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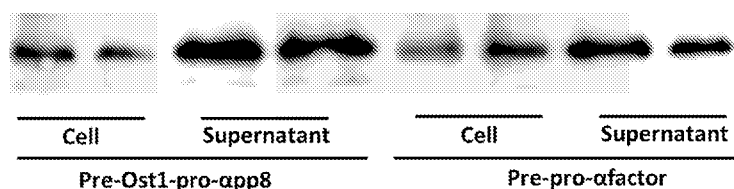


FIG. 6

(57) Abstract: Aspects of the disclosure relate to compositions and methods for producing recombinant proteins in yeast expression systems. The disclosure is based, in part, on expression vectors encoding certain oxalate oxidase proteins and methods of purifying such proteins. In some embodiments, expression constructs described herein comprise one or more N-terminal signal sequences, such as secretion signal sequences.



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RECOMBINANT OXALATE OXIDASE AND USES THEREOF

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. 119(e) of the filing date of US provisional Application Serial Number 62/988,603, filed March 12, 2020, entitled

5 “RECOMBINANT OXALATE OXIDASE AND USES THEREOF”, the entire contents of which are incorporated herein by reference.

BACKGROUND

Urinary oxalate, an integral part of the 24-hour urine stone profile, is used to calculate
10 mineral supersaturations and estimate kidney stone risk. However, there is no commercially available source of recombinant oxalate oxidase - an enzyme that could be used to create a diagnostic test strip to identify the presence of kidney stones.

SUMMARY

15 Aspects of the disclosure relate to compositions and methods for producing recombinant proteins in yeast expression systems. The disclosure is based, in part, on expression vectors encoding certain oxalate oxidase (OxOx) proteins and methods of purifying such proteins. In some embodiments, expression vectors described herein comprise a chimeric secretion signal encoding sequence. In some embodiments, transforming yeast cells with expression constructs comprising a
20 chimeric secretion signal linked to a recombinant OxOx protein surprisingly results in increased recovery of secreted heterologous protein (e.g., recombinant OxOx protein) from the yeast cells.

Accordingly, in some aspects, the disclosure provides an isolated nucleic acid comprising an expression construct comprising the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9.

25 In some embodiments, an isolated nucleic acid encodes a protein comprising the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11.

In some embodiments, an expression construct further comprises an alpha factor (α -factor) signal sequence operably linked to a nucleic acid sequence (e.g., the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9). In some embodiments, an α -factor signal sequence is

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positioned 5' relative to a nucleic acid sequence (e.g., the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9). In some embodiments, an α -factor signal sequence comprises the nucleic acid sequence set forth in SEQ ID NO: 12. In some embodiments, an expression construct comprises a non-yeast signal sequence that encodes a peptide comprising the sequence set forth in
5 any one of SEQ ID NOs: 17-19.

In some embodiments, an expression construct further comprises one or more purification tag sequences. In some embodiments, one or more purification tag sequences are positioned 3' relative to a nucleic acid sequence (e.g., the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9). In some embodiments, one or more purification tag sequences encodes a c-
10 myc epitope tag or a polyhistidine tag. In some embodiments, an expression construct does not comprise (e.g., lacks) a purification tag sequence.

In some aspects, the disclosure provides an isolated nucleic acid comprising an expression construct comprising a nucleic acid sequence encoding an oxalate oxidase (OxOx) protein operably linked to a non-yeast protein transduction domain sequence.

15 In some embodiments, an OxOx protein comprises the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11. In some embodiments, a nucleic acid sequence encoding a protein comprises the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9.

In some embodiments, a protein transduction domain sequence comprises bacterial transduction domain sequence. In some embodiments, a bacterial transduction domain sequence
20 comprises a twin-arginine translocation (Tat) pathway signal sequence. In some embodiments, a Tat pathway signal sequence comprises the nucleic acid sequence set forth in SEQ ID NO: 19.

In some embodiments, an expression construct further comprises a promoter operably linked to an α -factor signal sequence or a nucleic acid sequence (e.g., the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9). In some embodiments, a promoter is an AOX1 promoter.

