein, the term "NAD(P)H" means NADH or NADPH is the cofactor and, for enzymes capable of using both cofactors, means both NADH and NADPH.

The methods of the present invention include isolation of NOX proteins, and methods
 10 for enzymatic reactions comprising NOX. As used herein, NOX is understood to include the NAD(P)H oxidases disclosed herein, including bacterial oxidases that use NADH and NADPH as a cofactor, the enzymes that were isolated from *Borrelia burgdorferi* (BNOX) and from *Lactobacillus sanfranciscensis*, any recombinant sequences derived from bacterial oxidases that use NADH and NADPH as a cofactor and those found in *Borrelia burgdorferi*
 15 (BNOX) and in *Lactobacillus sanfranciscensis* (SFNOX) and recombinant proteins expressed by those sequences in heterologous hosts, and any nucleic acid or amino acid variants with the oxidase activity of SFNOX and BNOX, and any mutants of bacterial oxidases that use NADH and NADPH as a cofactor and those found in *Borrelia burgdorferi* (BNOX) and in *Lactobacillus sanfranciscensis* or nucleic acids thereof.

20 In general, NADH oxidases (E.C. 1.6.-.-) catalyze the oxidation of NADH by simultaneously reducing molecular O₂ to either hydrogen peroxide, H₂O₂, in a two-electron reduction (reaction 2), or directly to water in a four-electron reduction (reaction 3).



25 NADH oxidases contain a second cofactor, presumably covalently bound FAD, as evidenced by the consensus sequence GXT(H/S)AG near the N-terminus, and are widespread among different, evolutionary distinct organisms, such as humans, vertebrates, plants, *Drosophila* and different strains of bacteria. Bacteria harbor both H₂O₂-forming and H₂O-forming NADH-oxidases. Owing to the deactivation of almost all proteins upon the exposure to
 30 H₂O₂, the H₂O-forming enzymes are superior as biocatalysts. Addition of catalase could potentially destroy the H₂O₂ formed, however, catalase itself features a very high K_M-value of 1.1 M [45], so that the enzyme is not particularly active at low H₂O₂ concentrations. Thermophilic bacteria usually only feature peroxide-producing NADH oxidases, which,

despite their superior stability, renders them unfavorable for catalytic purposes. Water-producing NADH-oxidases can be found in various organisms, such as *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Mycobacterium*, *Methanococcus*, or *Leuconostoc*. These organisms can contain both water- as well as peroxide-producing enzymes.

5 Various H₂O-producing NADH-oxidases have been found and described in the literature (see Table 1). None of them, however, has been characterized with respect to all of the properties relevant to use as a biocatalyst. In most cases, kinetic properties have not been reported.

10	Table 1:	NADH oxidases			
	Bacteria	Enzyme	Accession Code	Sequence data	Reference
	<i>Leuconostoc mesenteroides</i>	Nox, H ₂ O			Koike, 1985 [46]
	<i>Enterococcus faecalis</i>	NPX	P37062 (SwissProt)	Protein, Nucleotide	Ross et al. 1991 [47]
	<i>Enterococcus faecalis</i>	Nox, H ₂ O	P37061 (SwissProt)	Protein, Nucleotide	Ross et al., 1992 [48]
	<i>Mycoplasma genitalis</i>	Nox, H ₂ O	Q49408 (EMBL)	Protein, Nucleotide	Peterson, 1993 [49]
	<i>Streptococcus mutans</i>	Nox, H ₂ O	D49951 (EMBL)	Protein, Nucleotide	Matsumoto, 1996 [50]
	<i>Mycoplasma pneumoniae</i>	Nox, H ₂ O	P75389 (SwissProt)	Protein, Nucleotide	
	<i>Methanococcus japonicus</i>	Nox, H ₂ O	Q58065 (EMBL)	Protein, Nucleotide	Bult, 1996 [51]

Sequence analysis of the water-producing enzymes in all the organisms listed above reveals the same highly conserved cysteine residue, compared to a rather modest overall sequence similarity. This suggests that all these flavoproteins constitute a distinct class of FAD-dependent oxidoreductases, different from others such as glutathione reductase and thioredoxin reductase. Other properties of the enzymes listed above are similar: the molecular weight of the subunit hovers around 50 kD, all enzymes are dimers and contain 1 FAD per subunit, and all are inactivated by hydrogen peroxide.

The present invention comprises compositions comprising NOX and methods of making and using NOX, wherein the NOX comprise bacterial oxidases that use NADH and NADPH as a cofactor, and NOX that were isolated from *Borrelia burgdorferi* (BNOX) and from *Lactobacillus sanfranciscensis*, (SFNOX). As NAD(P)H oxidases, both BNOX and SFNOX function to regenerate NAD⁺, (See Fig.1A) and SFNOX has both NAD⁺ and NADP⁺ regeneration activity (See Fig. 1B). The ability of SFNOX to oxidize both cofactors

renders it an extremely useful catalyst for coupled enzymatically-catalyzed oxidations. The present invention comprises bacterial oxidases that regenerate both NADP⁺ and NAD⁺. The present invention comprises novel NAD(P)H oxidases that reduce oxygen directly to water, which also makes them useful in coupled enzymatic reactions.

5 The NOX of the present invention participate in reactions where there is a complete conversion of one of the enantiomers in a racemic mixture, such as an alcohol to a ketone, leaving a highly enantiomer-enriched unreacted optical antipode of the original molecule, such as an alcohol. Dehydrogenases are capable of very specific enantiomeric selection and are used to prepare enantiomerically pure alcohols, hydroxy acids and amino acids as well as
10 the corresponding ketones and keto acids. The dehydrogenase reaction requires the regeneration of the NADH or NADPH for cofactor activity, and thus, the NOX of the present invention have utility in coupled reactions with dehydrogenases including, but not limited to, alcohol dehydrogenase, lactate dehydrogenase and amino acid dehydrogenase. Products from such reactions include the resolution of racemic mixtures, such resolution dependent on the
15 selectivity of the dehydrogenase used, and resulting in the unreacted racemate from the original racemic mixture, and the product of the enzyme reaction. For example, from a racemic mixture of an R/S-alcohol, in a reaction with an S-alcohol dehydrogenase, the resulting products are the unreacted enantiomer, the R-alcohol, and the resulting product, e.g, a ketone.

20 The NOX of the present invention are important in synthesis methods comprising enzyme reactions where the reactants have one or more chiral centers. An embodiment of the present invention comprises methods for enzyme reactions, comprising reacting at least one enzyme selective for one enantiomer of at least one chiral center of a compound with one or more chiral centers, with a reactant composition comprising the compound with one or more
25 chiral centers, wherein the at least one enzyme requires a nicotinamide-based cofactor, and reacting the nicotinamide-based cofactor with one or more of the NOX of the present invention. In such methods where NAD⁺ is the cofactor, both SFNOX and BNOX individually or in combination could be used. In such methods where NADP⁺ is the cofactor, bacterial oxidases that use NADH and/or NADPH as a cofactor, such as SFNOX,
30 could be used. Bacterial oxidases that use NADH and/or NADPH as a cofactor, including SFNOX, could be also used alone in methods where NADP⁺ and NAD⁺ are cofactors, as well as combinations of enzymes, such as bacterial oxidases that use NADH and NADPH as a cofactor, SFNOX and BNOX could be used in reactions where NAD⁺ and NADP⁺ are cofactors for enzymes in the reactions.

Embodiments of the present invention comprise isolated bacterial oxidases that use NADH and NADPH as a cofactor. Embodiments of the present invention also comprise SFNOX (SEQ ID NOs 2, 4 and 6) and BNOX (SEQ ID NOs 8, 10 and 12). The present invention also comprises nucleic acids of SFNOX (SEQ ID NOs 1, 3 and 5) and BNOX (SEQ ID NOs 7, 9 and 11).

SEQ ID 1 SFNOX

ATGAAAGTTATTGTAGTAGGTTGTACTCACGCTGGCACTTTTGCAGTTAAGCAAACGATT
 GCCGATCACCCCGATGCAGATGTGACTGCATATGAAATGAATGATAACATTTCTTTTTTA
 10 TCATGTGGAATCGCCCTTTACTTAGGTAAAGAAATTAATAA
 ACAATGATCCCCGAGGGCTTTTCTACTCAAGTCCAGAAGAATTAAGCAATCTTGGAGCTAACGTCCAA
 ATGCGTCATCAA
 GTTACAAACGTTGATCCAGAAACAAAAACAATCAAAGTTAAAGATTTAATCACCAACGAAGAAAAAAC
 AGAAGCATATGA
 15 CAAATTAATTATGACCACTGGCTCTAAGCCTACTGTTCTTCCAATCCCTGGAATCGATAGTAGTCGCG
 TTTACCTTTGTATA
 AAAACTATAACGATGCTAAAAAGTTATTTGAAGAAGCTCCCAAAGCTAAAACGATTACTATCATTGGT
 TCCGGTTATATTT
 GGTGCCGAACCTGGCTGAAGCCTACTCAAACCAAAATTATAACGTTAATTTAATTGATGGTCATGAACG
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 TAGCTGCTTTTGAAGAAGTCGATGATGAAATTATCACTAAAACCCCTAGATGGTAAAGAAATTAAATCT
 GATATTGCAATT
 25 CTTTGTATCGGTTTCCGCCCTAACACTGAATTACTTAAAGGTAAAGTTGCCATGTTGGATAACGGTGC
 AATCATTTACTGA
 TGAATACATGCATTCATCAAATCGCGACATTTTGTCTGCTGGTGATAGTGCCGCCGTTCACTACAACC
 CCACTAATTCTA
 ACGCTACATTCCTTTAGCTACCAACGCCGTACGCCAAGGGAGATTAGTTGGCCTAAATCTGACTGAA
 30 GACAAAGTAAAA
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 CAACGGATGAAG
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 GATGTTTTCGCAA
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 GTTATTCCAACC
 ACAATTTGATCGTCCGTTTAACTACTTAAACATTCTAGGCCAAGCTGCTCAAGCACAAGCTGACAAAG
 40 CACATAAAataa

SEQ ID 2 SF

MKVIVVGCTHAGTFAVKQTI
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 LDGKEIKSDIAI
 50 LCIGFRPNTELLKGKVAMLDNGAIITDEYMHSSNRDIFAAGDSAAVHYNPTNSNAYIPLATNAVRQGR
 LVGLNLTEDKVK
 DMGTQSSSGLKLYGRITYVSTGINTALAKANNLKVSEVIIADNYRPEFMLSTDEVLMISLVYDPKTRVIL

GGALSSMHDVSQ
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SEQ ID 3 SFNOXK2

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ATGCGTCATCAA
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AGAAGCATATGA
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TTTTAATGTCATAGTGTATGATCCTAAGACTCGTGTAATTTTGGGAGGGGCGCTTCAAGTATGCAC
GATGTTTCGCAA
35 TCAGCGAACGCTCTATCAGTATGTATTCAAATAAAAAACACGATTGACGATTTAGCAATGGTGGATAT
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ACAATTTGATCGTCCGTTTAACTACTTAAACATTCTAGGCCAAGCTGCTCAAGCACAAGCTGACAAAG
CACATAAAtaa

40 SEQ ID 4 SFNOXK2

MKVIVVGCTHAGTFVAVKQTIADHPDADVTVYEMNDNISFLSCGIALYLGKEIKNNDPRGLFYSSPEEL
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50 GGALSSMHDVSQ
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SEQ ID NO.: 5 SFNOX K6

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 30 TTTTAATGTCAATTAGTGTATGATCCTAAGACTCGTGTAATTTTGGGAGGGGCGCTTTCAAGTATGCAC
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 GTTATTCCAACC
 ACAATTTGATCGTCCGTTTAACTACTTAAACATTCTAGGCCAAGCTGCTCAAGCACAAGCTGACAAAG
 35 CACATAAAataa

SEQ ID NO.: 6 SFNOXK6

MKVIVVGCTHAGTFVAVKQTIADHPDADVTVYEMNDNISFLSCGIALYLGKEIKNNDPRGLFYSSPEEL
 40 SNLGANVQMRHQ
 VTNVDPETKTIKVKDLITNEERTEAYDKLIMTTGSKPTVPPIPGIDSSRVYLCKNYNDAKKLFEEAPK
 AKTITTIIGSGYI
 GAELAEAYSNQNYNVNLDGHERVLYKYFDKEFTDILAKDYEAHGVNLVLGSKVAAFEEDDEIITKT
 LDGKEIKSDIAI
 45 LCIGFRPNTGLLKGKVAMLDNGAIIITDEYMHSSNRDIFAAGDSAAVHYNPTNSNAYIPLATNAVRQGR
 LVGLNLTEDKVK
 DMGTQSSSGLKLYGRTYVSTGINTALAKANNLKVSEVIIADNYRPEFMLSTDEVLMMLVYDPKTRVIL
 GGALSSMHDVSQ
 SANVLSVCIQNKNTIDDLAMVDMLFQPQFDRPFNYLNLGQAAQAQADKAHK
 50

SEQ ID NO.: 7 BNOX

ATGATGAAAATAATAATTATTGGGGGCACATCAGCAGGAAGTAGTGCCGCAGCTAAAGCA
 AACCCTTAAACAAAAAGCTAGACATTACTATCTATGAAAAACAAATATTGTATCTTTT
 5 GGAACCTGTGGCCTGCCTTACTTTGTGGGGGGATTCTTTGACAACCCCAATACAATGATC
 TCAAGAACACAAGAAGAATTCGAAAAAACTGGAATCTCTGTTAAACTAACCACGAAGTT
 ATCAAAGTAGATGCAAAAAACAATACAATTGTAATAAAAAATCAAAAAACAGGAACCAT
 TTTAACAATACTTACGATCAACTTATGATAGCAACTGGTGCAAAACCTATTATTCCACCA
 ATCAATAATATCAATCTAGAAAAATTTTCATACTCTGAAAAATTTAGAAGACGGTCAAAAA
 10 ATAAAAAAATTAATGGATAGAGAAGAGATTAAAAATATAGTGATAATTGGTGGTGATAC
 ATTGGAATTGAAATGGTAGAAGCAGCAAAAAATAAAAGAAAAAATGTAAGATTAATTCAA
 CTAGATAAGCACATACTCAT
 AGATTCCCTTTGACGAAGAAATAGTCACAATAATGGAAGAAGAACTAACAAAAAGGGGGTTAATCTTC
 ATACAAATGAGT
 15 TTGTAAAAAGTTTAATAGGAGGAAAAAAGGCAGAAGGAGTAGTAACAAACAAAAATACTTATCAAGCT
 GACGCTGTTATA
 CTTGCTACCGGAATAAAACCTGACACTGAATTTTTAGAAAACAGCTTAAACTACTAAAAATGGAGC
 AATAATTGTAAA
 TGAGTATGGCGAAACTAGCATAAAAAATATTTTTTCTGCAGGAGATTGTGCAACTATTTATAATATAG
 20 TAAGTAAAAAAA
 ATGAATACATACCCTTGGCAACAACAGCCAACAACTTGAAGAATAGTTGGTGAAAAATTAGCTGGG
 AATCATAACAGCA
 TTTAAAGGCACATTGGGCTCAGCTTCAATTAAAATACTATCTTTAGAAGCTGCAAGAACAGGACTTAC
 AGAAAAAGATGC
 25 AAAAAAGCTCCAAATAAAATATAAAACGATTTTTGTAAAGGACAAAAATCATACAAATTATTATCCAG
 GCCAAGAAGATC
 TTTATATTAAATTAATTTATGAGGAAAAATACCAAAATAATCCTTGGGGCACAAGCAATAGGAAAAAAT
 GGAGCCGTAATA
 AGAATTCATGCTTTATCAATTGCAATCTATTCAAAGCTTACAACAAAAGAGCTAGGGATGATGGATT
 30 CTCATATTCCCCACCCTTCTCAAGAACTTGGGATATATTAAATATTGCTGGCAATGCTGCCAAAtag

SEQ ID NO.: 8 BNOX

MMKIIIIIGGTSAGTSAAAKA
 35 NRLNKKLDITIYEKTNIVSF
 GTCGLPYFVGFFDNPNMTI
 SRTQEEFEKTGISVKNHEV
 IKVDAKNNTIVIKNQKTGTI
 FNNTYDQLMIATGAKPIIPP
 40 INNINLENFHTLKNLEDGQK
 IKKLMDREEIKNIVIIGGGY
 IGIEMVEAAKNRKNVRLIQ
 LDKHILIDSFDEEIVTIMEE
 45 ELTKKGVNLHTNEFVKSLIGGKKAEGVVTKNNTYQADAVI
 LATGIKPDTEFLENQLKTTKNGAIIVNEYGETSIKNIFSAGDCATIYNIVSKKNEYIPLATTANKLGR
 IVGENLAGNHTA
 FKGTLGSAASIKILSLEAARTGLTEKDAKKLQIKYKTIFVKDNHTNYYPGQEDLYIKLIYEENTKIIL
 GAQAIGKNGAVI
 50 RIHALSIAIYSKLTTKELGMMDFSYSPFSSRTWDILNIAGNAAK

SEQ ID NO.: 9 BNOX K1

ATGATGAAAATAATAATTATTGGGGGCACATCAGCAGGAAGTAGTGCCGCAGCTAAAGCAAACCGCTT
 AAACAAAAAGCT
 AGACATTACTATCTATGAAAAACAAATATTGTATCTTTTGGAACCTGCGGCCTGCCTTACTTTGTGG
 5 GGGGATTCTTTG
 ACAACCCCAATACAATGATCTCAAGAACACAAGAAGATTGCAAAAACTGGAATCTCTGTTAAAACT
 AACCACGAAGCT
 ATCAAAGTAGATGCAAAAAACAATACAATTGTAATAAAAAATCAAAAAACAGGAACCATTTTTTAACAA
 TACTTACGATCA
 10 ACTTATGATAGCAACTGGTGCAAAACCTATTATTCCACCAATCAATAATATCAATCTAGAAAATTTTC
 ATACTCTGAAAA
 ATTTAGAAGACGGTCAAAAAATAAAAAATTAATGGATAGAGAAGAGATTAAAAATATAGCGATAATT
 GGTGGTGGATAC
 ATTGGAATTGAAATGGTAGAAGCAGCAAAAAATAAAAGAAAAAATGTAAGATTAATTCAACTAGATAA
 15 GCACATACTCAT
 AGATTCTTTTACGAAGAAATAGTCACAATAATGGAAGAAGAACTAACAAAAAGGGGGTTAATCTTC
 ATACAAATGAGT
 TTGTAAAAAGTTTAATAGGAGAAAAAAGGCAGGAGGAGTAGTAACAAACAAAAATACTTATCAAGCT
 GACGCTGTTATA
 20 CTTGCTACCGGAATAAAACCTGACACTGAATTTTTTAGAAAACCAGCTTAAAACTACTAAAAATGGAGC
 AATAATTGTAAA
 TGAGTATGGCGAAACTAGCATAAAAAATATTTTTCTGCAGGAGATTGTGCAACTATTTATAATATAG
 TAAGTAAAAAAA
 ATGAATACATACCCTTGGCAACAACAGCCAACAACTTGAAGAATAGTTGGTGAAAATTTAGCTGGG
 25 AATCATACAGCA
 TTTAAAGGCACATTGGGCTCAGCTTCAATTAAAATACTATCTTTAGAAGCTGCAAGAACGGGACTTAC
 AGAAAAAGATGC
 AAAAAGGCTCCAAATAAAATATAAAACGATTTTTGTAAAGGACAAAAATCATACAAATTATTATCCAG
 GCCAAGAAGATC
 30 TTTATATTAAATTAATTTATGAGGAAAATACCAAAATAATCCTTGGAGCACAAGCAACAGGAAAAAAT
 GGAGCCGTAATG
 AGAATTATCATGCTTTATCAATTGCAATCTATTCAAACTTACAACAAAAGAGCTAAGGATGATGGATTT
 CTCATATTCCCCACCTTCTCAAGAAGCTGGGATATATTAAATATTGCTGGCAATGCTGCCAAAtag

35 SEQ ID NO.: 10 BNOX K1

MMKIIIIIGGTSAGTSAAKA
 NRLNKKLDITIIYEKTNIVSF
 GTCGLPYFVGGFFDNPNTMI
 SRTQEEFEKGTGISVKTNHEA
 40 IKVDAKNNTIVIKNQKTGTIFNNTYDQLMIATGAKPIIPPINNINLENFHTLKNLEDGQKIKKLMRE
 EIKNIAIIGGGY
 IGIEMVEAAKNRKNVRLIQLDKHILIDSFDEEIVTIMEEELTKKGVNLHTNEFVKSLIGEKKAGGVV
 TNKNTYQADAVI
 LATGIKPDTEFLENQLKTTKNGAIIIVNEYGETSIKNIFSAGDCATIYNIVSKKNEYIPLATTANKLGR
 45 IVGENLAGNHTA
 FKGTLGSAKILSLEAARTGLTEKDAKRLQIKYKTIFVKDNHTNYYPGQEDLYIKLIYEENTKIIL
 GAQATGKNGAVM
 RIHALSIAIYSKLTTKELRMDFSYSPPFRTWDILNIAGNAAK

50

SEQ ID NO.: 11 BNOX K6

ATGATGAAAATAATAATTATTGGGGGCACATCAGCAGGAAGTAGTGCCGCAGCTAAAGCAAACCGCTT
 AAACAAAAAGCT
 AGACATTACTATCTATGAAAAACAAATATTGTATCTTTTGGAACCTGTGGCCTGCCTTACTTTGTGG
 55 GGGGATTCTTTG

ACAACCCCAATACAATGATCTCAAGAACACAAGAAGAATTCGAAAAAACTGGAATCTCTGTTAAAACT
 AACCACGAAGTT
 ATCAAAGTAGATGCAAAAAACAATACAATTGTAATAAAAAATCAAAAAACAGGAACCATTTTAAACAA
 TACTTACGATCA
 5 ACTTATGATAGCAACTGGTGCAAAACCTATTATTCCACCAATCAATAATATCAATCTAGAAAATTTTC
 ATACTCTGAAAA
 ATTTAGAAGACGGTCAAAAAATAAAAAATTAATGGATAGAGAAGAGATTAAAAATATAGTGATAATT
 GGTGGTGGATAC
 ATTGGAATTGAAATGGTAGAAGCAGCAAAAAATAAAAGAAAAAGTGTAAAGATTAATTCAACTAGATAA
 10 GCACATACTCAT
 AGATTCTTTGACGAAGAAATAGTCACAATAATGGAAGAAGAACTAACAAAAAGGGGGTTAATCTTC
 ATACAAATGAGT
 TTGTAAAAAGTTTAATAGGAGGAAAAAGGCAGAAGGAGTAGTAACAAACAAAAATACTTATCAAGCT
 GACGCTGTTATA
 15 CTTGCTACCGGAATAAACCTGACACTGAATTTTTAGAAAACCAGCTTAAAACTACTAAAAATGGAGC
 AATAATTGTAAA
 TGAGTATGGCGAAACTAGCATAAAAAATATTTTTCTGCAGGAGATTGTGCAACTATTTATAATATAG
 TAAGTAAAAAAA
 ATGAATACATACCCCTTGGCAACAACAGCCAACAACTTGAAGAATAGTTGGTGAAAATTTAGCTGGG
 20 AATCATAACAGCA
 TTTAAAGGCACATTGGGCTCAGCTTCAATTAAATACTATCTTTAGAAGCTGCAAGAACAGGACTTAC
 AGAAAAAGATGC
 AAAAAAGCTCCAAATAAAATATAAACGATTTTTGTAAAGGACAAAAATCATACAAATTATTATCCAG
 GCCAAGAAGATC
 25 TTTATATTAAATTAATTTATGAGGAAATACCAAAATAATCCTTGGGGCACAAGCAATAGGAAAAAT
 GGAGCCGTAATA
 AGAATTCATGCTTTATCAATTGCAATCTATTCAAAGCTTACAACAAAAGAGCTAGGGATGATGGATTT
 CTCATATTCCCCACCCCTTCTCAAGAACTTGGGATATATTAAATATTGCTGGCAATGCTGCCAAAtag

30

SEQ ID NO.: 12 BNOX K6

B6 protein sequence

35 MMKIIIIIGGTSAGTSAAAKA
 NRLNKKLDITIYEKTNIVSF
 GTCGLPYFVGGFFDNPNTMI
 SRTQEEFEKTGISVKTNHEV
 IKVDAKNNTIVIKNQKTGTIFNNTYDQLMIATGAKPIIPPINNINLENFHTLKNLEDGQKIKKLMRE
 40 EIKNIVIIGGGY
 IGIEMVEAAKNKRKSVRLIQLDKHILIDSFDEEIVTIMEEELTKKGVNLHTNEFVKSLIGGKKAEGVV
 TNKNYQADAVI
 LATGIKPDTEFLENQLKTTKNGAIIVNEYGETSIKNIFSAGDCATIYNIVSKKNEYIPLATTANKLGR
 IVGENLAGNHTA
 45 FKGTLSASIKILSLEAARTGLTEKDAKKLQIKYKTIFVKDKNHTNYYPGQEDLYIKLIYEENTKIIL
 GAQAIGKNGAVI
 RIHALSIAIYSKLTTKELGMMDFSYPFSSRTWDILNIAGNAAK

SFNOX and BNOX disclosed herein only share a modest 32% amino acid sequence
 50 identity in between themselves and only 34% identity to the NOXs of either *Enterococcus*
faecalis [48] or *Streptococcus mutans* [50], except for 55% between SFNOX and *E. faecalis*.
 The NOX coding genes from *Borrelia burgdorferi* (BNOX) and *Lactobacillus*

sanfranciscensis (SFNOX) were isolated from the genomic DNA using gene specific primers derived from the coding sequence, SEQ. ID. NO.: 13-16.

In Fig. 2A-2C, the complete nucleotide sequences of BNOX, BNOXK1 and BNOXK6 (SEQ ID NO.: 7, 9 and 11) as well as the respective deduced amino acid sequences (SEQ ID NO.: 8, 10 and 12) are shown. The nucleotide sequences are compared to the annotated sequence available in the data bank, BNOX. In Fig.2D-2F, both nucleotide (SEQ ID NO.: 1, 3 and 5) and deduced amino acid sequences (SEQ ID NO.: 2, 4 and 6) of SFNOX, SFNOXK2 and SFNOXK6 (SEQ ID NO.: 1, 3 and 5) are shown and are similarly compared to the annotated nucleotide sequence in the data bank, SFNOX. The decoration box indicates an exact match to the annotated sequences.

Comparison of the amino acid sequences between SFNOX and BNOX revealed a rather modest sequence identity of 32%. The consensus sequences are the FAD-binding site motif GXT(H/S)AG in position 8-14 (counted from the BNOX N-terminus), the putative catalytic cysteine residue in position 42, and the NAD-binding site GXGYIG in positions 156-161. Alignment with the sequences of the NADH oxidases of *Enterococcus faecalis* [48] and *Streptococcus mutans* [50] demonstrated at most 34% identity between any two including the two novel proteins, except for 55% between SFNOX and the enzyme from *E. faecalis*.

The present invention also comprises nucleic acids that hybridize under stringent conditions with the single-stranded (ss) nucleic acids or their complementary ss nucleic acids of the present invention. Stringent conditions are well known to those skilled in the art; see Sambrook et al., (Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), 1.101-1.104). Stringent conditions are established by conditions such as salt concentrations, temperature and amount of time for washing of the hybridized nucleic acids. For example, conditions include washing of hybridized nucleic acids in 0.1%SDS and 1.0x to 0.2xSSC, at temperatures from 50° C to 68° C, for times of 0.5 to 1.0 hours.

The present invention also comprises protein and nucleic acid sequences that exhibit a homology (exclusive of natural degeneration) greater than 80%, preferably greater than 90%, 91%, 92%, 93% or 94%, more preferably greater than 95% or 96%, and most preferably greater than 97%, 98% or 99% with SEQ. ID. NO.: 1-16, provided that the enzymatic activity is retained or the purpose of the sequence is retained, e.g. coding for a protein having this specific enzymatic activity or a protein fragment having a particular binding capability or immunogenic capability. Homology is defined by the equation $H(\%) = [1 - V/X] \times 100$, where H is homology, X is the total number of nucleotide bases or amino acids of the comparison

sequence, and V is the number of different nucleotide sequences or amino acids of the sequence of the comparison sequence. The term "nucleic acids coding for amino acid sequences" includes all nucleic acid sequences that could code for the amino acid sequences according to the degeneration of the genetic code. Additionally, nucleic acid sequences comprising modified, complexed or rare replacement nucleotides are comprised within the term nucleic acids. Nucleic acids comprise all types of nucleic acids, including single-stranded, double-stranded, nucleoproteins, sequences made with either deoxyribose or ribose (DNA or RNA) or mixtures thereof. Nucleic acids also comprise all corresponding interfering sequences, such as RNAi sequences, and antisense molecules.

In other embodiments, the present invention comprises primers for producing the gene sequences disclosed herein, for example, by amplification using methods known to those skilled in the art such as polymerase chain reaction. The primers include the sense and antisense primers coding for the corresponding amino acid sequences. Suitable primers may in principle be obtained by methods known to the person skilled in the art. The discovery of primers according to the invention is carried out by comparison with known DNA sequences or by translation of the visually detected amino acid sequences into the codon of the organism under consideration (e.g. for *Streptomyces*: Wright et al., *Gene* 1992, 113, 55-65). Common features in the amino acid sequence of proteins of so-called superfamilies are also of use for this purpose (Firestine et al., *Chemistry & Biology* 1996, 3, 779-783). Further information relating to the above may be found in "Oligonucleotide synthesis: a practical approach", edited by M. J. Gait, IRL Press Ltd, Oxford Washington D.C., 1984; PCR Protocols: A guide to methods and applications, edited by M. A. Innis, D. H. Gelfound, J. J. Sninsky and T. J. White. Academic Press, Inc., San Diego, 1990. The following primers are most preferred: Restriction sites used are underlined.

Primer sequences:

N- and C-terminal primers for *L. sanfranciscensis*

SEQ ID NO.:13 5' gcg c gaattc atg aaa gtt att gta gta ggt tgt act 3'

sanfransec, T_m 67.2°C

SEQ ID NO.:14 5' gcg c aagctt tta ttt atg tgc ttt gtc agc ttg tgc 3'

Sanfranashind, T_m 62.8°C

N- and C-terminal primers for *B. burgdorferi*

SEQ ID NO.:15 5' gcg c gg atc c at gat gaa aat aat aat tat tgg ggg 3'

Borrnoxs, T_m 69.5°C

SEQ ID NO.:16 5' gcg c aa gct t ct att tgg cag cat tgc cag caa tat t 3'

Borroxas, T_m 70.6°C

5 The compositions of the present invention comprise vectors, plasmids or constructs comprising one or more of the NOX of the present invention. The terms vectors, plasmids or constructs are used interchangeably to mean nucleic acid sequences having at least all or a portion of SFNOX (SEQ. ID NO.: 1, 3 and 5) or BNOX (SEQ.ID NO.: 7, 9 and 11), or all or a portion of a combination of any of SEQ ID NO.: 1, 3, 5, 7, 9 and 11. Such constructs may also have other sequences such as antibiotic resistance, the same or different promoters for
10 SFNOX or BNOX, and other sequences known to those skilled in the art.

Use of plasmids, vectors or constructs and different types of plasmids, vectors or constructs are well known in the art and the present invention contemplates inclusion of these uses and types with the sequences disclosed herein. Such art includes, but is not limited to, Sambrook, supra, or brochures from companies such as Novagen, Promega, New England
15 Biolabs, Clontech or Gibco BRL. Well known plasmids include pBTac (Roche Biochemicals), pKK-223 (Stratagene) or pET (Novagen).

The compositions of the present invention also comprise combinations of all or a portion of SEQ ID NO.: 1, 3, 5, 7, 9 and 11.with other nucleic acid sequences to encode chimera proteins, or the nucleic acids of NOX combined with proteins or attached to solid
20 supports such as beads. Such chimera proteins or other combinations may or may not retain the enzyme activity of SFNOX and/or BNOX. For example, a nucleic acid construct that codes for a chimera protein is constructed from SEQ. ID NO.: 1 and sequences for an antibody protein or binding fragment thereof. Such a chimera is used in antibody labeling experiments.

25 The present invention also comprises compositions comprising the NOX enzymes disclosed herein that include immobilization of the enzymes on heterogeneous substrates. For example, the enzymes may be immobilized or attached to other proteins, through methods such as chemical linking of the proteins, attached to inert substrates such as microtiter plates, chromatography materials, balls, beads or other substances. The invention
30 contemplates the use of such immobilized enzymes in methods of synthesis, measurement, analysis or other methods wherein enzymes are used. These methods for immobilizing and using such immobilized enzymes are known to those skilled in the art.

The compositions of the present invention also comprise antibodies and other specific binding partners, such as substrates, of SFNOX and BNOX, and immunogenic epitopes

thereof. Such antibodies may be polyclonal or monoclonal, and include fragments such as Fab, FC, heavy chains, light chains, constant, variable, or hypervariable fragments or regions, and any type of antibody include but are not limited to IgM, IgG, IgA, IgD, and IgE.

The compositions of the present invention also contemplate the inclusion of any
5 cofactors, metals or other compounds or molecules necessary for activity or stability of the NOX of the present invention.

The present invention also comprises microorganisms comprising the nucleic acids disclosed herein, particularly SEQ ID NO.: 1, 3, 5, 7, 9 and 11. The microorganisms in which the nucleic acids are cloned are useful for propagation and production of a sufficient
10 amount of the recombinant enzyme or enzymes. The methods for cloning, propagating and producing recombinant proteins in cellular systems are well known in the art. Examples of such microorganisms include, but are not limited to, prokaryotes or eucaryotes, such as Pseudomonas, Streptomyces, Arthrobacter, Bacillus, Staphylococcus, Enterococcus, especially E. coli, Candida, Hansenula, Pichia and baculovirus systems. Plasmids, vectors or
15 constructs containing the gene constructs of SEQ.ID.NO.: 1 and/or 3 are cloned into host organisms, such as those above.

The nucleic acids disclosed herein that code for the NAD(P)H oxidase (NOX) as described herein, are preferably suitable for the production of whole-cell catalysts. The invention provides a whole-cell catalyst containing a cloned gene for a dehydrogenase and a
20 cloned gene for an NAD(P)H oxidase. The whole-cell catalyst according to the invention should contain an NAD(P)H oxidase (NOX), preferably a bacterial oxidase that can regenerate NAD⁺ and NADP⁺. More preferably, the NAD(P)H oxidase is one or more of the NOX disclosed herein and coded for by SEQ ID NO.: 1, 3, 5, 7, 9 and 11.. The production of such an organism is known to the person skilled in the art (PCT/EP00/08473;
25 PCT/US00/08159).

The advantage of such an organism is the simultaneous expression of at least two different enzymes, and then only the whole cell catalyst recombinant organism is used for the enzymatic reaction. In order to match the expression of the enzymes with respect to their reaction rates, the coding nucleic acids may be carried on various plasmids having different
30 copy numbers and/or promoters of different strengths may be used. In one embodiment, the enzymes are encoded on plasmids with similar copy numbers in a host cell; and/or under the control of promoters of similar strength. With enzyme systems matched in this way there is advantageously no accumulation of a possible inhibiting intermediate compound(s), and the

reaction under consideration may proceed at an optimal overall rate. This is described in PCT/EP00/08473; and Gellissen et al., Appl. Microbiol. Biotechnol. 1996, 46, 46-54.

Methods of the present invention comprise methods for growing and isolating NOX proteins, particularly bacterial oxidases capable of regenerating NAD⁺ and NADP⁺. One embodiment comprises growing host organisms, *Lactobacillus sanfranciscensis* or *Borrelia burgdorferi*, and isolating the NOX enzyme by methods known to those skilled in the art, such as ammonium or acid precipitation, or chromatography, and other protein purification techniques. An embodiment comprises growing bacteria and isolating bacterial NOX that are capable of regenerating NAD⁺ and NADP⁺. Another embodiment comprises growing and isolating recombinant NOX proteins.