25 In some embodiments, an expression construct further comprises a transcription terminator sequence. In some embodiments, a transcription terminator sequence is an AOX1 transcription terminator sequence.

In some aspects, the disclosure provides a plasmid or a host cell comprising an isolated nucleic acid as described herein. In some embodiments, a plasmid comprises the nucleic acid
30 sequence set forth in any one of SEQ ID NOs: 14-16.

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In some embodiments, a host cell is a yeast cell. In some embodiments, a yeast cell is a *Pichia pastoris* cell (X-33 or GS115 strains) or *Saccharomyces cerevisiae* cell.

In some aspects, the disclosure provides a container housing a population of yeast cells, wherein at least one yeast cell comprises an isolated nucleic acid or plasmid as described herein.

5 In some aspects, the disclosure provides a method for producing a recombinant protein, the method comprising introducing into a cell an isolated nucleic acid as described herein under conditions under which the cell expresses a recombinant oxalate oxidase (OxOx) protein.

In some embodiments, a recombinant OxOx protein comprises the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11, or is encoded by the nucleic acid sequence set
10 forth in any one of SEQ ID NOs: 1, 2, and 7-9.

In some embodiments, a method further comprises purifying a recombinant OxOx protein from a cell. In some embodiments, purifying comprises lysing a cell to produce cell lysate and an insoluble fraction. In some embodiments, the insoluble fraction is further treated to separate recombinant OxOx protein from yeast cellular membrane.

15 In some embodiments, purifying comprises contacting a cell lysate or insoluble fraction with an anion exchange media. In some embodiments, an anion exchange media comprises a Q-Sepharose anion exchange column. In some embodiments, purifying comprises providing conditions under which recombinant OxOx protein binds to a column.

In some embodiments, a method further comprises eluting bound recombinant OxOx protein
20 from a column to produce a purified protein fraction. In some embodiments, a purified protein fraction comprises >90% recombinant OxOx protein.

In some aspects, the disclosure provides an isolated nucleic acid comprising a nucleic acid sequence encoding a protein comprising a chimeric secretion signal having the amino acid sequence set forth in SEQ ID NO: 26.

25 In some embodiments, a chimeric secretion signal comprises a pre region encoded by the nucleic acid sequence set forth in SEQ ID NO: 21. In some embodiments, a chimeric secretion signal comprises a pro region encoded by the nucleic acid sequence set forth in SEQ ID NO: 23.

In some embodiments, a protein comprises an oxalate oxidase (OxOx) protein. In some embodiments, an OxOx protein is a barley OxOx protein. In some embodiments, a protein
30 comprises the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11.

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In some embodiments, an isolated nucleic acid further comprises a promoter operably linked to a nucleic acid sequence encoding a protein. In some embodiments, a promoter comprises an AOX1 promoter.

In some embodiments, an isolated nucleic acid further comprises a transcription terminator
5 sequence, optionally wherein the transcription terminator sequence is an AOX1 transcription terminator sequence.

In some aspects, the disclosure provides a protein comprising a chimeric secretion signal having the amino acid sequence set forth in SEQ ID NO: 26.

In some embodiments, a protein comprises an oxalate oxidase (OxOx) protein, optionally a
10 barley OxOx protein or a wheat OxOx protein. In some embodiments, a protein comprises the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, or 11.

In some embodiments, a protein further comprises a purification tag (e.g., a poly Histidine tag, etc.).

In some aspects, the disclosure provides a cell comprising an isolated nucleic acid or protein
15 (e.g., protein comprising a chimeric secretion signal) as described herein. In some embodiments, a cell is a yeast cell. In some embodiments, a yeast cell is a *Pichia pastoris* cell.

BRIEF DESCRIPTION OF DRAWINGS

FIGs. 1A-1C show OxOx plasmid construction and confirmation. FIG. 1A shows synthesis
20 of a codon optimized barley-derived oxalate oxidase gene and cloned into the EcoRI/XbaI sites of a pPICZαA expression vector. FIG. 1B shows sequencing of the OxOx gene insert. FIG. 1C shows the NCBI nucleotide BLAST® alignment tool was used to confirm the integrity of the inserted OxOx gene. SEQ ID NO: 2 is shown.

FIGs. 2A-2D show OxOx expression and purification. FIG. 2A shows accumulation of
25 recombinant OxOx in the insoluble fraction of yeast lysate. FIG. 2B shows *P. pastoris* culture media sample western blotting using an anti-OxOx antibody. FIG. 2C shows purification of recombinant OxOx from culture media that was dialyzed overnight against distilled water, pH adjusted to 9.0 using a tris buffer, applied to a Q-sepharose anion exchange column, and purified eluted with 1M NaCl. FIG. 2D shows the eluted fraction separated by SDS-PAGE and total protein
30 was visualized with Coomassie stain to determine purity.

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FIG 3 shows Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Analysis. Following trypsin digestion, LC-MS/MS was used to confirm the identity of the major band from the Coomassie gel in FIG. 2C. A peptide “AGETFVIPR” (SEQ ID NO: 20) was identified with 100% accuracy that is unique to the barley-derived oxalate oxidase (OxOx) protein and that is not present in the *P. pastoris* proteome.

FIGs. 4A-4B show representative data relating to reaction kinetics of oxalate oxidase (OxOx). FIG. 4A shows a Michaelis-Menten saturation curve. Kinetic analysis was performed in a 96-well plate at 37°C. Purified OxOx (400 µg/ml) was added to buffer pH 3.1 containing MBTH (0.11 mM), DMAB (1.6 mM), and HRP (0.1 U/ml) at designated potassium oxalate concentrations. Absorbance at 590 nm was measured and plotted against substrate concentration. FIG. 4B shows a Lineweaver-Burk plot. A linear transformation for the data described in FIG. 1A was plotted. Enzyme K_m was estimated to be 256 µM.

FIG. 5 shows a schematic representing expression vectors comprising yeast secretion signals. The top construct comprises a yeast α -factor secretion signal containing a “pre” region and a “pro” region. The bottom construct comprises a chimeric secretion signal containing a yeast OST “pre” region and a yeast pro α pp-8 “pro” region.

FIG. 6 shows representative Western blot data comparing secreted protein (“Supernatant”) and cell-bound protein (“Cell”) extracted from yeast cells transformed with expression constructs encoding recombinant OxOx linked to either a chimeric secretion signal (left) or a wild-type yeast α -factor secretion signal. Data indicate increased protein secretion from expression constructs comprising the chimeric secretion signal.

DETAILED DESCRIPTION

Aspects of the disclosure relate to compositions and methods for producing recombinant proteins in yeast expression systems, for example expression vectors encoding certain oxalate oxidase (OxOx) proteins and methods of purifying such proteins. The disclosure is based, in part, on the recognition that recombinant OxOx proteins comprising certain signal sequence tags surprisingly accumulate in the cellular membrane of yeast cells used for expressing such OxOx proteins.

Recombinant Oxalate Oxidase

Oxalate oxidase (OxOx) is a germin protein found in plants such as barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*). Germins are developmentally-regulated plant enzymes that generate hydrogen peroxide (H₂O₂) from the oxidative catabolism of oxalate. Germins typically require the presence of manganese (Mn) for enzymatic activity. Structurally, germins comprise a β-barrel domain containing two conserved histidine-containing sequence motifs. In some embodiments, a *Hordeum vulgare* oxalate oxidase protein comprises the nucleic acid sequence set forth in NCBI Accession Number Y14203.1, or the amino acid sequence set forth in NCBI Accession No. CAA74595.1. In some embodiments, a *Triticum aestivum* oxalate oxidase protein comprises the nucleic acid sequence set forth in NCBI Accession Number M63223.1, or the amino acid sequence set forth in NCBI Accession No. AAA34270.1. In the context of biotechnological applications, OxOx proteins are useful, in some embodiments, for detection of calcium oxalate kidney stones in a sample or subject. However, there is currently no commercially available source of oxalate oxidase that could be used to manufacture such diagnostic indications.