The nucleic acids according to the invention can be used for the production of recombinant (rec) NAD(P)H oxidase, which is included herein in the term NOX. Recombinant techniques known in the art can be used to produce the enzymes described herein in an amount sufficient for an industrial process from host cells carrying the nucleic acids encoding the enzyme. The production of the rec-enzymes according to the invention is carried out by genetic engineering processes as described in, for example, Sambrook supra, Balbas P & Bolivar F. 1990; Design and construction of expression plasmid vectors in *E. coli*, Methods Enzymology 185, 14-37; Vectors: A Survey of Molecular Cloning Vectors and Their Uses. R. L. Rodriguez & D. T. Denhardt, Eds: 205-225). With regard to the general procedure (PCR and fusion PCR, inverse PCR, cloning, expression etc.), reference may be made to the following literature and the references cited therein: Riley J, Butler R, Finnear R, Jenner D, Powell S, Anand R, Smith J C, Markham A F (1990). A novel, rapid method for the isolation of terminal sequences from yeast artificial chromosome (YAC) clones. Nucl Acids Res. 18, 8186; Triglia T, Peterson M G, Kemp D J (1988). A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res. 16, 8186; Sambrook J, Fritsch E F, Maniatis T (1989). Molecular Cloning. Cold Spring Harbour Laboratory Press; Vectors: A Survey of Molecular Cloning Vectors and Their Uses. R. L. Rodriguez & D. T. Denhardt, I I.

The bacterial oxidase enzymes described herein may be used in the free form as homogeneously purified compounds, or as enzymes produced by recombinant technology. Furthermore the enzymes may also be employed as a constituent of an intact host organism or in conjunction with the macerated cell mass of the host organism purified to an arbitrarily high degree. It is also possible to use the enzymes in immobilized form (Bhavender P. Sharma, Lorraine F. Bailey and Ralph A. Messing, "Immobilisierte Biomaterialien--

Techniken und Anwendungen", Angew. Chem. 1982, 94, 836-852). The immobilization is preferably carried out by lyophilisation (Dordick et al. J. Am. Chem. Soc. 194, 116, 5009-5010; Okahata et al. Tetrahedron Lett. 1997, 38, 1971-1974; Adlercreutz et al. Biocatalysis 1992, 6, 291-305). It is most particularly preferred to carry out the lyophilisation in the presence of surfactants such as aerosol OT, polyvinylpyrrolidone, polyethylene glycol (PEG) or Brij 52 (diethyleneglycolmonocetyl ether) (Goto et al. Biotechnol. Techniques 1997, 11, 375-378). The use as CLECs is also possible (St Clair et al. Angew Chem Int Ed Engl 2000 Jan, 39(2), 380-383).

The present invention comprises methods for isolating NOX. One embodiment comprises methods of isolation comprising the purification table shown in Table 2. The procedure results in a strong single prominent band at 50 kDa in the protein gel analysis, is scalable, and results in high yields. Acid precipitation as the first resolution eliminates buffer/salt exchanges and leaves the final protein preparation in stabilizing levels of ammonium sulfate. [63]

Table 2: Purification table resulting in scalability and high yield

Step	Vol (ml)	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification factor	Σ U
Lysate (pH5.0)	10.2	424.4	14.3	29.7	100.0	1.0	4329.3
Dialysis (60kDa MWCO)/Acid Precip pH 4.8	10.5	277.4	5.4	51.8	67.3	1.7	2912.7
Mono-Q	1.0	476.7	5.1	93.1	57.8*	3.1	476.7
45% ammonium sulfate dialysis	0.35	1114.1	8.4	132.6	47.3*	4.5	390.0

*estimated theoretical yield for entire preparation.

Another embodiment comprises the steps in a different sequence (lysate - 45% ammonium sulfate precipitation - acid precipitation (pH 5, 30°C) - Q-Sepharose FF) which, in one experiment, resulted in the same specific activity to within 0.5%. The yield of this alternative purification sequence was 33.6%.

Another embodiment for isolation of NOX comprises displacement chromatography after dialysis and acid precipitation, (like that described above). The displacer was naphthalene-1,3,6-trisulfonic acid, which provides for a method of isolation that can be scaled-up for industrial use. As the results in Table 3 reveal, purity in excess of 95% at 26%

yield was achieved with a specific activity of 221 U/mg protein. The highly pure and active fractions can then be pooled and stored in 45% ammonium sulfate solution at 4°C to preserve the enzyme's stability. Displacement chromatography generally improves at higher loadings [52] and the novel displacer, naphthalene-1,3,6-trisulfonic acid, is an inexpensive reagent in contrast to many other reported displacers [53].

A method for isolating NOX comprises, a) precipitating with an acid solution of pH 4.5 to 6.0, a bacterial cellular lysate; and b) isolating NOX proteins from the solution of b) after precipitation occurs. In general, precipitation in an acid solution inactivates most proteins in the cellular lysate, but not the NOX proteins. Precipitation begins as soon as the lysate is placed in the acidic solution. The range of pH of the acidic solution is from approximately 4.5 to approximately 6.0, preferably pH 4.5 to 5.5, and more preferably pH 5.0. The time for precipitation can be in a range from minutes to hours, including 10 minutes to 24 hours. Precipitating activity can occur at the same time as other activities such as salt removal in dialysis systems. The precipitated material contains inactivated proteins and the resulting solution contains the NOX proteins. Isolation of the NOX protein from the solution can be accomplished any number of different methods known to those skilled in the art. For example, NOX may be isolated by centrifugation of the solution, or centrifugation followed by other techniques such as displacement chromatography, sizing chromatography, affinity chromatography, molecular sieving, cofactor binding, or other techniques that isolate the NOX from the solution. These isolation methods are well known in the art and all applicable methods are contemplated as part of the present invention.

Table 3: Purification of NADH oxidase from *L. sanfranciscensis*

Step	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Σmg	ΣU	Purification Factor
Lysate (pH 5.0)	768.6	21.7	35.4	-	661.9	23,443	1.0
Dialysis (60 kDa MWCO membrane)/ Acid precip pH 5.0	582.2	9.0	65.0	79.5	286.7	18,629	1.8
Displacement Source 30Q	136.2	0.6	220.9	26.2	27.75	6,131	6.2

The ability of bacterial oxidases, such as SFNOX, to oxidize both cofactors, NADPH and NADH, renders such enzymes as an extremely useful catalyst for coupled enzymatically-catalyzed oxidations. To demonstrate the regeneration to either NAD^+ or NADP^+ by NAD(P)H oxidase SFNOX, the enzyme was combined with (R)-ADH from *L. brevis* to produce acetophenone and (S)-phenylethanol from racemic (RS)-phenylethanol. (R)-ADH from *L. brevis* [54] was picked for the following advantages: i) (R)-1-phenylethanol is a very good substrate, on a par with the best substrates of the enzyme, ii) whereas the wildtype is mainly NADPH-dependent, the G37D mutant strongly prefers NADH over NADPH [55], albeit at reduced specific activity; iii) lastly, (R)-ADH from *L. brevis* has been explored extensively for the enzymatic generation of several pharmaceutically interesting chiral alcohols [56-59,25].

In experiments described herein, after 12 h, nearly complete conversion (maximally 50% of racemic phenylethanol) was achieved in all but the case of the G37D mutant ADH with NAD^+ . The very high K_M -value of the mutant ADH for NAD^+ in comparison with the wildtype for NADP^+ is a possible explanation for the lower rate (less than $v_{\max}$) and thus lower conversion after 12 hr. The number of turnovers ($[\text{acetophenone}]/[\text{cofactor}]$) of up to more than 100 clearly demonstrates catalysis by both enzymes involved.

Methods of the present invention comprise coupled enzymatic reactions wherein bacterial oxidases, including but not limited to, SFNOX, provide both NAD^+ and NADP^+ to one or more enzymes. An embodiment of methods of using bacterial oxidases, including but not limited to, SFNOX, comprises use in analytical determinations such as in measuring the total amount of reducing equivalents from NAD^+ and NADP^+ in a cell by measuring the reactions of bacterial oxidases, including but not limited to, SFNOX, and NAD/NADP. Such measurements can be important to estimate the ability of a cell to achieve reduction of a given substrate. The reducing equivalent amount can provide an identifying characteristic of a cell or cell types. For example, such measurement could differentiate between normal, precancerous and cancerous cells, or between normal cells and cells entering apoptosis, or between different cellular types. Another embodiment comprises using bacterial oxidases, including but not limited to, SFNOX as a standard in NAD/NADP experiments.

One embodiment of the present invention comprises methods and compositions comprising recombinant NOX and nucleic acids encoding recombinant NOX that have been altered from genomic or recombinant sequences by mutation. One method comprises the production of improved rec-NOX and rec-NOX obtained thereby or nucleic acids coding

therefor, starting from the nucleic acids according to the invention coding for an NAD(P)H oxidase (NOX), such method comprising,

- a) mutating nucleic acids,
- b) cloning the nucleic acids obtained from a) in a vector, plasmid or construct;
- 5 and
- c) isolating the proteins expressed.

This process may be executed once or any desired number of times in succession. Preferably, the mutated nucleic acids code for proteins having a property different from the proteins encoded by the nucleic acids disclosed herein, more preferably, the mutated rec-NOX have
10 enhanced desired properties such as faster cofactor turnover or more stability in reaction conditions.

Embodiments of mutations of the present invention comprise individual amino acid substitutions, and its concomitant changes in the nucleic acid sequence. Preferred
15 embodiments comprise mutated sequences comprising at least a substitution at position 42 of SFNOX. For example, mutations of position of 42 of C to S, C to M, C to A and C to F. (for example, SEQ ID NO. 17-20), and the nucleic acids, including degenerate bases, that encode such amino acids. Embodiments of the present invention comprise other amino acid substitutions at this site, and such mutations include substitution or unnatural amino acids, such as homoserine, or unnatural nucleic acids.

20

SEQ ID NO. 17

MKVIVVGCTHAGTFAVKQTI
ADHPDADVTAYEMNDNISFL
SSGIALYLGKEIKNNDPRGLFYSSPEELSNLGANVQMRHQ
25 VTNVDPETKTIKVKDLITNEEKTEAYDKLIMTTGSKPTVPPIPGIDSSRVYLCKNYNDAKKLFEEAPK
AKTITIIGSGYI
GAELAEAYSNQNYNVNLIDGHERVLYKYFDKEFTDILAKDYEAHGVNLVLGSKVAAFEEDDEIITKT
LDGKEIKSDIAI
LCIGFRPNTELLKGKVAMLDNGAIITDEYMHSSNRDIFAAGDSAAPHYNPTNSNAYIPLATNAVRQGR
30 LVGLNLTEDKVK
DMGTQSSSGLKLYGRYVSTGINTALAKANNLKVSEVIIADNYRPEFMLSTDEVLMMLVYDPKTRVIL
GGALSSMHDVSQ
SANVLSVCIQNKNTIDDLAMVDMLFQPQFDRPFNYLNILGQAAQAQADKAHK

35 SEQ ID NO. 18

MKVIVVGCTHAGTFAVKQTI
ADHPDADVTAYEMNDNISFL
SMGIALYLGKEIKNNDPRGLFYSSPEELSNLGANVQMRHQ
VTNVDPETKTIKVKDLITNEEKTEAYDKLIMTTGSKPTVPPIPGIDSSRVYLCKNYNDAKKLFEEAPK
40 AKTITIIGSGYI
GAELAEAYSNQNYNVNLIDGHERVLYKYFDKEFTDILAKDYEAHGVNLVLGSKVAAFEEDDEIITKT
LDGKEIKSDIAI

LCIGFRPNTELLKGKVAMLDNGAIITDEYMHSSNRDIFAAGDSA AVHYNPTNSNAYIPLATNAVRQGR
 LVGLNLTEDKVK
 DMGTQSSSGLKLYGRITYVSTGINTALAKANNLKVSEVIIADNYRPEFMLSTDEVLM SLVYDPKTRVILGGALSSM
 HDVSQ

5 SANVLSVCIQNKNTIDDLAMVDMLFQPQFDRPFNYLNILGQAAQAQADKAHK

SEQ ID NO. 19

MKVIVVGCTHAGTFAVKQTI

ADHPDADVTAYEMNDNISFL

10 SAGIALYLKGEIKNNDPRGLFYSSPEELSNLGANVQMRHQ

VTNVDPETKTIKVKDLITNEEKTEAYDKLIMTTGSKPTVPPIPGIDSSRVYLCKNYNDAKKLFEEAPK

AKTITIIIGSGYI

GAELAEAYSNQNYNVNLIDGHERVLYKYFDKEFTDILAKDYEAHGVNLVLGSKVAAFEEDDEIITKT

LDGKEIKSDIAI

15 LCIGFRPNTELLKGKVAMLDNGAIITDEYMHSSNRDIFAAGDSA AVHYNPTNSNAYIPLATNAVRQGR

LVGLNLTEDKVK

DMGTQSSSGLKLYGRITYVSTGINTALAKANNLKVSEVIIADNYRPEFMLSTDEVLM SLVYDPKTRVIL

GGALSSMHDVSQ

SANVLSVCIQNKNTIDDLAMVDMLFQPQFDRPFNYLNILGQAAQAQADKAHK

20

SEQ ID NO. 20

MKVIVVGCTHAGTFAVKQTI

ADHPDADVTAYEMNDNISFL

SFGIALYLKGEIKNNDPRGLFYSSPEELSNLGANVQMRHQ

25 VTNVDPETKTIKVKDLITNEEKTEAYDKLIMTTGSKPTVPPIPGIDSSRVYLCKNYNDAKKLFEEAPK

AKTITIIIGSGYI

GAELAEAYSNQNYNVNLIDGHERVLYKYFDKEFTDILAKDYEAHGVNLVLGSKVAAFEEDDEIITKT

LDGKEIKSDIAI

LCIGFRPNTELLKGKVAMLDNGAIITDEYMHSSNRDIFAAGDSA AVHYNPTNSNAYIPLATNAVRQGR

30 LVGLNLTEDKVK

DMGTQSSSGLKLYGRITYVSTGINTALAKANNLKVSEVIIADNYRPEFMLSTDEVLM SLVYDPKTRVIL

GGALSSMHDVSQ

SANVLSVCIQNKNTIDDLAMVDMLFQPQFDRPFNYLNILGQAAQAQADKAHK

35 Embodiments of mutations of the sequences and resulting proteins disclosed herein
 also include, but are not limited to, substitutions at other sites, insertions, deletions, additions
 and reversions, changes due to recombination of NOX sequences or sequences comprising
 NOX sequences with other nucleic acids, and other mutations known to those skilled in the
 art.

40 The procedure for mutating the enzymes of the present invention by mutagenesis
 methods has long been known to the person skilled in the art. As mutagenesis methods there
 may be used all methods for this purpose available to the person skilled in the art. In
 particular these include saturation mutagenesis, random mutagenesis, shuffling methods as
 well as site-directed mutagenesis (Eigen M. and Gardinger W. (1984) Evolutionary molecular
 45 engineering based on RNA replication. Pure & Appl. Chem. 56(8), 967-978; Chen & Arnold
 (1991) Enzyme engineering for nonaqueous solvents: random mutagenesis to enhance
 activity of subtilisin E in polar organic media. Bio/Technology 9, 1073-1077; Horwitz, M.

and L. Loeb (1986) "Promoters Selected From Random DNA-Sequences" Proceedings Of The National Academy Of Sciences Of The United States Of America 83(19): 7405-7409; Dube, D. and L. Loeb (1989) "Mutants Generated By The Insertion Of Random Oligonucleotides Into The Active-Site Of The Beta-Lactamase Gene" Biochemistry 28(14): 5703-5707; Stemmer PC (1994). Rapid evolution of a protein in vitro by DNA shuffling. Nature. 370; 389-391 and Stemmer P C (1994) DNA shuffling by random fragmentation and reassembly: In vitro recombination for molecular evolution. Proc Natl Acad Sci USA. 91; 10747-10751).

The new nucleic acid sequences that are obtained are cloned according to the methods known to those skilled in the art in a host organism and the expressed enzymes are detected and then isolated using suitable screening methods (Roberts J., Stella V. J. and Decedue C. J. (1985) A colorimetric assay of pancreatic lipase: rapid detection of lipase and colipase separated by gel filtration. Lipids 20(1): 42-45; Pratt R. F., Faraci W. S. and Govardhan C. P. (1985) A direct spectrophotometric assay for D-alanine carboxypeptidases and for the esterase activity of beta-lactamases. Anal. Biochem. 144(1): 204-206; Bruckner, H., R. Wittner, and H. Godel (1991) Fully automated high-performance liquid chromatographic separation of DL-amino acids derivatized with o-Phthaldialdehyde together with N-isopropyl-cysteine. Application to food samples).

The present invention also comprises using NAD(P)H oxidase (NOX), bacterial oxidases with NAD⁺ and NADP⁺ regeneration activity, SFNOX, BNOX and the proteins encoded by SEQ ID NOs 1-12 and 17-20 and any mutations thereof, for the production of chiral enantiomer-enriched organic compounds such as, for example, alcohols or amino acids, in coupled enzymatic reactions. Such compounds are useful in pharmaceutical preparations, in agricultural uses, for food, and crop protection industries as well as building blocks for novel compounds not accessible through fermentation and for asymmetric synthesis templates. Embodiments of such methods comprise a method of organic synthesis, comprising, reacting a bacterial NAD(P)H oxidase with NADH or NADPH in a coupled enzyme reaction, and isolating the products of the reaction. Such methods of use include the synthesis of enantomerically-enriched chiral compounds, synthesis of chiral compounds, synthesis of physiologically effective compounds that are used in treatments of humans, animals, plants, insects, microbiological organisms, and other eukaryotes and prokaryotes. For example, compounds are produced that are effective in treatment of humans and other animals for hypertension, diabetes, cardiovascular disease, cancer, and conditions involving the brain, eyes, heart, lungs, liver, immune system, urinary organs, reproductive organs, integumentary system, nervous system and

other conditions where pharmaceutical agents are effective. Compositions that are effective in such methods include compositions comprising at least a bacterial oxidase that regenerates NADP⁺ or NAD⁺, and preferably comprise at least a bacterial oxidase that regenerates NADP⁺ and NAD⁺.

5 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of molecular biology. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein. All publications, patent applications, patents, and other references
10 mentioned herein are incorporated by reference in their entirety. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

The present application claims priority to U.S. Provisional Patent Application 60/399, 850, the entire contents of which are incorporated herein by reference. Additionally, the references cited herein are each hereby incorporated by reference in its entirety.

15 Having generally described this invention, a further understanding can be obtained by reference to certain specific examples. The examples are not intended to limit, and should not be interpreted as limiting, the scope of what the inventors regard as their invention.

EXAMPLES

20

EXAMPLE 1

Bacterial strains, media and growth conditions

The genomic DNA from *Borrelia burgdorferi* (ATCC 35210) and the strain *Lactobacillus sanfranciscensis* (ATCC 27651) were obtained from ATCC and grown in MRS
25 medium (Gibco) at pH 6.5 under facultatively anaerobic conditions at 30°C in quiescent culture. For expression of wild-type NADH oxidase, the *L. sanfranciscensis* strain was grown in the same medium, but under aeration with 120 rpm in an Infors shaker at 30°C.

Host strains of *E.coli* were grown in Luria-Bertani medium at pH 7.5 and 37°C, for cloning purposes or routine growth and plasmid production the host strain XL1 blue
30 (Stratagene, La Jolla) was used. For expression purposes an HB101 strain (Stratagene, La Jolla) or M15 strain including the pREP4 plasmid (Qiagen, Hilden) was employed. These *E.coli* strains were grown at 30°C under agitation for optimized expression levels. Ampicillin was added to the medium at a final concentration of 100µg/ml to maintain

selection pressure. To the M15 strain, 25 μ g/ml kanamycin was added to maintain the additional helper plasmid.

Plasmids used: for cloning and sequencing, target genes were cloned into pBluescript (Stratagene, La Jolla); for expression either the pkk223-3 (Amersham) or pBTac2 (Roche) were chosen.

EXAMPLE 2

Manipulation and amplification of DNA

The nox DNA sequences were identified using a search of the NCBI Genebank (Accession files AB035801 for SFNOX and NC_001318 for BNOX). The corresponding specific 5' and 3' primers were synthesized at MWG Biotech (High Point, NC).

Primer optimization was performed using a primer design program (webbased design, <http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer>). The nox genes from *L.sanfranciscensis* and *B. burgdorferi* were amplified using PCR and the gene-specific primers. Restriction sites used are underlined.

Primer sequences:

N- and C-terminal primers for *L.sanfranciscensis*

5' gcg c gaattc atg aaa gtt att gta gta ggt tgt act 3' sanfransec0 T_m 67.2°C

5' gcg c aagctt tta ttt atg tgc ttt gtc agc ttg tgc 3' sanfranashind T_m 62.8°C

20 N- and C-terminal primers for *B. burgdorferi*

5' gcg c gg atc c at gat gaa aat aat aat tat tgg ggg 3' bormnoxs T_m 69.5°C

5' gcg c aa gct t ct att tgg cag cat tgc cag caa tat t 3' bormnoxas T_m 70.6°C

Amplification of the target DNA was performed using the protocol from the failsafe PCR kit (Epicentre, Madison). Twelve reactions using 12 different buffers were set up and tested for optimal conditions. Setting up the PCR reactions involved final DNA concentration of 100 ng (*L.sanfranciscensis*) and 3.4 ng (*B. burgdorferi*), 200 μ M of each dNTP, 10 μ M of each primer and 1 U of Taq polymerase (Epicentre, Madison) in a final volume of 25 μ l. To each of these reactions, 25 μ l of each of the twelve doubly concentrated reaction buffer was added. DNA was amplified successfully for 30 cycles in an Eppendorf Gradient Thermocycler (Eppendorf, Hamburg) using the following conditions: each cycle involved a denaturing step at 30 sec 94°C, an annealing step at 30 sec 60°C or 68°C, and an extension step at 2 min 72°C. Of the final reaction mixture, 50 μ l was analyzed on 1% agarose gels stained with 0.05% ethidium bromide. Prior to any further use, these PCR products were gel purified using the gel extraction kit (Qiagen, Hilden).

Amplification in PCR succeeded using the PCR failsafe kit (Epicentre, Madison) to yield products of the predicted size of 1335bp for bnox and 1356bp for sfnox in several of the 12 buffers provided with the kit. The primers were designed to contain convenient restriction sites (EcoR1 and HindIII for SFNOX and BamH1 + HindIII as well for BNOX) at both ends to facilitate the following cloning step.

DNA electrophoresis on a 1% agarose gel demonstrates amplification of the nox genes under different PCR-buffer conditions. As expected, single bands in each lane were found at around the 1300 bp band for BNOX and between 1300 and 1400bp for the SFNOX. Depending on buffer conditions, strong or weak amplification was observed with both NOX genes. Each one of the strongest bands was cut out of the 1% agarose gel and purified using the gel purification kit (Qiagen, Hilden).

Both gene products as well as the pbluescript vector (Stratagene) were restricted with the following enzymes: BNOX with BamH1 and HindIII and SFNOX with EcoR1 and HindIII. Following restriction, the genes and the vector were purified through gel electrophoresis and subsequent elution (gel purification kit, Qiagen, Hilden). Both genes were separately cloned into the pbluescript vector (Stratagene, La Jolla) and transformed into the *E.coli* XL1blue strain (Stratagene, La Jolla). Positive clones were screened using colony PCR and restriction analysis.

Nucleotide data corresponding to the 1335bp of BNOX and 1356bp for SFNOX, starting with ATG, were obtained through cycle sequencing using an ABI prism sequencer. Nucleotide sequence and deduced open reading frames are shown in Figure 2. Sequencing templates were the pbluescript-constructs. The open reading frame for both noxes is capable of encoding a protein with a molecular mass of 48.8 kD for SFNOX and 48 kD for BNOX. SDS-PAGE of the proteins derived from the expressed genes exhibited a prominent band at around 45-50 kD. The GC content of the genes coding for BNOX and SFNOX are very low, 32 % and 37 %, respectively, consistent with the range reported by Ross and Claiborne (1992).[48]

EXAMPLE 3

Cloning

Nox-specific DNA from *L.sanfranciscensis* was ligated into pBluescript (Stratagene, La Jolla) using EcoR1 (5') and HindIII (3') restriction sites and accordingly, nox from *B. burgdorferi* with BamH1(5') and HindIII (3') restriction sites. For all necessary ligations the Rapid Ligation kit protocol (Roche, Penzberg) was followed. The same pmol amounts of

DNA were ligated, concentrations were calculated accordingly using the spectrophotometrically determined 260/280 nm ratio. Wildtype or mutant expression clones were constructed with the same restriction sites of Nox-*L-sanfranciscensis* (Lsfnox) into pkk223-3 (Amersham, Piscataway, NJ) and nox-*B-burgdorferi* (Bnox) into pBtac2 (Roche, Penzberg). Positive clones were tested either through colony PCR or restriction digest after plasmid preparation using the Miniprep Spin kit (Qiagen, Hilden).

EXAMPLE 4

Colony PCR

Colonies of the transformation plate were picked and first transferred onto a master plate, then suspended into 50 μ l of lysis buffer containing Triton-X-100 (20 mM Tris pH 8.5 + 5 mM EDTA + 1% Triton X-100). After denaturing at 95°C for 15 min, the solution was vortexed for 10 seconds and then 5 μ l of the extract was tested in PCR (total volume 50 μ l) using the gene specific primers.

EXAMPLE 5

Plasmid preparation

5 ml LB_{amp} was inoculated with a colony and grown overnight at 37°C. Cells were harvested by centrifugation (10000 rpm, 5 min, Eppendorf centrifuge, Hamburg) and plasmid DNA was isolated following the manufacturer's protocol (Miniprep Spin Kit, Qiagen, Hilden). Plasmid DNA was eluted into 50 μ l water and 5 μ l were digested with the corresponding restriction enzymes at the sites used for cloning and ligating.

EXAMPLE 6

Sequencing

20 μ g of plasmid DNA (using the pBluescript vector) was sent off for sequencing using the same primers as for amplification in PCR. The templates were labeled with Applied Biosystems' "BigDye Terminator v3.0 Cycle Sequencing Ready Reaction" Kit for 25 cycles. Excess dye terminator molecules were removed with Qiagen Dye-Ex Spin Columns (Qiagen, Hilden). The samples were analyzed on the Applied Biosystems 3100 Genetic Analyzer (Perkin-Elmer-AB, Boston).

Sequence analysis of both SFNOX and BNOX genes revealed differences when compared to the annotated nucleotide sequences derived from the NCBI databank (accession files AB035801 for SFNOX and NC_001318 for BNOX). Both fully sequenced SFNOX

clones, SFNOXK2 and SFNOXK6, featured an amino acid change from alanine to valine at position 30 (A30V). SFNOXK6 showed an additional change from lysine to arginine at position 102 (K102R). Both constructs, when overexpressed, showed comparable activity. Though not wishing to be bound by any particular theory, it is believed that position 102 does not diminish enzyme activity and that SFNOXK2 with its sequence difference in position 30 shows the correct sequence for a NADH oxidase from *L. sanfranciscensis* rather than the sequence annotated in the databases.

EXAMPLE 7

10 Expression of the nox genes

The pbluescript constructs were used to cut out the desired gene and subclone it into the expression vector pkk223-3 (Amersham) or pBTac2 (Roche), respectively. With this method no additional PCR was required and risk for additional PCR errors was avoided. Subcloning was successful using the Rapid DNA ligation kit (Roche) and the ligation was transformed into competent HB101 (Stratagene, La Jolla) or M15 *E.coli* strains (Qiagen, Hilden). Colonies formed were tested for successful incorporation through colony PCR.

Two successful clones of each construct were expressed at 37°C and harvested after 4 h of IPTG induction (SFNOXK2 and SFNOXK6 for *L.sanfranciscensis* and BNOXK1 and BNOXK6 for *B. burgdorferi*). Cell density was equalized to an OD₆₀₀ of 5.0 and then ultrasonicated in 200 µl of 100 mM TEA pH 7.5 buffer. Equal amounts of each fraction, soluble and insoluble, induced and uninduced, were loaded onto a 12.5 % SDS-PAGE. At 37°C, the SFNOXK6 clone demonstrates a high level of overexpression in the insoluble fraction, possibly owing to the additional mutation. BNOXK1 does not show an overexpression, and the expression level of BNOXK6 is slightly lower than that of SFNOXK2. In the case of SFNOXK2, the addition of helper plasmid pREP4 resulted in less uninduced expression when compared to the same clone without the helper plasmid.

Heterologous expression of the nox genes in *E.coli* was performed as follows: 5 ml starter LB_{amp} cultures were inoculated with aliquots from frozen stock cultures containing either bnox-pBTac2 or sfnox-pkk223-3 and grown overnight at 37°C. These starter cultures were used to inoculate 200 ml cultures (1% v/v) or 1 L cultures (1 % v/v), which were vigorously aerated until A₆₀₀ reached 0.5 -0.6, at which point the cultures were induced with 1 mM IPTG (final concentration) and protein expression was performed for 4 h. Cells were harvested and pellets frozen away at -20°C or used directly for enzyme activity assays.

For SDS-PAGE, 5 ml cultures were grown up to A_{600} of 0.5 and then induced with 1 mM IPTG for 4h. Cells were harvested, resuspended in 200 μ l TE50/50 (50mM Tris, 50 mM EDTA pH 8.0), and sonicated for 2 x 15 sec with ice cooling. Supernatant (representing the soluble fraction) was separated after centrifugation and the insoluble fraction was resuspended in 200 μ l TE 50/50 and shortly sonicated (5 sec) to dissolve the pellet. 10 % SDS sample buffer was added (10% Glycerol, 2% SDS, 0.063 M Tris/HCl pH 6.8, 0.1% Bromphenolblue + either 10 % β -Mercaptoethanol or 75 mM DTT) and 30 μ l analyzed on a 12.5% SDS-PAGE, stained with Coomassie blue (Pierce gel code staining solution, Pierce, Rockford, IL). Standard proteins used for molecular mass determination were obtained from New England Biolabs (Beverly, MA; broad range molecular weight markers, prestained).

Protein Gel Analysis

Prior to SDS-PAGE, protein samples were diluted to 2 mg/mL concentration in deionized water if the initial concentration was above 2 mg/mL. The 50 μ L diluted samples were then mixed with 50 μ L of 2X sample buffer composed of 125 mM Tris-HCl, pH 6.8, 4% SDS, 50% glycerol, 0.02% bromophenol blue, and 10% 2-mercaptoethanol. Mixed samples were incubated at 100°C for 5 minutes and then placed on ice. 10 to 20 μ L of the samples were loaded onto a 12% PAGER™ Gold precast gel and run in a Hoefer SE260 chamber at 125V for 2 hours (running buffer: 25 mM Tris base, 192 mM glycine, 0.1% SDS) with chilling water circulating at 4°C. Molecular weight standards ProSieve® from BMA or Precision Plus Protein™ standards from Bio-Rad were added to lanes immediately adjacent to the sample lanes.

Determination of protein concentration

Protein concentration was determined by the Bradford method utilizing Coomassie Plus Protein assay reagent and pre-diluted protein assay standards-BSA (Pierce Chemical) for the calibration curve. Coomassie blue from Pierce (gelcode blue stain reagent, Pierce, Rockford, IL) were used in staining.

EXAMPLE 8

Enzyme assays

Nox activity assay: Cell-free extracts of the recombinant sfnox and bnox *E. coli* strains were prepared using ultrasonication described above in 0.1 M TEA pH 7.5 + 5 mM DTT or β -mercaptoethanol. Nox activities were assayed at 30°C in a total volume of 1 mL at 340 nm using the following conditions: in 0.1 M TEA pH 7.5 a final concentration of 0.2 mM

NADH was dissolved and 10 μ l enzyme solution was added. Enzyme reaction was followed for 1 min, activity was calculated using an extinction coefficient ϵ of NADH of 6.22 L/(mol-cm).

5 EXAMPLE 9

Fermentation

Production strains were grown in 5 ml cultures at 37°C and 250 rpm in 15 ml disposable culture tubes to 1.0 OD 600 nm in LB media + 100 ug/ml ampicillin. One liter cultures of LB medium supplemented with 5 g/L glycerol were seeded with 1 ml of the starter
10 culture and grown at 30°C and 200 rpm. Both baffled and unbaffled Fernbach shake flask were used for fermentation. When the cultures reached 1.0 OD 600 nm the flask were induced by addition of 0.5 mM IPTG and grown for an additional 3-4 hours. Additional ampicillin, 200 μ g/ml, was added at induction and every hour thereafter to maintain selection pressure on the culture. When helper plasmids were present in the strains 50 μ g/ml
15 kanamycin was also added to the culture. Cultures were harvested by centrifugation at 5000 rpm in 1 L centrifuge containers (Beckman J2-M) and the resulting cell pellet was frozen at -80C.

EXAMPLE 10

20 Purification of sfnoxK2 enzyme

Frozen cell pellets were thawed and resuspended in 10 ml of 100 mM potassium phosphate buffer pH 6.8 +1 mM EDTA + 5 mM DTT + 5mM spermine. The cell slurry was then sonicated with a Fisher Scientific 60 Sonic dismembrator for 6x2 minutes while floating the tube in ice water for cooling. The resulting lysate was centrifuged at 18,000 rpm in a
25 Beckman J2-21M for 45 minutes at 4°C. The clarified lysate was then loaded into Spectro/Por® regenerated cellulose dialysis membrane tubing (60K MWCO) and dialyzed against 1 L of 45% ammonium sulfate + 50mM potassium phosphate buffer pH 6.8 + 1mM EDTA + 5 mM DTT. After four hours the sample was transferred to a second freshly prepared 45% ammonium sulfate solution. Following an additional 8 hours of dialysis
30 (overnight), the sample was centrifuged at 18,000 rpm for 15 minutes at 4°C. The resulting solution was transferred to a Pierce Slide-A-Lyzer® dialysis cassette (10K MWCO) and dialyzed versus 20 mM 1-methylpiperazine buffer pH 5.0 and 30°C + 5mM DTT. The sample was dialyzed versus a liter of buffer for two hours at 30°C with stirring (200 rpm) on

a digital magnetic stirplate/heater with a temperature probe to maintain the solution at 30°C. A buffer exchange was performed after one hour of dialysis. The sample was then transferred and centrifuged at 18,000 rpm for 15 minutes at 4°C. The resulting solution was then loaded onto a Amersham Pharmacia HiPrep 16/10 Q FF column on an AKTA system at 5 4°C. A gradient separation was performed from 0 to 100% 1M NaCl with the running buffer 20 mM 1-methylpiperazine buffer pH 5.0 at 4°C. 5 ml fractions were collected over the course of the run and the nine most active fractions were pooled.

A second purification protocol utilized 100 mM 1-methylpiperazine buffer pH 5.0 in the lysis buffer. Frozen cell pellets were thawed and resuspended in 10 ml of 100 mM - 10 methylpiperazine buffer pH 5.0 +1 mM EDTA + 5 mM DTT + 5 mM spermine. The cell 10 slurry was then sonicated with a Fisher Scientific 60 Sonic dismembrator for 6x2 minutes while floating the tube in ice water for cooling. The resulting lysate was centrifuged at 18,000 rpm in a Beckman J2-21M for 45 minutes at 4°C. The clarified lysate was then loaded into Spectro/Por® regenerated cellulose dialysis membrane tubing (60K MWCO) and 15 dialyzed with 1 L of 20 mM 1-methylpiperazine buffer pH 5.0 at 35°C + 5mM DTT. The sample was dialyzed versus 1 L of buffer for two hours at 35°C with stirring (200 rpm) on a digital magnetic stirplate/heater with a temperature probe to maintain the solution at 35°C. A buffer exchange was performed after one hour of dialysis. The sample was then transferred and centrifuged at 18,000 rpm for 15 minutes at 4°C. The resulting solution was then loaded 20 onto a Amersham Pharmacia Mono-Q column on an AKTA system at 4°C. A gradient separation over 10 column volumes was performed from 0 to 100% 1M NaCl with the running buffer 20 mM 1-methylpiperazine buffer pH 5.0 at 4°C. 1 mL fractions were collected over the course of the run and the most active fraction was dialyzed versus 45% ammonium sulfate + 50 mM potassium phosphate buffer pH 6.2 + 1 mM EDTA + 5 mM 25 DTT. After four hours the sample was transferred to a second freshly prepared 1.5 liters of 45% ammonium sulfate solution. Following an additional 4 hours of dialysis the sample was centrifuged at 18,000 rpm for 15 minutes at 4°C.

EXAMPLE 11

Hydrogen peroxide assay

A novel use of an assay for H_2O_2 , based on fluorescence of resorufin rather than on UV-VIS spectroscopy of ABTS (2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]) or o-dianisidine, has been employed successfully to demonstrate that both NADH oxidases of the present invention form H_2O instead of H_2O_2 as co-product. With its detection limit of 100 nM, the test based on 9-acetyl-resorufin ("Amplex Red") is much more sensitive than the other assays mentioned. Horseradish peroxidase (HRP)-catalyzed oxidation of 9-acetyl resorufin ("Amplex Red") to fluorescent resorufin was the assay. Amplex Red reacts with H_2O_2 according to a strict 1:1 stoichiometry. The resorufin assay (λ_{max} : 587 nm (emission), $\epsilon = 54,000 \text{ L}(\text{mol}\cdot\text{cm})^{-1}$) with its extremely low detection limit of 100 nM resorufin product is much more sensitive than other assays, such as ABTS or o-dianisidine.[60-62].