Thus, in some aspects, the disclosure provides isolated nucleic acids and vectors (e.g., plasmids, etc.) encoding a recombinant oxalate oxidase (OxOx) protein. As used herein “nucleic acid” refers to a DNA or RNA molecule. An “isolated nucleic acid” or “recombinant nucleic acid” refers to a nucleic acid (e.g., DNA or RNA) that has been prepared *in vitro*, for example by recombinant technology. Nucleic acids are polymeric macromolecules comprising a plurality of nucleotides. In some embodiments, the nucleotides are deoxyribonucleotides or ribonucleotides. In some embodiments, the nucleotides comprising the nucleic acid are selected from the group consisting of adenine, guanine, cytosine, thymine, uracil and inosine. In some embodiments, the nucleotides comprising the nucleic acid are modified nucleotides. Non-limiting examples of natural nucleic acids include genomic DNA and plasmid DNA. A “recombinant protein” refers to a protein expressed by an isolated nucleic acid or recombinant nucleic acid. In some embodiments, the nucleic acids of the instant disclosure are synthetic. As used herein, the term “synthetic nucleic acid” refers to a nucleic acid molecule that is constructed via joining nucleotides by a synthetic or non-natural method. One non-limiting example of a synthetic method is solid-phase oligonucleotide synthesis. In some embodiments, the nucleic acids of the instant disclosure are isolated.

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In some embodiments, recombinant oxalate oxidase (OxOx) protein is encoded by a nucleic acid having a sequence that is at least 50%, 60%, 70%, 80%, 90%, 95%, 99% or more identical to the nucleic acid sequence set forth in NCBI Accession Number Y14203.1. In some embodiments, a recombinant OxOx protein comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99% or more identical the amino acid sequence set forth in NCBI Accession No. CAA74595.1. In some embodiments, a recombinant OxOx protein is encoded by a nucleic acid that is at least 50%, 60%, 70%, 80%, 90%, 95%, 99% or more identical the nucleic acid sequence set forth in NCBI Accession Number M63223.1. In some embodiments, a recombinant OxOx protein comprises an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99% or more identical the amino acid sequence set forth in NCBI Accession No. AAA34270.1.

In some embodiments, an isolated nucleic acid encoding a recombinant OxOx protein comprises a codon-optimized (e.g., codon-optimized for expression in yeast cells, codon-optimized for expression in mammalian cells, etc.) coding sequence for the OxOx protein. In some embodiments, an isolated nucleic acid comprises a nucleic acid sequence that is at least 50%, 60%, 70%, 80%, 90%, 95%, 99% or more identical to the nucleic acid sequence set forth in SEQ ID NO: 1 or 4. In some embodiments, an isolated nucleic acid encodes a protein comprising an amino acid sequence that is at least 70%, 80%, 90%, 95%, 99% or more identical to the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11.

In some embodiments, a recombinant OxOx protein comprises one or more amino acid substitutions, insertions, or deletions relative to a wild-type OxOx protein (e.g., a protein comprising the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11). In some embodiments, a recombinant OxOx protein comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 50, 75, 80, 80, or 100 amino acid substitutions, insertions, or deletions relative to a wild-type OxOx protein (e.g., a protein comprising the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11). In some embodiments, a recombinant OxOx protein comprises more than 100 (e.g., 120m 150, 250, etc.) amino acid substitutions, insertions, or deletions relative to a wild-type OxOx protein (e.g., a protein comprising the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11).

In some embodiments, a recombinant OxOx protein retains functional activity. For example, a recombinant OxOx protein may retain the ability to catalyze the breakdown of oxalate into

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hydrogen peroxide (e.g., in the presence of Mn). The activity of a recombinant OxOx protein may vary. In some embodiments, a recombinant OxOx protein is between 2-fold and 100-fold (e.g., 2, 3, 4, 5, 10, 20, 50, 75, 100-fold, etc.) more active (e.g., by measurement of enzyme kinetics, substrate binding efficiency, etc.) than a wild-type OxOx protein (e.g., OxOx protein that has been isolated, extracted, and/or purified from barley or wheat). In some embodiments, a recombinant OxOx protein is more than 100-fold (e.g., 200, 500, 1000-fold, etc.) more active (e.g., by measurement of enzyme kinetics, substrate binding efficiency, etc.) than a wild-type OxOx protein (e.g., OxOx protein that has been isolated, extracted, and/or purified from barley or wheat). In some embodiments, a recombinant OxOx protein is between 2-fold and 100-fold (e.g., 2, 3, 4, 5, 10, 20, 50, 75, 100-fold, etc.) less active (e.g., by measurement of enzyme kinetics, substrate binding efficiency, etc.) than a wild-type OxOx protein (e.g., OxOx protein that has been isolated, extracted, and/or purified from barley or wheat). In some embodiments, a recombinant OxOx protein is more than 100-fold (e.g., 200, 500, 1000-fold, etc.) less active (e.g., by measurement of enzyme kinetics, substrate binding efficiency, etc.) than a wild-type OxOx protein (e.g., OxOx protein that has been isolated, extracted, and/or purified from barley or wheat).

Expression Constructs

Aspects of the disclosure relate to expression constructs encoding a recombinant OxOx protein. As used herein, an “expression construct” refers to an isolated nucleic acid that is engineered to express one or more nucleic acid sequences or genes of interest. An expression cassette generally includes one or more control elements and nucleic acid sequences or genes of interest to be expressed. As used herein, the term “engineered to express” refers to an isolated nucleic acid that directs expression of a sequence or gene of interest (e.g., a gene encoding recombinant OxOx protein) and, optionally, one or more expression control sequences. Examples of expression control sequences include but are not limited to promoter sequences, enhancer sequences, repressor sequences, poly A tail sequences, internal ribosomal entry sites, Kozak sequences, antibiotic resistance genes (e.g., *ampR*, *kanR*, a chloramphenicol resistance gene, a β -lactamase resistance gene, *etc.*), an origin of replication (*ori*), transcriptional start sites, transcriptional terminator sites, *etc.*

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One or more control elements (e.g., expression control sequences) are typically operably linked to the nucleic acid sequences or genes of interest. As used herein, a nucleic acid sequence (e.g., coding sequence) and regulatory control sequences are said to be “operably linked” when they are covalently linked in such a way as to place the expression or transcription of the nucleic acid sequence under the influence or control of the regulatory sequences. In some embodiments, a control sequence is directly operably linked (e.g., immediately upstream, such as 5’, or downstream, such as 3’) relative to a nucleic acid sequence or gene encoding a recombinant OxOx protein. In some embodiments, a control sequence is indirectly operably linked (e.g., upstream, such as 5’, or downstream, such as 3’, with intervening nucleic acid sequence, such as another control sequence) relative to a nucleic acid sequence or gene encoding a recombinant OxOx protein.

In some embodiments, one or more isolated nucleic acid is operably linked to a promoter sequence. A promoter can be a constitutive promoter or an inducible promoter. In some embodiments, a promoter is a constitutive promoter. Examples of constitutive promoters include but are not limited to constitutive *E. coli* σ^{70} promoters, constitutive *E. coli* σ^S promoters, constitutive *E. coli* σ^{32} promoters, constitutive *E. coli* σ^{54} promoters, constitutive *B. subtilis* σ^A promoters, constitutive *B. subtilis* σ^B promoters, constitutive bacteriophage T7 promoters, constitutive bacteriophage SP6 promoters, constitutive yeast promoters, *etc.* In some embodiments, the promoter is a yeast promoter. Examples of yeast promoters include but are not limited to AOX1, SAS, FLD1, ICL1, PHO89, THI11, ADH1, ENO1, GUT1, GAP, TEF1, PGK1, GCW14, G1, G6, *etc.*