For the sensitive assay of putative hydrogen peroxide formation during the reaction of NADH oxidase the horseradish peroxidase-catalyzed oxidation of 9-acetyl resorufin was employed. The Amplex Red hydrogen peroxide assay kit (A-22188) from molecular probes was utilized for these assays. Following the protocols outlined in the kit instructions, a standard curve of H_2O_2 was prepared in the reaction buffer (50 mM sodium phosphate buffer pH 7.4) from the peroxide stock. The prepared concentrations were 20, 10, 5, and 2.5 μM H_2O_2 and 0 as a control. A working solution of 100 μM Amplex Red reagent and 0.2 U/ml horseradish peroxidase (HRP) was prepared in the reaction buffer as per kit instructions. As NADH and other reducing reagents are known to interfere with the amplex red assay, the NADH oxidase enzymes were allowed to react with the substrate immediately prior to the Amplex Red analysis. Reaction buffer from the kit was utilized in running enzyme test with 300 μM NADH as well as the controls without NADH. The enzyme conversions were performed by adding 3 μl of enzyme prep to 3 ml of reaction buffer with 300 μM NADH, mixing, and following the conversion until completion by absorbance at 340 nm. 50 μl of the final reaction mixture and standard curve solutions were added to each well in a 96 well fluorescence plate (costar, black, pp). Five replicates were made per sample and standard curve point. 50 μl of the Amplex Red reagent was added to each well and incubated for 30 minutes at 30°C. Fluorescence readings were performed in a BMG FLUOstar Galaxy micro plate reader with 544ex/590em filter settings.

0.6 μM resorufin above background was detected (and thus an equal concentration of H_2O_2 formed) upon conversion of 300 μM NADH with SFNOX (0.2% yield) but could not detect any resorufin above background in our experiment with BNOX. The value found for SFNOX was above the detection limit of 0.25 μM [60] so it might indicate leakage of H_2O_2 which is formed during the operation of NADH oxidase. Nevertheless, any H_2O_2 formed only constitutes a very minor component of the product flux of SFNOX, showing that water indeed is the co-product formed during the NADH oxidase reaction of SFNOX and BNOX.

EXAMPLE 12

10 Kinetics of cofactor substrates

The kinetic analysis was performed on ammonium sulfate fractions from both the sfnox and bnox strains in 50 mM HEPES buffer pH 7.0 at 30°C. The initial enzyme fractions were diluted in 25 mM HEPES pH 7.0 at 30°C + 25% glycerol + 5mM DTT to approximately -0.05 A_{340nm}/min and retained on ice during analysis. Conversion of NAD(P)H was followed by change of absorbance at 340 nm in a Jasco V-530 spectrophotometer. 3 mL methylacrylate disposable cuvettes were used for all experiments and all runs were performed in triplicate at 30°C. Reactions with NADH were started by adding 3 μL of enzyme preparation, 9 μL for NADPH, to the cuvette and mixing by inversion with parafilm three times. Varying concentrations of NAD(P)H substrate were made by preparing 100 mL of a 300 μM solution in a volumetric flask. Dilutions of this solution were then made to provide the differing substrate concentrations for the kinetic profile.

Regarding the kinetics SFNOX, it was found that both NADH and NADPH bind rather tightly, as judged by the low K_M value of 6.7 μM . Surprisingly, however, NADPH turned out to be almost as good a substrate as NADH: its v_{max} of 11 U/mg (at pH 7.0) is about a quarter of the value for NADH with 39.3 U/mg. SFNOX is much more active in comparison with BNOX: at comparable degree of purity, the latter only has a v_{max} of 2.03 U/mg, and furthermore does not accept NADPH as a substrate.

Investigation of kinetic parameters with NADH and NADPH cofactors as substrates was performed with the supernatant of the 45% ammonium sulfate cut (40% for BNOXK6) in air-saturated solution at 30°C and pH 7.0 in 0.1 M HEPES buffer. Figure 3 A-C demonstrates that not only does the SFNOX accept NADPH as a substrate with good reactivity (v_{max} = 11 U/mg), about 30% of activity towards NADH (v_{max} = 39.3 U/mg), but nearly identical K_M values of 6.7 and 6.1 μM indicate similar binding affinity. In contrast,

BNOX only accepts NADH and at a higher K_M value of 22.0 μM than SFNOX. Chi values and error bars reveal high accuracy with < 10% error in most cases. Fig. 3 A-C shows the kinetics of SFNOX and BNOX with NAD(P)H cofactor in air-saturated solution at pH 7 and 30° C.

- 5 The activity profile as a function of pH showed a surprising feature: instead of a bell-shaped curve a bimodal curve with a minimum around pH 5.5 was found. As this pH value is very close to the calculated pI value of pH 5.4, it is believed that the enzyme is not active and/or not stable at its pI value. As a pH optimum in the acidic range is not very common, the superposition of pH optimum and pI does not happen frequently.
- 10 With the supernatant of the 45% ammonium sulfate cut, an activity- pH profile was measured for SFNOX (Figure 4). The pH optimum of activity was found at pH 5.2. Below pH 5, activity decreased markedly and reached zero at pH 4.5. Rates at pH 4.5 to 5.2 are reported as net rates, with the chemical decomposition rate at low pH subtracted. At pH values above 5.2, activity falls off sharply before recovering significantly at pH 6.0, reaching
- 15 a peak at pH 7.0, and then gradually leveling off up to pH 8.5. The sharp activity decline between pH 5.2 and 6.0 coincides with the enzyme's pI, calculated to be pH 5.4. At pH 5.5, samples instantaneously lose activity, except for a very small residual activity.

EXAMPLE 13

20 Activity-pH profile of sfnoxK2

- The pH profile was performed on ammonium sulfate fractions from the sfnoxK2. 100 mM buffer solutions at 30°C and 200 μM NADH were used for activity analysis as monitored by absorbance at 340 nm. All samples were tested in triplicate in 3 mL methylacrylate disposable cuvettes. The following buffers were utilized within the buffering
- 25 range of 1 pH unit from their pKa: acetate, N-methylpiperazine, MES (2-[N-morpholino]ethanesulfonic acid hydrate), and bis-tris-propane. Sodium hydroxide or hydrochloric acid were used in preparation of the respective buffers.

EXAMPLE 14

30 Purification of NADH oxidase

 A modified purification strategy was employed to obtain highly purified NADH oxidase. Frozen cell pellets, 13 g WCP, were thawed and resuspended in 30 mL of 100 mM 1-methylpiperazine buffer pH 5.0 + 1mM EDTA + 5 mM DTT + 5mM Spermine. The

resulting cell slurry was sonicated with a Fisher Scientific 60 Sonic dismembrator for 6 X 2 minutes while floating the tube in ice/water for cooling. The resulting lysate was centrifuged at 20,000 rpm in a Beckman J2-21M for 45 minutes at 4°C. The clarified lysate was then loaded into Specto/Por[®] regenerated cellulose dialysis membrane tubing (60 K MWCO) and dialyzed with 1.5 L of 20 mM 1-methylpiperazine pH 5.0 at 30°C +1mM EDTA+ 10 mM β-mercaptoethanol. This step comprises the acid precipitation step along with concurrent dialysis for salt removal. The sample was dialyzed versus 1.5 L of buffer for two hours at 30C and 200 rpm stirring before exchanging the dialysis buffer and dialyzing for two more hours under the same conditions. Temperature and stirring conditions were maintained by a digital stir plate with and external temperature probe. The sample was then transferred and centrifuged at 20,000 rpm for 45 minutes at 4°C. The resulting clarified solution was then loaded onto a Amersham Pharmacia Hiprep 16/10 Source™ 30Q column on an AKTAexplorer system at 4°C. The protein was then eluted with displacement chromatography utilizing 5mM naphthalene-1,3,6-trisulfonic acid. After sample loading the column was washed with 10 column volumes of 20 mM 1-methylpiperazine pH 5.0 at 4°C + 5 mM DTT. The protein elution phase was then started by switching to 20 mM 1-methylpiperazine pH 5.0 at 4°C + 5 mM DTT +5mM naphthalene-1,3,6-trisulfonic acid. 5 mL fractions were collected at a flow rate of 5 ml/min. Fractions with a tested specific activity of over 200 were pooled and dialyzed at 4°C against 2 L of 45 % Ammonium sulfate + 50 mM potassium phosphate buffer pH 6.8 + 1 mM EDTA +10 mM β-mercaptoethanol using Specto/Por[®] regenerated cellulose dialysis membrane tubing (14 kD MWCO). The total dialysis time was 12 hours with one buffer exchange after 6 hours. The resulting concentrated preparation of 23 mL total volume and 1.3 mg/mL was stored at 4°C. No additional purification or loss of activity was apparent in the 45% ammonium sulfate preparation. The preparation was measured to have an activity of 137 U/mL or 221 U/mg protein on NADH on the day the coupled experiments were started.

Samples of the purified enzyme preparations were run on a 12% Tris-Glycine SDS-PAGE gel (PAGEr[®] gold precast gel). The running buffer and sample were prepared according to the manufacturers protocol. The NADH oxidase sample was diluted 1:10 in DI water prior to mixing with sample loading buffer. 20 µL of the wildtype ADH, G37D ADH mutant, and NADH oxidase (dil) samples were mixed with an equal volume of 2x sample loading buffer, vortexed, and then incubated in a water bath at 95 °C for fifteen minutes. Due to the presence of 50% glycerol in the purified wildtype ADH and G37D ADH mutant

samples, sample-loading buffer without glycerol was utilized. Samples were then centrifuged at 14,000 g for 5 minutes and placed on ice prior to loading on the gel. 15 to 30 μ L of each sample was loaded into the wells with blank sample buffer added to the empty wells. The gel was run on a Hoefer Mighty Small™ (SE260) with circulated cooling water at 4 °C. The gel was run under constant voltage (125V) for 2.5 hours. At the completion of the electrophoresis run, the gel was washed with three changes of DI water. The gel was then stained with Pierce Gelcode blue for 1 hour and then transferred to DI water to destain for an additional hour. Images were taken in an Alpha Innotech AlphaImager 3300 for gel documentation.

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EXAMPLE 15

Cofactor regenerating assay:

Application of NADH oxidase in cofactor regeneration is performed using a batch conversion with R-ADH as the production enzyme. All reactions were run at 30°C with standard buffer composed: 50 mM HEPES pH 7.0 at 30°C and 150 mM total ionic strength by addition of 138 mM NaCl, 5 mM DTT, 1 mM $MgCl_2$, and 100 mM racemic phenylethanol. Cofactors and enzymes were then added to 100 μ L of buffer as outlined in Table 3 and vortexed. 30 μ L of the mixed solution was then added to 0.65 mL polypropylene PCR reaction tubes, capped, and floated in a water bath. Three identical vials were prepared for each condition. Time point samples were taken by centrifuging for 1 min at 14,000 rpm in a Microfuge and adding 270 μ L methanol to the reaction vial.

The results of coupled reactions after 12 hours, as analyzed by selective ion monitoring (SIM) mass spectrometry, are shown in Table 4. The standard curves used for SIM mass spectrometry are shown in Figure 5. Measured degrees of conversion values were normalized using the mass balance of acetophenone and phenylethanol to correct for manual injection error. Satisfactory linearity was obtained for both phenylethanol and acetophenone up to 100 mM concentration.

Table 4: Coupled alcohol-ketone conversion with cofactor regeneration

Sample#	Cofactor (4 mM)	ADH (U/ml)	ADH mut (U/ml)	NADH ox (U/ml)	Normalized Conversion (%)	Turnovers
1	NAD		2.0	8.0	43.6	10.9
2	NADH		2.0	8.0	35.0	8.7
3	NADP	2.0		8.0	38.2	9.5
4	NADPH	2.0		8.0	40.1	10.0
5	NAD			8.0	-2.3	-0.6
6	NAD		2.0		1.7	0.4
7	NADP			8.0	-0.7	-0.2
8	NADP	2.0			2.3	0.6
9	NAD*		2.0	8.0	43.6	109.0
10	NADP*	2.0		8.0	40.2	100.5
11	NAD*		2.0	4.0	27.9	69.8
12	NADP*	2.0		4.0	41.7	104.1

*These samples utilized 0.4 mM concentrations of cofactor.

Standard conditions: 30°C, pH 7.0 (50 mM HEPES), 5 mM DTT, 1 mM MgCl₂, 150 mM total ionic strength (addition of 138 mM NaCl), and 100 mM racemic phenylethanol.

The coupled reaction results shown in Table 4 are consistent with expected results from successfully coupled reactions. The comparison of reduced versus oxidized cofactor (runs 1 & 2 as well as 3 & 4) indicate that the starting oxidation state of the cofactor does not significantly impact the results. Given the higher stability and lower cost, the oxidized cofactor would be the reagent of choice for typical coupled reactions. The controls (runs 5-8) demonstrated that no conversion occurs without ADH (runs 5 & 7) and that slightly less than stoichiometric conversion was observed in the absence of NADH oxidase (runs 6 & 8) to regenerate the cofactor. Conversions in excess of stoichiometry would have indicated a potential NAD(P)H-oxidizing impurity in the ADH preparations. Reducing the cofactor concentration to 0.4 mM (runs 9-12) still indicated effective conversion with concomitant higher number of turnovers of cofactor; however, a lower degree of conversion was observed for the mutant ADH in the presence of 4 U/mL instead of 8 U/mL NADH oxidase. After 12 h, nearly complete conversion (maximally 50% of racemic phenylethanol) was achieved in all but the case of the mutant ADH with NAD⁺.

EXAMPLE 16

GC/MS analysis

Samples and a prepared standard curve were submitted to the IBB central mass spectroscopy facility for GC/selective ion analysis. The separate standard curves were
5 prepared for the $\pm$ phenylethanol and acetophenone. The $\pm$ phenylethanol curve consisted of 100 mM, 10 mM, and 1 mM in the coupled reaction base buffer, diluted 1:10 in methanol. The acetophenone curve consisted of 50 mM, 10 mM, and 1 mM in the coupled reaction base buffer, diluted 1:10 in methanol. Total mass areas were reported for ions of mass 120 (acetophenone) and 122 ($\pm$ phenylethanol). Sample concentrations from the coupled reaction
10 were estimated by interpolation on these standard curves (R^2 for both curves > 0.90).

Whereas this invention has been described in detail with particular reference to its most preferred embodiments, it will be apparent to those skilled in the art that various modifications and variations can be made in the present invention in light of the above teachings without departing from the scope or spirit of the invention. Other embodiments of
15 the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

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- 20 [65] B.R. Riebel, P.R. Gibbs, W.B. Wellborn and A.S. Bommarius, Cofactor regeneration of both NAD⁺ from NADH and NADP⁺ from NADPH: NADH oxides from *Lactobacillus sanfranciscensis*", *Adv. Synth. Catal.* 2003, 345, 707-712.

Claims

- 5 What is claimed is:
1. An isolated bacterial NADPH oxidase.
 2. The oxidase of Claim 1, wherein the isolated bacterial oxidase regenerates NADP+
10 and NAD+.
 3. The isolated bacterial NAD(P)H oxidase of Claims 1 or 2, which is obtained from
Lactobacillus sanfranciscensis.
 - 15 4. The isolated bacterial NADPH oxidase of Claim 3, which comprises SEQ ID NO:2, 4,
6, 17, 18, 19, or 20.
 5. An isolated nucleic acid, which encodes the isolated bacterial oxidase of Claim 1 or 2.
 - 20 6. A vector comprising the isolated nucleic acid of Claim 5.
 7. A host cell comprising the isolated nucleic acid of Claim 5.
 8. An isolated nucleic acid, which encodes the isolated bacterial oxidase of Claims 3.
 - 25 9. A vector comprising the isolated nucleic acid of Claim 8.
 10. A host cell comprising the isolated nucleic acid of Claim 8.
 - 30 11. The isolated nucleic acid of Claims 5 or 8, which comprises SEQ ID NOs: 1, 3 or 5.
 12. A vector comprising the isolated nucleic acid of claim 11.
 13. A host cell comprising the isolated nucleic acid of claim 11.

14. A nucleic acid, which hybridizes under stringent conditions to the nucleic acid according to claim 11, and which encodes a protein with NAD(P)H oxidase activity.
- 5 15. An isolated NAD(P)H oxidase obtained from *Borrelia*.
16. The isolated NADH oxidase of Claim 15, which is obtained from *Borrelia burgdorferi*.
- 10 17. The isolated NAD(P)H oxidase of claim 15, which comprises SEQ ID NO:8, 10 or 12.
18. An isolated nucleic acid, which encodes the isolated oxidase of claim 15.
- 15 19. A vector comprising the isolated nucleic acid of claim 18.
20. A host cell comprising the isolated nucleic acid of claim 18.
21. An isolated nucleic acid, which encodes the isolated oxidase of claim 17.
- 20 22. A vector comprising the isolated nucleic acid of Claim 21.
23. A host cell comprising the isolated nucleic acid of Claim 21.
- 25 24. The isolated nucleic acid of Claim 15, comprising SEQ ID NOs: 7, 9 or 11..
25. A vector comprising the isolated nucleic acid of Claim 24.
26. A host cell comprising the isolated nucleic acid of Claim 24.
- 30 27. A nucleic acid, which hybridizes under stringent conditions to the nucleic acid of Claim 24, and which encodes a protein with NAD(P)H oxidase activity.
28. An isolated bacterial NAD(P)H oxidase that oxidizes both NADH and NADPH.

29. The isolated bacterial NAD(P)H oxidase of Claim 28, which is obtained from *Lactobacillus sanfranciscensis*.

5 30. A method for producing a recombinant-NAD(P)H oxidase, comprising cultivating the host cell of Claims 7, 10 or 13 to express the nucleic acid; and collecting the rec- NAD(P)H oxidase produced.

10 31. The recombinant NAD(P)H oxidase produced by the method of Claim 29.

32. A process for producing a recombinant NAD(P)H oxidase, comprising cultivating the host cell of Claims 20, 23 or 26 to express the nucleic acid; and collecting the rec- NAD(P)H oxidase produced.

15 33. The recombinant NAD(P)H oxidase produced by the method of Claim 31.

34. A method for producing a recombinant NAD(P)H oxidase, comprising mutating one or more of the nucleic acids of SEQ ID NOs. 1, 3, 5, 7, 9, or 11; expressing the one ore more proteins encoded by the mutated nucleic acids, detecting a mutated recombinant NAD(P)H
20 oxidase with altered activity; and isolating the one ore more mutated nucleic acids that encode the recombinant NAD(P)H oxidase with altered activity.