In some embodiments, a promoter is an inducible promoter (e.g., induced in the presence of a small molecule, such as IPTG or tetracycline). Examples of inducible promoters include but are not limited to a promoter comprising a tetracycline responsive element (TRE), a pLac promoter, a pBad promoter, alcohol-regulated promoters (e.g., AlcA promoter), steroid-regulated promoters (e.g., LexA promoter), temperature-inducible promoters (e.g., Hsp70- or Hsp90-derived promoters, light-inducible promoters (e.g., YFI), *etc.* In some embodiments, an inducible promoter is a methanol-regulated promoter, for example AOX1 promoter as described by Yang et al. *Scientific Reports* (2018) 8:1401. In some embodiments, an AOX1 promoter comprises the nucleic acid sequence set forth in SEQ ID NO: 13.

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In some embodiments, an isolated nucleic acid comprises a transcription initiation sequence operably linked to nucleic acid sequences or genes of interest (e.g., a gene encoding a recombinant OxOx protein). In some embodiments, an isolated nucleic acid comprises a transcription terminator sequence. Examples of transcription terminator sequences are described by Vogl et al. (2014) *ACS Synth Biol* 3(3):1880191. In some embodiments, an isolated nucleic acid comprises a transcriptional terminator sequence. Examples of transcriptional terminator sequences include but are not limited to AOX1, CYC1, TEF1, PGK1, FUM1, SED1, GND1, GYP7, CYC7, YGR127W, PSY4, GSY2, MRP4, VPS13, TPS1, ECM10, YOLO36W, GRE3, HUG1, TIP1, AIP1, YJR085C, HSP26, PDC6, ADH1, UBX6, SPO1, GAT2, LSC2, PRM5, IDP1, SPG5, HIS5, CPS1, PRM9, etc.

In some embodiments, the transcriptional terminator sequence is an AOX1 transcriptional terminator sequence, for example as described by Vogl et al. (2018) *Applied and Environmental Microbiology* 84 (6) e02712-17. An isolated nucleic acid described by the disclosure may further comprise a polyadenylation (poly A) sequence.

Aspects of the disclosure relate inclusion of one or more signal sequences (e.g., signal peptide sequences) in an expression construct encoding a recombinant OxOx protein. In some embodiments, a signal sequence is a secretion signal sequence. Secretion signals are typically short peptides that are present at the N-terminus of a protein and assist in directing the protein into the secretory pathway of a cell. In some embodiments, a signal sequence facilitates translocation of the protein across cellular membrane. A secretion signal may be a yeast secretion signal. Examples of yeast secretion signals include but are not limited to PHO1, α -MF, USC2, PHA-E, KILM1, pGKL, CLY-L8, SCW, DSE, EXG, Pir1, HBF1, etc. In some embodiments, a secretion signal is a yeast alpha-factor (α -factor) signal sequence. Yeast alpha-factor (α -factor), as described, for example by Lin-Cereghino et al. (2013) *Gene* 519(2):311-317. In some embodiments, a yeast α -factor signal sequence is encoded by a nucleic acid comprising the sequence set forth in SEQ ID NO: 12.

Aspects of the disclosure relate to expression constructs comprising one or more (e.g., 1, 2, 3, 4, 5, or more) chimeric signal sequences. A “chimeric signal sequence” generally refers to a nucleic acid sequence encoding a signal peptide that comprises a first region from one gene and a second region from a different gene. For example, in the context of yeast secretion signals, a chimeric signal sequence may comprise a first nucleic acid sequence encoding a “pre” region of a first gene and a second nucleic acid sequence encoding a “pro” region of a second gene.

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In some embodiments, an expression construct comprises an Oligosaccharyl transferase subunit 1 (OST1) “pre” region, such as a *S. cerevisiae* “pre” region, or a variant thereof, for example as described by Barrero et al. *Microb. Cell Fact.* (2018) 17:161. A variant of a “pre” region may have 1, 2, 3, 4, 5, 6, or more amino acid substitutions relative to a wild-type “pre” region. In some embodiments, an OST1 “pre” region is encoded by the nucleic acid sequence set forth in SEQ ID NO: 21. In some embodiments, an OST1 “pre” region comprises the amino acid sequence set forth in SEQ ID NO: 22.