35. A recombinant NAD(P)H oxidase obtained by the method of Claim 33.

25 36. An isolated nucleic acid encoding the recombinant NADH oxidase of Claim 34.

37. A vector comprising the nucleic acid of Claim 35.

30 38. A host cell comprising the nucleic acid of Claim 35.

39. A method of producing a chiral enantiomer-enriched organic compound, comprising contacting the organic compound with a NAD(P)H oxidase of Claim 1, 3, 15, 28, 30, 32, or 34.

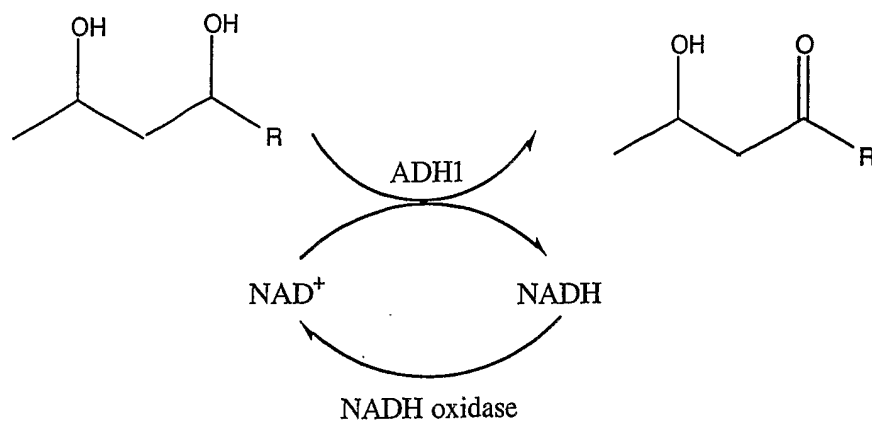
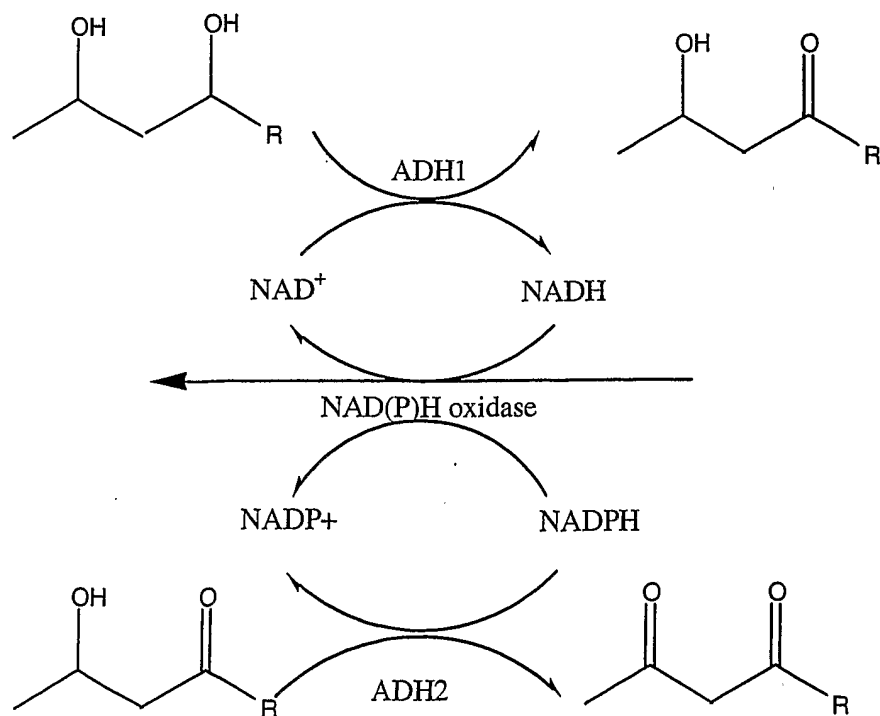
40. The method of Claim 38, wherein the organic compound is an alcohol, an amino acid or a hydroxy acid.
41. A whole-cell catalyst, comprising the isolated recombinant NAD(P)H oxidase of
5 Claims 4, 17, 30, 32 or 34.
42. The whole-cell catalyst of Claim 40, wherein the recombinant NAD(P)H oxidase is obtained from *Lactobacillus sanfranciscensis*.
- 10 43. The whole-cell catalyst of Claim 40, wherein the recombinant NAD(P)H oxidase is obtained from *Borrelia burgdorferi*.
44. A whole cell catalyst, comprising a nucleic acid of at least one of SEQ. ID NOs.: 1, 3, 5, 7, 9 or 11, and a nucleic acid, which encodes a dehydrogenase.
- 15 45. A method for enzyme reactions, comprising reacting at least one enzyme selective for one enantiomer of at least one chiral center of a compound with one or more chiral centers, with a reactant composition comprising the compound with one or more chiral centers, wherein the at least one enzyme requires a nicotinamide-based cofactor, and reacting the
20 nicotinamide-based cofactor with one or more of the NOX of the present invention.
46. The method of Claim 44, further comprising, obtaining a reaction product.
47. The method of Claim 44, further comprising, obtaining a chiral enantiomer-enriched
25 compound.
48. A method for isolating NAD(P)H oxidases, comprising
a) precipitating with an acid solution of pH 4.5 to 6.0, a bacterial cellular
lysate; and
30 b) isolating NAD(P)H oxidases from the solution of b) after precipitation occurs.
49. An NAD(P)H oxidase, of Claims 1, 3, 4, 15, 17, 28, 30, 32, or 34, whereby the enzyme is immobilized on a heterogenous substrate.

50. A method of synthesizing chiral compounds, comprising, using at least one NAD(P)H oxidase of Claims 1, 3, 4, 15, 17, 28, 30, 32, or 34 in an enzyme reaction.

5 51. A method of analyzing NAD(P)H in a cellular environment, comprising reacting the NADH or NADPH with at least one NAD(P)H oxidase of Claims 1, 3, 4, 15, 17, 28, 30, 32, or 34.

10

15

**Figure 1A****Figure 1B**

1	H	M	K	I	I	I	I	G	G	T	S	A	G	T	S	A	A	A	K	A	borrellianox
1	ATG	ATG	AAA	ATA	ATA	ATT	ATT	GGG	GGC	ACA	TCA	GCA	GGA	ACT	AGT	GCC	GCA	GCT	AAA	GCA	b6blue
1	M	M	K	I	I	I	I	G	G	T	S	A	G	T	S	A	A	A	K	A	b1blue
1	ATG	ATG	AAA	ATA	ATA	ATT	ATT	GGG	GGC	ACA	TCA	GCA	GGA	ACT	AGT	GCC	GCA	GCT	AAA	GCA	b1blue
1	M	M	K	I	I	I	I	G	G	T	S	A	G	T	S	A	A	A	K	A	b1blue
1	ATG	ATG	AAA	ATA	ATA	ATT	ATT	GGG	GGC	ACA	TCA	GCA	GGA	ACT	AGT	GCC	GCA	GCT	AAA	GCA	b1blue
61	N	R	L	N	R	K	L	D	I	T	I	Y	E	K	F	N	I	V	S	F	borrellianox
61	AAC	CGC	TTA	AAC	AAA	ARG	CTA	GAC	ATT	ACT	ATC	TAT	GAA	AAA	ACA	AAT	ATT	GTA	TCT	TTT	b6blue
61	N	R	L	N	R	K	L	D	I	T	I	Y	E	K	F	N	I	V	S	F	b1blue
61	AAG	CGC	TTA	AAC	AAA	ARG	CTA	GAC	ATT	ACT	ATC	TAT	GAA	AAA	ACA	AAT	ATT	GTA	TCT	TTT	b1blue
61	N	R	L	N	R	K	L	D	I	T	I	Y	E	K	F	N	I	V	S	F	b1blue
61	AAG	CGC	TTA	AAC	AAA	ARG	CTA	GAC	ATT	ACT	ATC	TAT	GAA	AAA	ACA	AAT	ATT	GTA	TCT	TTT	b1blue
121	G	I	C	G	L	P	Y	E	V	G	G	F	F	D	N	P	N	T	M	I	borrellianox
121	GGA	ACC	TGT	GGC	CTG	CCT	TAC	TTC	TTC	GGG	GGA	TTC	TTC	GAC	AAC	CCC	AAT	ACA	ATG	ATC	b6blue
121	G	I	C	G	L	P	Y	E	V	G	G	F	F	D	N	P	N	T	M	I	b1blue
121	GGA	ACC	TGT	GGC	CTG	CCT	TAC	TTC	TTC	GGG	GGA	TTC	TTC	GAC	AAC	CCC	AAT	ACA	ATG	ATC	b1blue
121	G	I	C	G	L	P	Y	E	V	G	G	F	F	D	N	P	N	T	M	I	b1blue
121	GGA	ACC	TGT	GGC	CTG	CCT	TAC	TTC	TTC	GGG	GGA	TTC	TTC	GAC	AAC	CCC	AAT	ACA	ATG	ATC	b1blue
181	S	R	T	O	E	F	E	R	T	G	I	S	V	K	T	N	H	E	V		borrellianox
181	TCA	AGA	ACA	CAA	GAA	TTC	GAA	AAA	ACT	GGA	ATC	TCT	GTT	AAA	ACT	AAC	CAC	GAA	GTT	b6blue	
181	S	R	T	O	E	F	E	R	T	G	I	S	V	K	T	N	H	E	V		b1blue
181	TCA	AGA	ACA	CAA	GAA	TTC	GAA	AAA	ACT	GGA	ATC	TCT	GTT	AAA	ACT	AAC	CAC	GAA	GTT	b1blue	
181	S	R	T	O	E	F	E	R	T	G	I	S	V	K	T	N	H	E	V		b1blue
181	TCA	AGA	ACA	CAA	GAA	TTC	GAA	AAA	ACT	GGA	ATC	TCT	GTT	AAA	ACT	AAC	CAC	GAA	GTT	b1blue	
241	I	K	V	D	A	K	N	H	T	I	V	I	K	N	O	K	T	G	T	I	borrellianox
241	ATC	AAA	GTA	GAT	GCA	AAA	AAC	AAT	ACA	ATT	GTA	ATA	AAA	AAT	CAA	AAA	ACA	GGG	ACC	ATT	b6blue
241	I	K	V	D	A	K	N	H	T	I	V	I	K	N	O	K	T	G	T	I	b1blue
241	ATC	AAA	GTA	GAT	GCA	AAA	AAC	AAT	ACA	ATT	GTA	ATA	AAA	AAT	CAA	AAA	ACA	GGG	ACC	ATT	b1blue
241	I	K	V	D	A	K	N	H	T	I	V	I	K	N	O	K	T	G	T	I	b1blue
241	ATC	AAA	GTA	GAT	GCA	AAA	AAC	AAT	ACA	ATT	GTA	ATA	AAA	AAT	CAA	AAA	ACA	GGG	ACC	ATT	b1blue
301	F	N	N	T	Y	D	O	L	H	I	A	T	G	A	K	P	I	I	P	P	borrellianox
301	TTT	AAC	AAT	ACT	TAC	GAT	CAA	CTT	ATG	ATA	GCA	ACT	GGT	GGA	AAA	CCT	ATT	ATT	CCA	CCA	b6blue
301	F	N	N	T	Y	D	O	L	H	I	A	T	G	A	K	P	I	I	P	P	b1blue
301	TTT	AAC	AAT	ACT	TAC	GAT	CAA	CTT	ATG	ATA	GCA	ACT	GGT	GGA	AAA	CCT	ATT	ATT	CCA	CCA	b1blue
301	F	N	N	T	Y	D	O	L	H	I	A	T	G	A	K	P	I	I	P	P	b1blue
301	TTT	AAC	AAT	ACT	TAC	GAT	CAA	CTT	ATG	ATA	GCA	ACT	GGT	GGA	AAA	CCT	ATT	ATT	CCA	CCA	b1blue

Figure 2A

361	I	N	N	I	N	L	E	N	F	H	T	L	K	N	L	E	D	G	Q	K		borrellianox
361	ATC	AAT	AAT	ATC	AAT	CTA	GAA	AAT	TTT	CAT	ACT	CTG	AAA	AAT	TTA	GAA	GAC	GGT	CAA	AAA		b6blue
361	I	N	N	I	N	L	E	N	F	H	T	L	K	N	L	E	D	G	Q	K		b1blue
361	ATG	AAT	AAT	ATC	AAT	CTA	GAA	AAT	TTT	CAT	ACT	CTG	AAA	AAT	TTA	GAA	GAC	GGT	CAA	AAA		
361	I	N	N	I	N	L	E	N	F	H	T	L	K	N	L	E	D	G	Q	K		
361	ATC	AAT	AAT	ATG	AAT	CTA	GAA	AAT	TTT	CAT	ACT	CTG	AAA	AAT	TTA	GAA	GAC	GGT	CAA	AAA		
421	I	K	K	L	M	D	R	E	E	I	K	N	I	V	I	I	G	G	G	Y		borrellianox
421	ATA	AAA	AAA	TTA	ATG	GAT	AGA	GAA	GAG	ATT	AAA	AAT	ATA	GTG	ATA	ATT	GGT	GGT	GGA	TAC		b6blue
421	I	K	K	L	M	D	R	E	E	I	K	N	I	V	I	I	G	G	G	Y		b1blue
421	ATA	AAA	AAA	TTA	ATG	GAT	AGA	GAA	GAG	ATT	AAA	AAT	ATA	GTG	ATA	ATT	GGT	GGT	GGA	TAC		
421	I	K	K	L	M	D	R	E	E	I	K	N	I	A	I	I	G	G	G	Y		
421	ATA	AAA	AAA	TTA	ATG	GAT	AGA	GAA	GAG	ATT	AAA	AAT	ATA	GCG	ATA	ATT	GGT	GGT	GGA	TAC		
481	I	G	I	E	M	V	E	A	A	K	N	K	R	K	N	V	R	L	I	Q		borrellianox
481	ATT	GGA	ATT	GAA	ATG	GTA	GAA	GCA	GCA	AAA	AAT	AAA	AGA	AAA	AAT	GTA	AGA	TTA	ATT	CAA		b6blue
481	I	G	I	E	M	V	E	A	A	K	N	K	R	K	S	N	R	L	I	Q		
481	ATT	GGA	ATT	GAA	ATG	GTA	GAA	GCA	GCA	AAA	AAT	AAA	AGA	AAA	AGT	GTA	AGA	TTA	ATT	CAA		b1blue
481	I	G	I	E	M	V	E	A	A	K	N	K	R	K	N	V	R	L	I	Q		
481	ATT	GGA	ATT	GAA	ATG	GTA	GAA	GCA	GCA	AAA	AAT	AAA	AGA	AAA	AAT	GTA	AGA	TTA	ATT	CAA		
541	L	D	K	H	E	L	I	D	S	F	D	E	E	I	V	T	I	M	E	E		borrellianox
541	CTA	GAT	AAG	CAC	ATA	CTC	ATA	GAT	TCC	TTT	GAC	GAA	GAA	ATA	GTC	ACA	ATA	ATG	GAA	GAA		b6blue
541	L	D	K	H	E	L	I	D	S	F	D	E	E	I	V	T	I	M	E	E		b1blue
541	CTA	GAT	AAG	CAC	ATA	CTC	ATA	GAT	TCC	TTT	GAC	GAA	GAA	ATA	GTC	ACA	ATA	ATG	GAA	GAA		
541	L	D	K	H	E	L	I	D	S	F	D	E	E	I	V	T	I	M	E	E		
541	CTA	GAT	AAG	CAC	ATA	CTC	ATA	GAT	TCC	TTT	GAC	GAA	GAA	ATA	GTC	ACA	ATA	ATG	GAA	GAA		
601	E	L	T	K	K	G	V	N	L	H	T	N	E	F	V	K	S	L	I	G		borrellianox
601	GAA	CTA	ACA	AAA	AAG	GGG	GTT	AAT	CTT	CAT	ACA	AAT	GAG	TTT	GTA	AAA	AGT	TTA	ATA	GGA		b6blue
601	E	L	T	K	K	G	V	N	L	H	T	N	E	F	V	K	S	L	I	G		b1blue
601	GAA	CTA	ACA	AAA	AAG	GGG	GTT	AAT	CTT	CAT	ACA	AAT	GAG	TTT	GTA	AAA	AGT	TTA	ATA	GGA		
601	E	L	T	K	K	G	V	N	L	H	T	N	E	F	V	K	S	L	I	G		
601	GAA	CTA	ACA	AAA	AAG	GGG	GTT	AAT	CTT	CAT	ACA	AAT	GAG	TTT	GTA	AAA	AGT	TTA	ATA	GGA		
661	E	K	K	A	E	G	V	V	T	N	K	N	T	Y	Q	A	D	A	V	I		borrellianox
661	GAA	AAA	AAG	GCA	GAA	GGA	GTA	GTA	ACA	AAC	AAA	AAT	ACT	TAT	CAA	GCT	GAC	GCT	GTT	ATA		b6blue
661	G	K	K	A	E	G	V	V	T	N	K	N	T	Y	Q	A	D	A	V	I		
661	GGA	AAA	AAG	GCA	GAA	GGA	GTA	GTA	ACA	AAC	AAA	AAT	ACT	TAT	CAA	GCT	GAC	GCT	GTT	ATA		b1blue
661	E	K	K	A	G	G	V	V	T	N	K	N	T	Y	Q	A	D	A	V	I		
661	GAA	AAA	AAG	GCA	GGA	GGA	GTA	GTA	ACA	AAC	AAA	AAT	ACT	TAT	CAA	GCT	GAC	GCT	GTT	ATA		
721	L	A	T	G	I	K	P	D	T	E	F	L	E	N	Q	L	K	T	T	K		borrellianox
721	CTT	GCT	ACC	GGA	ATA	AAA	CCT	GAC	ACT	GAA	TTT	TTA	GAA	AAC	CAG	CTT	AAA	ACT	ACT	AAA		b6blue
721	L	A	T	G	I	K	P	D	T	E	F	L	E	N	Q	L	K	T	T	K		b1blue
721	CTT	GCT	ACC	GGA	ATA	AAA	CCT	GAC	ACT	GAA	TTT	TTA	GAA	AAC	CAG	CTT	AAA	ACT	ACT	AAA		
721	L	A	T	G	I	K	P	D	T	E	F	L	E	N	Q	L	K	T	T	K		
721	CTT	GCT	ACC	GGA	ATA	AAA	CCT	GAC	ACT	GAA	TTT	TTA	GAA	AAC	CAG	CTT	AAA	ACT	ACT	AAA		

Figure 2B

781	N G A I I V N E Y G E T S I K N I F S A	borrelianox
781	AAT GGA GCA ATA ATT GTA AAT GAG TAT GGC GAA ACT AGC ATA AAA AAT ATT TTT TCT GCA	
781	N G A I I V N E Y G E T S I K N I F S A	b6blue
781	AAT GGA GCA ATA ATT GTA AAT GAG TAT GGC GAA ACT AGC ATA AAA AAT ATT TTT TCT GCA	
781	N G A I I V N E Y G E T S I K N I F S A	b1blue
781	AAT GGA GCA ATA ATT GTA AAT GAG TAT GGC GAA ACT AGC ATA AAA AAT ATT TTT TCT GCA	
841	G D C A T I Y N I V S K K N E Y I P L A	borrelianox
841	GGA GAT TGT GCA ACT ATT TAT AAT ATA GTA AGT AAA AAA AAT GAA TAC ATA CCC TTG GCA	
841	G D C A T I Y N I V S K K N E Y I P L A	b6blue
841	GGA GAT TGT GCA ACT ATT TAT AAT ATA GTA AGT AAA AAA AAT GAA TAC ATA CCC TTG GCA	
841	G D C A T I Y N I V S K K N E Y I P L A	b1blue
841	GGA GAT TGT GCA ACT ATT TAT AAT ATA GTA AGT AAA AAA AAT GAA TAC ATA CCC TTG GCA	
901	T T A N K L G R I V G E N L A G N H T A	borrelianox
901	ACA ACA GCC AAC AAA CTT GGA AGA ATA GTT GGT GAA AAT TTA GCT GGG AAT CAT ACA GCA	
901	T T A N K L G R I V G E N L A G N H T A	b6blue
901	ACA ACA GCC AAC AAA CTT GGA AGA ATA GTT GGT GAA AAT TTA GCT GGG AAT CAT ACA GCA	
901	T T A N K L G R I V G E N L A G N H T A	b1blue
901	ACA ACA GCC AAC AAA CTT GGA AGA ATA GTT GGT GAA AAT TTA GCT GGG AAT CAT ACA GCA	
961	F K G T L G S A S I K I L S L E A A R T	borrelianox
961	TTT AAA GGC ACA TTG GGC TCA GCT TCA ATT AAA ATA CTA TCT TTA GAA GCT GCA AGA ACA	
961	F K G T L G S A S I K I L S L E A A R T	b6blue
961	TTT AAA GGC ACA TTG GGC TCA GCT TCA ATT AAA ATA CTA TCT TTA GAA GCT GCA AGA ACA	
961	F K G T L G S A S I K I L S L E A A R T	b1blue
961	TTT AAA GGC ACA TTG GGC TCA GCT TCA ATT AAA ATA CTA TCT TTA GAA GCT GCA AGA ACA	
1021	G L T E K D A K K L Q I K Y R T I F V K	borrelianox
1021	GGA CTT ACA GAA AAA GAT GCA AAA AAG CTC CAA ATA AAA TAT AAA ACG ATT TTT GTA AAG	
1021	G L T E K D A K K L Q I K Y R T I F V K	b6blue
1021	GGA CTT ACA GAA AAA GAT GCA AAA AAG CTC CAA ATA AAA TAT AAA ACG ATT TTT GTA AAG	
1021	G L T E K D A K K L Q I K Y R T I F V K	b1blue
1021	GGA CTT ACA GAA AAA GAT GCA AAA AAG CTC CAA ATA AAA TAT AAA ACG ATT TTT GTA AAG	
1081	D K N H T N Y Y P G Q E D L Y I K L I Y	borrelianox
1081	GAC AAA AAT CAT ACA AAT TAT TAT CCA GGC CAA GAA GAT CTT TAT ATT AAA TTA ATT TAT	
1081	D K N H T N Y Y P G Q E D L Y I K L I Y	b6blue
1081	GAC AAA AAT CAT ACA AAT TAT TAT CCA GGC CAA GAA GAT CTT TAT ATT AAA TTA ATT TAT	
1081	D K N H T N Y Y P G Q E D L Y I K L I Y	b1blue
1081	GAC AAA AAT CAT ACA AAT TAT TAT CCA GGC CAA GAA GAT CTT TAT ATT AAA TTA ATT TAT	
1141	E E N T K I I L G A Q A I G K N G A V I	borrelianox
1141	GAG GAA AAT ACC AAA ATA ATC CTT GGC GCA CAA GCA ATA GGA AAA AAT GGA GCC GTA ATA	
1141	E E N T K I I L G A Q A I G K N G A V I	b6blue
1141	GAG GAA AAT ACC AAA ATA ATC CTT GGC GCA CAA GCA ATA GGA AAA AAT GGA GCC GTA ATA	
1141	E E N T K I I L G A Q A T G K N G A V M	b1blue
1141	GAG GAA AAT ACC AAA ATA ATC CTT GGA GCA CAA GCA ACA GGA AAA AAT GGA GCC GTA ATG	
1201	R I H A L S I A I Y S K L T T K E L G M	borrelianox
1201	AGA ATT CAT GCT TTA TCA ATT GCA ATC TAT TCA AAA CTT ACA ACA AAA GAG CTA GGG ATG	
1201	R I H A L S I A I Y S K L T T K E L G M	b6blue
1201	AGA ATT CAT GCT TTA TCA ATT GCA ATC TAT TCA AAG CTT ACA ACA AAA GAG CTA GGG ATG	
1201	R I H A L S I A I Y S K L T T K E L R M	b1blue
1201	AGA ATT CAT GCT TTA TCA ATT GCA ATC TAT TCA AAA CTT ACA ACA AAA GAG CTA AGG ATG	
1261	M D F S Y S P P F S R T W D I L N I A G	borrelianox
1261	ATG GAT TTC TCA TAT TCC CCA CCC TTC TCA AGA ACT TGG GAT ATA TTA AAT ATT GCT GGC	
1261	M D F S Y S P P F S R T W D I L N I A G	b6blue
1261	ATG GAT TTC TCA TAT TCC CCA CCC TTC TCA AGA ACT TGG GAT ATA TTA AAT ATT GCT GGC	
1261	M D F S Y S P P F S R T W D I L N I A G	b1blue
1261	ATG GAT TTC TCA TAT TCC CCA CCC TTC TCA AGA ACT TGG GAT ATA TTA AAT ATT GCT GGC	
1321	N A A K	borrelianox
1321	AAT GCT GCC AAA TAG	
1321	N A A K	b6blue
1321	AAT GCT GCC AAA TAG	
1321	N A A K	b1blue
1321	AAT GCT GCC AAA TAG	

Figure 2C

1	M	K	V	I	V	V	G	C	T	H	A	G	T	T	A	V	K	O	T	I	noxsanfran
1	ATG	AAA	GTT	ATT	GTA	GTA	GGT	TGT	ACT	CAC	GCT	GAC	ACT	TTT	GCA	GTT	AAG	CAA	ACG	ATT	sfk6seq
1	M	K	V	I	V	V	G	C	T	H	A	G	T	T	A	V	K	O	T	I	sfk2seq
1	ATG	AAA	GTT	ATT	GTA	GTA	GGT	TGT	ACT	CAC	GCT	GAC	ACT	TTT	GCA	GTT	AAG	CAA	ACG	ATT	
1	M	K	V	I	V	V	G	C	T	H	A	G	T	T	A	V	K	O	T	I	
1	ATG	AAA	GTT	ATT	GTA	GTA	GGT	TGT	ACT	CAC	GCT	GAC	ACT	TTT	GCA	GTT	AAG	CAA	ACG	ATT	
61	A	D	H	P	D	A	D	V	T	A	Y	E	M	N	D	N	I	S	F	L	noxsanfran
61	GCC	GAT	GAC	CCG	GAT	GCA	GAT	GTG	ACT	GCA	TAT	GAA	ATG	AA	GAT	AAC	ATT	TGC	TTT	TTA	sfk6seq
61	A	D	H	P	D	A	D	V	T	A	Y	E	M	N	D	N	I	S	F	L	
61	GCC	GAT	GAC	CCG	GAT	GCA	GAT	GTG	ACT	GCA	TAT	GAA	ATG	AA	GAT	AAC	ATT	TGC	TTT	TTA	sfk2seq
61	A	D	H	P	D	A	D	V	T	A	Y	E	M	N	D	N	I	S	F	L	
61	GCC	GAT	GAC	CCG	GAT	GCA	GAT	GTG	ACT	GCA	TAT	GAA	ATG	AA	GAT	AAC	ATT	TGC	TTT	TTA	
121	S	C	G	I	A	L	Y	L	G	K	E	I	K	N	N	D	P	R	G	L	noxsanfran
121	TCA	TGT	GGA	ATC	GGC	CTT	TAC	TTA	GGT	AAA	GAA	ATT	AAA	AAC	AAT	GAT	CCC	CGA	GGG	CTT	sfk6seq
121	S	C	G	I	A	L	Y	L	G	K	E	I	K	N	N	D	P	R	G	L	
121	TCA	TGT	GGA	ATC	GGC	CTT	TAC	TTA	GGT	AAA	GAA	ATT	AAA	AAC	AAT	GAT	CCC	CGA	GGG	CTT	sfk2seq
121	S	C	G	I	A	L	Y	L	G	K	E	I	K	N	N	D	P	R	G	L	
121	TCA	TGT	GGA	ATC	GGC	CTT	TAC	TTA	GGT	AAA	GAA	ATT	AAA	AAC	AAT	GAT	CCC	CGA	GGG	CTT	
181	F	Y	S	S	P	E	E	L	S	N	L	G	A	N	V	O	M	R	H	O	noxsanfran
181	TTC	TAC	TCA	AGT	CCA	GAA	GAA	TTA	AGC	AAT	CTT	GGA	GCT	AAC	GTC	CAA	ATG	CGT	CAT	CAA	sfk6seq
181	F	Y	S	S	P	E	E	L	S	N	L	G	A	N	V	O	M	R	H	O	
181	TTC	TAC	TCA	AGT	CCA	GAA	GAA	TTA	AGC	AAT	CTT	GGA	GCT	AAC	GTC	CAA	ATG	CGT	CAT	CAA	sfk2seq
181	F	Y	S	S	P	E	E	L	S	N	L	G	A	N	V	O	M	R	H	O	
181	TTC	TAC	TCA	AGT	CCA	GAA	GAA	TTA	AGC	AAT	CTT	GGA	GCT	AAC	GTC	CAA	ATG	CGT	CAT	CAA	
241	V	T	N	V	D	P	E	T	K	T	I	K	V	K	D	L	I	T	N	E	noxsanfran
241	GTT	ACA	AAC	GTT	GAT	CCA	GAA	ACA	AAA	ACA	ATC	AAA	GTT	AAA	GAT	TTA	ATC	ACC	AAC	GAA	sfk6seq
241	V	T	N	V	D	P	E	T	K	T	I	K	V	K	D	L	I	T	N	E	
241	GTT	ACA	AAC	GTT	GAT	CCA	GAA	ACA	AAA	ACA	ATC	AAA	GTT	AAA	GAT	TTA	ATC	ACC	AAC	GAA	sfk2seq
241	V	T	N	V	D	P	E	T	K	T	I	K	V	K	D	L	I	T	N	E	
241	GTT	ACA	AAC	GTT	GAT	CCA	GAA	ACA	AAA	ACA	ATC	AAA	GTT	AAA	GAT	TTA	ATC	ACC	AAC	GAA	
301	E	K	T	E	A	Y	D	K	L	I	M	T	T	G	S	K	P	T	V	P	noxsanfran
301	GAA	AAA	ACA	GAA	GCA	TAT	GAC	AAA	TTA	ATT	ATG	ACC	ACT	GGT	TCT	ATG	CGT	ACT	GTT	CGT	sfk6seq
301	E	K	T	E	A	Y	D	K	L	I	M	T	T	G	S	K	P	T	V	P	
301	GAA	AAA	ACA	GAA	GCA	TAT	GAC	AAA	TTA	ATT	ATG	ACC	ACT	GGT	TCT	ATG	CGT	ACT	GTT	CGT	sfk2seq
301	E	K	T	E	A	Y	D	K	L	I	M	T	T	G	S	K	P	T	V	P	
301	GAA	AAA	ACA	GAA	GCA	TAT	GAC	AAA	TTA	ATT	ATG	ACC	ACT	GGT	TCT	ATG	CGT	ACT	GTT	CGT	

Figure 2D

361	P	I	P	G	I	D	S	S	R	V	Y	L	G	K	N	Y	N	D	A	K	noxsanfran	
361	CCA	ATC	CCT	GGA	ATC	GAT	AGT	AGT	CGC	GTT	TAC	CIT	TGT	AAA	AAC	TAT	AAC	GAT	GCT	AAA	sfk6seq	
361	P	I	P	G	I	D	S	S	R	V	Y	L	C	K	N	Y	N	D	A	K	sfk2seq	
361	CCA	ATC	CCT	GGA	ATC	GAT	AGT	AGT	CGC	GTT	TAC	CTT	TGT	AAA	AAC	TAT	AAC	GAT	GCT	AAA		
361	P	I	P	G	I	D	S	S	R	V	Y	L	C	K	N	Y	N	D	A	K		
361	CCA	ATC	CCT	GGA	ATC	GAT	AGT	AGT	CGC	GTT	TAC	CTT	TGT	AAA	AAC	TAT	AAC	GAT	GCT	AAA		
421	K	L	F	E	E	A	P	K	A	K	T	I	T	I	I	G	S	G	Y	I	noxsanfran	
421	AAG	TIA	TTT	GAA	GAA	GCT	CGC	AAA	GCT	AAA	ACG	ATT	ACT	ATC	ATC	ATT	GCT	TGT	GCT	TAT	ATT	
421	K	L	F	E	E	A	P	K	A	K	T	I	T	I	I	G	S	G	Y	I	sfk6seq	
421	AAG	TIA	TTT	GAA	GAA	GCT	CGC	AAA	GCT	AAA	ACG	ATT	ACT	ATC	ATC	ATT	GCT	TGT	GCT	TAT	ATT	
421	K	L	F	E	E	A	P	K	A	K	T	I	T	I	I	G	S	G	Y	I	sfk2seq	
421	AAG	TIA	TTT	GAA	GAA	GCT	CGC	AAA	GCT	AAA	ACG	ATT	ACT	ATC	ATC	ATT	GCT	TGT	GCT	TAT	ATT	
481	G	A	E	L	A	E	A	Y	S	N	Q	N	Y	N	Y	N	L	I	D	G	noxsanfran	
481	GGT	GCC	GAA	CTG	GCT	GAA	GCC	TAC	TCA	ACA	AAT	TAT	AAC	GTT	AAT	TTA	ATT	GAT	GGT			
481	G	A	E	L	A	E	A	Y	S	N	Q	N	Y	N	Y	N	L	I	D	G	sfk6seq	
481	GGT	GCC	GAA	CTG	GCT	GAA	GCC	TAC	TCA	ACA	AAT	TAT	AAC	GTT	AAT	TTA	ATT	GAT	GGT			
481	G	A	E	L	A	E	A	Y	S	N	Q	N	Y	N	Y	N	L	I	D	G	sfk2seq	
481	GGT	GCC	GAA	CTG	GCT	GAA	GCC	TAC	TCA	ACA	AAT	TAT	AAC	GTT	AAT	TTA	ATT	GAT	GGT			
541	H	E	R	V	L	Y	K	Y	F	D	K	E	F	T	D	I	L	A	K	D	noxsanfran	
541	CAT	GAA	CGA	GTT	CTT	TAC	AAG	TAT	TTT	GAT	ANA	GAA	TTT	ACT	GAT	ATT	TTA	GCC	AAA	GAT		
541	H	E	R	V	L	Y	K	Y	F	D	K	E	F	T	D	I	L	A	K	D	sfk6seq	
541	CAT	GAA	CGA	GTT	CTT	TAC	AAG	TAT	TTT	GAT	ANA	GAA	TTT	ACT	GAT	ATT	TTA	GCC	AAA	GAT		
541	H	E	R	V	L	Y	K	Y	F	D	K	E	F	T	D	I	L	A	K	D	sfk2seq	
541	CAT	GAA	CGA	GTT	CTT	TAC	AAG	TAT	TTT	GAT	ANA	GAA	TTT	ACT	GAT	ATT	TTA	GCC	AAA	GAT		
601	Y	E	A	H	G	V	N	L	V	L	G	S	K	V	A	A	F	E	E	V	noxsanfran	
601	TAT	GAA	GCT	CAT	GTT	AGT	CTG	GTT	CTT	CTT	TCA	AAA	GTA	GTA	GCT	GCT	TTT	GAA	GAA	GTC		
601	Y	E	A	H	G	V	N	L	V	L	G	S	K	V	A	A	F	E	E	V	sfk6seq	
601	TAT	GAA	GCT	CAT	GTT	AGT	CTG	GTT	CTT	CTT	TCA	AAA	GTA	GTA	GCT	GCT	TTT	GAA	GAA	GTC		
601	Y	E	A	H	G	V	N	L	V	L	G	S	K	V	A	A	F	E	E	V	sfk2seq	
601	TAT	GAA	GCT	CAT	GTT	AGT	CTG	GTT	CTT	CTT	TCA	AAA	GTA	GTA	GCT	GCT	TTT	GAA	GAA	GTC		
661	D	D	E	I	I	T	K	T	L	D	G	K	E	I	K	S	D	I	A	I	noxsanfran	
661	GAT	GAT	GAA	ATT	ATC	ACT	AAA	ACC	CTA	GAT	GGT	AAA	GAA	ATT	AAA	TCT	GAT	ATT	GCA	ATT		
661	D	D	E	I	I	T	K	T	L	D	G	K	E	I	K	S	D	I	A	I	sfk6seq	
661	GAT	GAT	GAA	ATT	ATC	ACT	AAA	ACC	CTA	GAT	GGT	AAA	GAA	ATT	AAA	TCT	GAT	ATT	GCA	ATT		
661	D	D	E	I	I	T	K	T	L	D	G	K	E	I	K	S	D	I	A	I	sfk2seq	
661	GAT	GAT	GAA	ATT	ATC	ACT	AAA	ACC	CTA	GAT	GGT	AAA	GAA	ATT	AAA	TCT	GAT	ATT	GCA	ATT		
721	L	C	I	G	F	R	P	N	T	E	L	L	L	K	G	K	V	A	M	L	D	noxsanfran
721	CTT	TGT	ATC	GGT	TTC	CGC	CCT	AAA	ACT	AAA	GTA	TTA	CTT	AAA	GGT	ANA	GTT	GCC	ATG	TTG	GAT	
721	L	C	I	G	F	R	P	N	T	E	L	L	L	K	G	K	V	A	M	L	D	sfk6seq
721	CTT	TGT	ATC	GGT	TTC	CGC	CCT	AAA	ACT	AAA	GTA	TTA	CTT	AAA	GGT	ANA	GTT	GCC	ATG	TTG	GAT	
721	L	C	I	G	F	R	P	N	T	E	L	L	L	K	G	K	V	A	M	L	D	sfk2seq
721	CTT	TGT	ATC	GGT	TTC	CGC	CCT	AAA	ACT	AAA	GTA	TTA	CTT	AAA	GGT	ANA	GTT	GCC	ATG	TTG	GAT	

Figure 2E

781	N G A I I T D E Y M H S S N R D I F A A	noxsanfran
781	AAC GGT GCA ATC ATT ACT GAT GAA TAC ATG CAT TCA TCA AAT CGC GAC ATT TTT GCT GCT	
781	N G A I I T D E Y M H S S N R D I F A A	sfk6seq
781	AAC GGT GCA ATC ATT ACT GAT GAA TAC ATG CAT TCA TCA AAT CGC GAC ATT TTT GCT GCT	
781	N G A I I T D E Y M H S S N R D I F A A	sfk2seq
781	AAC GGT GCA ATC ATT ACT GAT GAA TAC ATG CAT TCA TCA AAT CGC GAC ATT TTT GCT GCT	
841	G D S A A V H Y N P T N S N A Y I P L A	noxsanfran
841	GGT GAT AGT GCC GCC GTT CAC TAC AAC CCC ACT AAT TCT AAC GCC TAC ATT CCT TTA GCT	
841	G D S A A V H Y N P T N S N A Y I P L A	sfk6seq
841	GGT GAT AGT GCC GCC GTT CAC TAC AAC CCC ACT AAT TCT AAC GCC TAC ATT CCT TTA GCT	
841	G D S A A V H Y N P T N S N A Y I P L A	sfk2seq
841	GGT GAT AGT GCC GCC GTT CAC TAC AAC CCC ACT AAT TCT AAC GCC TAC ATT CCT TTA GCT	
901	T N A V R Q G R L V G L N L T E D K V K	noxsanfran
901	ACC AAC GCC GTA CGC CAA GGG AGA TTA GTT GGC CTA AAT CTG ACT GAA GAC AAA GTA AAA	
901	T N A V R Q G R L V G L N L T E D K V K	sfk6seq
901	ACC AAC GCC GTA CGC CAA GGG AGA TTA GTT GGC CTA AAT CTG ACT GAA GAC AAA GTA AAA	
901	T N A V R Q G R L V G L N L T E D K V K	sfk2seq
901	ACC AAC GCC GTA CGC CAA GGG AGA TTA GTT GGC CTA AAT CTG ACT GAA GAC AAA GTA AAA	
961	D M G T Q S S S G L K L Y G R T Y V S T	noxsanfran
961	GAC ATG GGA ACC CAA TCT TCA TCT GGT CTT AAA CTA TAC GGT CGG ACT TAT GTC TCA ACT	
961	D M G T Q S S S G L K L Y G R T Y V S T	sfk6seq
961	GAC ATG GGA ACC CAA TCT TCA TCT GGT CTT AAA CTA TAC GGT CGG ACT TAT GTC TCA ACT	
961	D M G T Q S S S G L K L Y G R T Y V S T	sfk2seq
961	GAC ATG GGA ACC CAA TCT TCA TCT GGT CTT AAA CTA TAC GGT CGG ACT TAT GTC TCA ACT	
1021	G I N T A L A K A N N L K V S E V I I A	noxsanfran
1021	GGA ATC AAT ACG GCT CTT GGT AAA GCC AAT AAT TTA AAA GTT AGC GAA GTA ATC ATA GCT	
1021	G I N T A L A K A N N L K V S E V I I A	sfk6seq
1021	GGA ATC AAT ACG GCT CTT GGT AAA GCC AAT AAT TTA AAA GTT AGC GAA GTA ATC ATA GCT	
1021	G I N T A L A K A N N L K V S E V I I A	sfk2seq
1021	GGA ATC AAT ACG GCT CTT GGT AAA GCC AAT AAT TTA AAA GTT AGC GAA GTA ATC ATA GCT	
1081	D N Y R P E F M L S T D E V L M S L V Y	noxsanfran
1081	GAT AAT TAT CGT CCA GAA TTT ATG TTA TCA ACG GAT GAA GTT TTA ATG TCA TTA GTG TAT	
1081	D N Y R P E F M L S T D E V L M S L V Y	sfk6seq
1081	GAT AAT TAT CGT CCA GAA TTT ATG TTA TCA ACG GAT GAA GTT TTA ATG TCA TTA GTG TAT	
1081	D N Y R P E F M L S T D E V L M S L V Y	sfk2seq
1081	GAT AAT TAT CGT CCA GAA TTT ATG TTA TCA ACG GAT GAA GTT TTA ATG TCA TTA GTG TAT	
1141	D P K T R V I L G G A L S S M H D V S Q	noxsanfran
1141	GAT CCT AAG ACT CGT GTA ATT TTG GGA GGG GCG CTT TCA AGT ATG CAC GAT GTT TCG CAA	
1141	D P K T R V I L G G A L S S M H D V S Q	sfk6seq
1141	GAT CCT AAG ACT CGT GTA ATT TTG GGA GGG GCG CTT TCA AGT ATG CAC GAT GTT TCG CAA	
1141	D P K T R V I L G G A L S S M H D V S Q	sfk2seq
1141	GAT CCT AAG ACT CGT GTA ATT TTG GGA GGG GCG CTT TCA AGT ATG CAC GAT GTT TCG CAA	
1201	S A N V L S V C I Q N K N T I D D L A M	noxsanfran
1201	TCA GCG AAC GTC TTA TCA GTA TGT ATT CAA AAT AAA AAC ACG ATT GAC GAT TTA GCA ATG	
1201	S A N V L S V C I Q N K N T I D D L A M	sfk6seq
1201	TCA GCG AAC GTC TTA TCA GTA TGT ATT CAA AAT AAA AAC ACG ATT GAC GAT TTA GCA ATG	
1201	S A N V L S V C I Q N K N T I D D L A M	sfk2seq
1201	TCA GCG AAC GTC TTA TCA GTA TGT ATT CAA AAT AAA AAC ACG ATT GAC GAT TTA GCA ATG	
1261	V D M L F Q P Q F D R P F N Y L N I L G	noxsanfran
1261	GTG GAT ATG TTA TTC CAA CCA CAA TTT GAT CGT CCG TTT AAC TAC TTA AAC ATT CTA GGC	
1261	V D M L F Q P Q F D R P F N Y L N I L G	sfk6seq
1261	GTG GAT ATG TTA TTC CAA CCA CAA TTT GAT CGT CCG TTT AAC TAC TTA AAC ATT CTA GGC	
1261	V D M L F Q P Q F D R P F N Y L N I L G	sfk2seq
1261	GTG GAT ATG TTA TTC CAA CCA CAA TTT GAT CGT CCG TTT AAC TAC TTA AAC ATT CTA GGC	
1321	O A A O A O A D K A H K	noxsanfran
1321	CAA GCT GCT CAA GCA CAA GCT GAC AAA GCA CAT AAA TAA	
1321	O A A O A O A D K A H K	sfk6seq
1321	CAA GCT GCT CAA GCA CAA GCT GAC AAA GCA CAT AAA TAA	
1321	O A A O A O A D K A H K	sfk2seq
1321	CAA GCT GCT CAA GCA CAA GCT GAC AAA GCA CAT AAA TAA	

Figure 2F

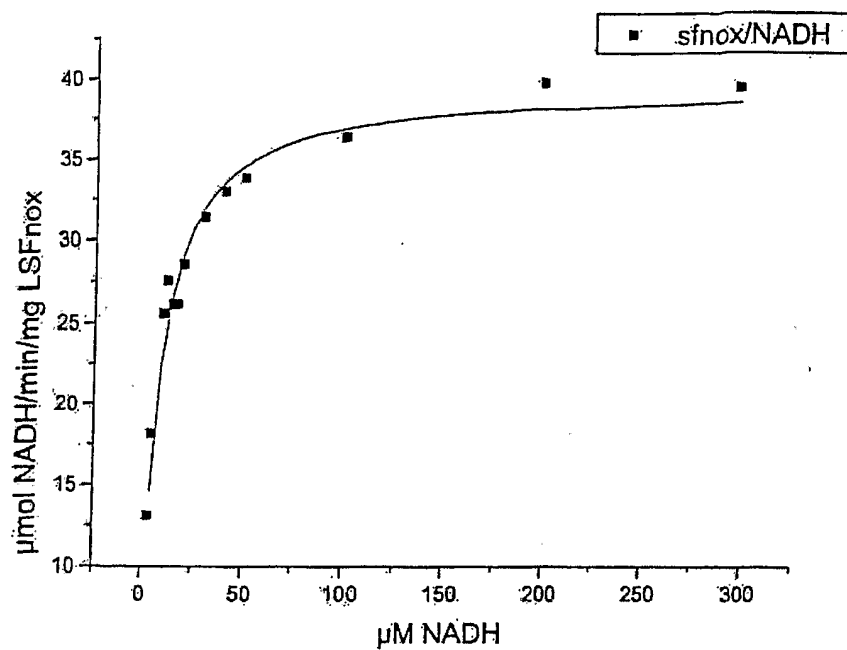


Figure 3A

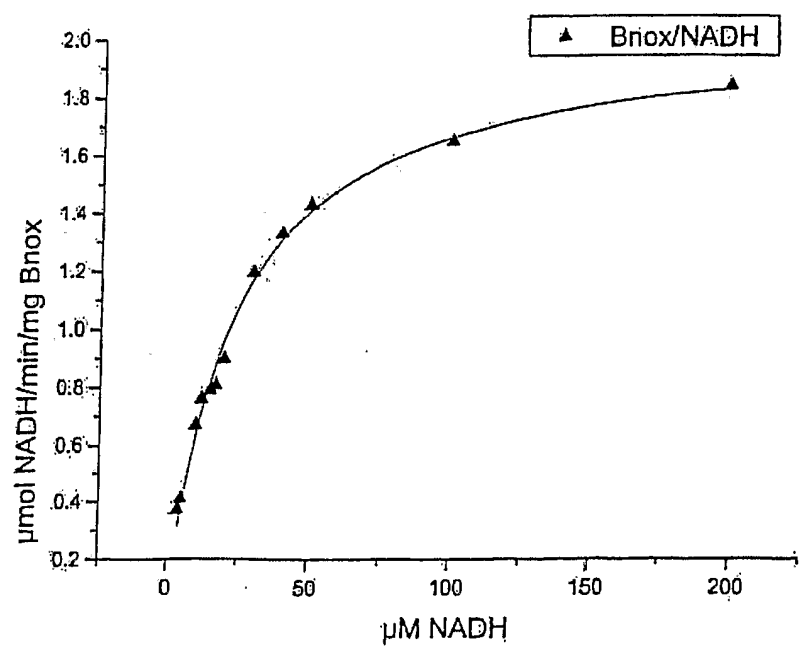
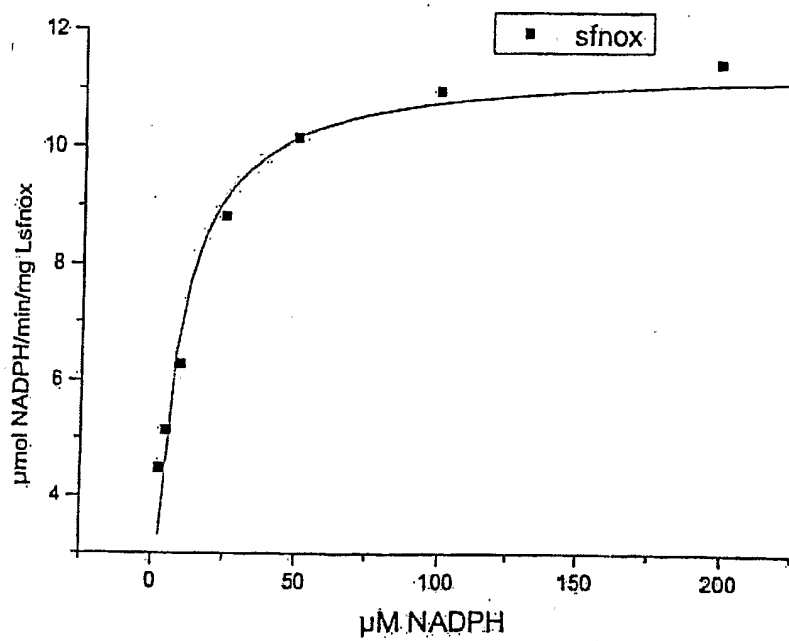
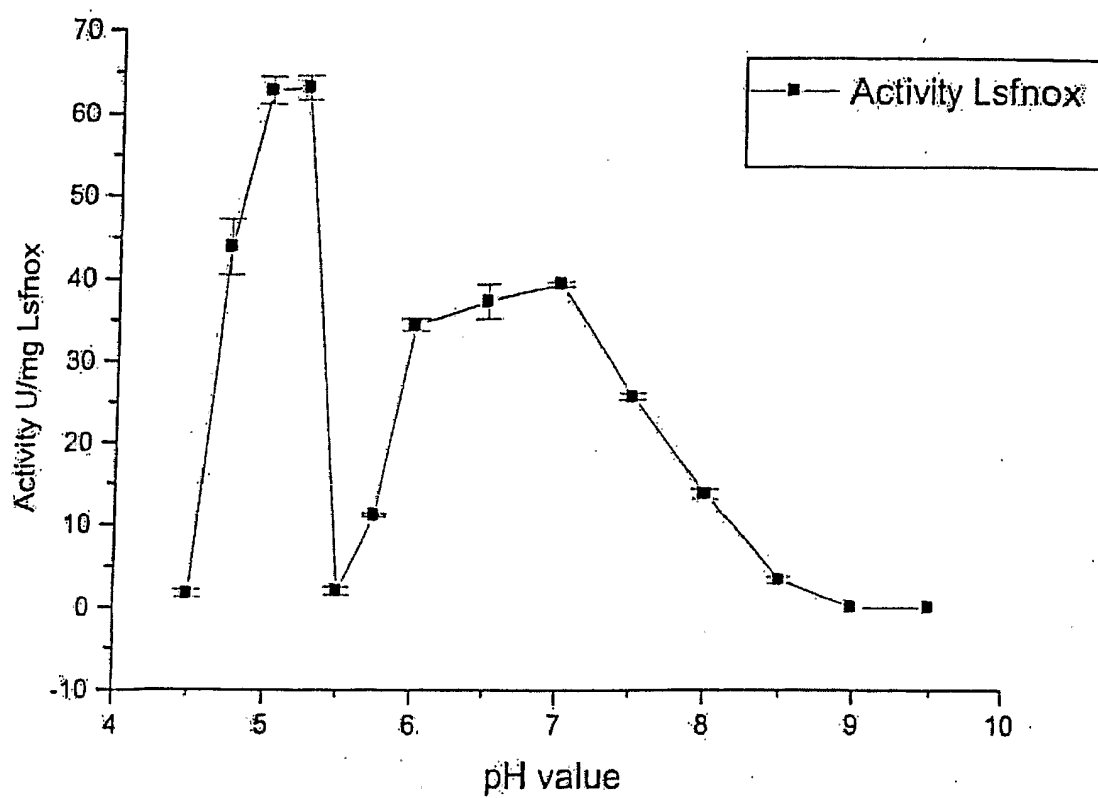


Figure 3B

**Figure 3C**

**Figure 4**

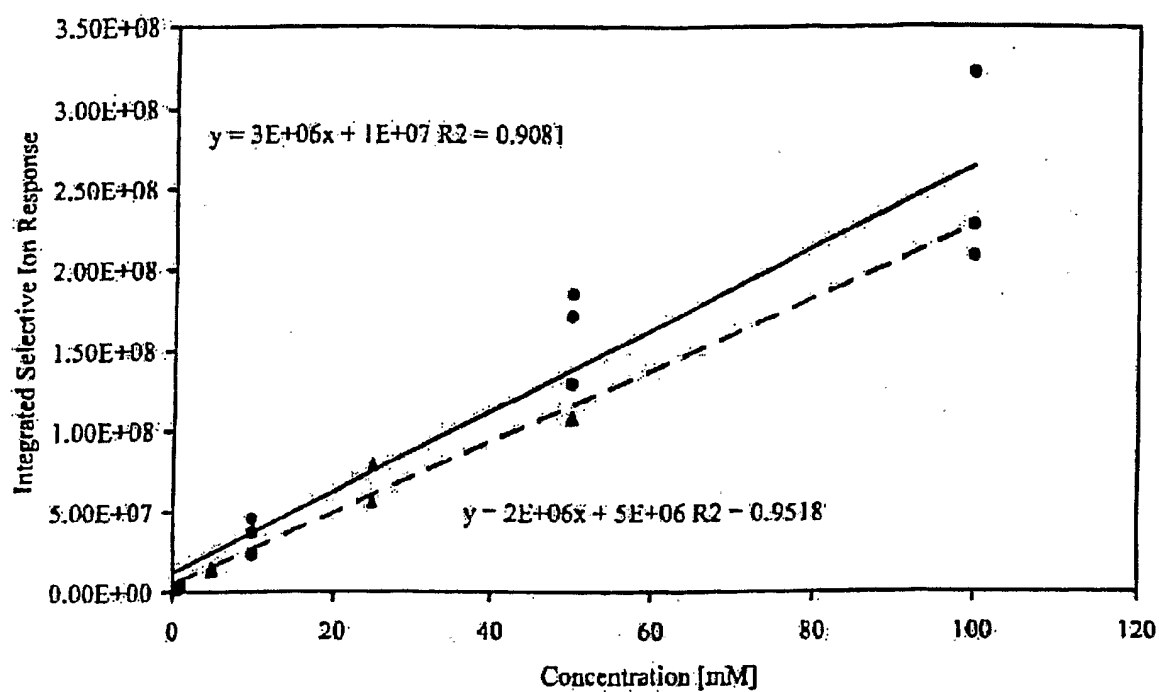


Figure 5