In some embodiments, an expression construct comprises a yeast α -factor “pro” region or a variant thereof, for example as described by Rakestraw et al. *Biotechnol Bioeng.* 2009 August 15; 103(6): 1192–1201. doi:10.1002/bit.22338. In some embodiments, a “pro” region variant comprises a pro α pp-8 “pro” region. In some embodiments, a “pro” region is encoded by the nucleic acid sequence set forth in SEQ ID NO: 23. In some embodiments, a “pro” region comprises the amino acid sequence set forth in SEQ ID NO: 24.

In some embodiments, an expression construct comprises a chimeric signal sequence comprising an OST1 “pre” region and a pro α pp-8 “pro” region. In some embodiments, an expression construct comprises a chimeric signal sequence encoded by the nucleic acid sequence set forth in SEQ ID NO: 25. In some embodiments, an expression construct comprises a chimeric signal sequence having the amino acid sequence set forth in SEQ ID NO: 26.

In some embodiments, a secretion signal sequence is a non-yeast secretion signal (e.g., a secretion signal that is not naturally present in yeast or is heterologous to yeast). The disclosure is based, in part on the recognition that inclusion of one or more signal sequences that are not native to yeast (e.g., that are not naturally present in yeast, or are heterologous to yeast) in expression constructs described herein improves expression of recombinant OxOx protein in yeast cells relative to expression constructs that do not include the one or more signal sequences. In some embodiments, an expression construct described herein comprises a bacterial signal peptide sequence (e.g., a secretion signal sequence naturally present in bacteria) operably linked to a nucleic acid encoding a recombinant OxOx protein. In some embodiments, the non-yeast signal sequence is a twin-arginine translocation pathway (Tat pathway) signal sequence. In some embodiments, a Tat pathway signal sequence is a *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Yersinia pseudotuberculosis*, or *E. coli O157:H7* Tat pathway signal sequence. In some embodiments, a Tat

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pathway signal sequence is encoded by the nucleic acid sequence set forth in SEQ ID NO: 19. In some embodiments, a protein comprising a Tat pathway signal sequence is transported (or capable of being transported) across a cellular membrane in a folded state.

A non-yeast signal sequence may also be derived from the same organism as the
5 recombinant gene of interest. For example, in some embodiments, an expression construct encoding recombinant OxOx protein may comprise a plant signal sequence. In some embodiments, the plant signal sequence is a barley or wheat plant signal sequence. In some embodiments, the plant signal sequence comprises the amino acid sequence set forth in SEQ ID NO: 17 or 18 (or a nucleic acid encoding such a peptide). Additional examples of non-yeast signal sequences include but are not
10 limited to twin arginine translocation (TAT) sequences, protein transduction domain (PTD) sequences, etc.

An expression construct may further comprise one or more nucleic acid sequences encoding a peptide purification tag. Examples of peptide purification tags include but are not limited to FLAG tag, c-myc epitope tag, polyhistidine (poly-His) tag, etc. The nucleic acid sequence
15 encoding the purification tag may be positioned upstream (e.g. 5' or N-terminal) or downstream (e.g., 3' or C-terminal) with respect to the nucleic acid sequence encoding the recombinant OxOx protein. In some embodiments, an expression construct encoding a recombinant OxOx protein lacks a protein purification tag.

In some embodiments, an isolated nucleic acid engineered to express a protein is a
20 component of a vector. Examples of vectors include plasmids, viral vectors, cosmids, and artificial chromosomes. In some aspects, one or more isolated nucleic acids engineered to express a protein (e.g., recombinant OxOx protein, *etc.*) are located (e.g., situated) on a plasmid, for example a bacterial plasmid or yeast plasmid. In some embodiments, the vector is a high-copy plasmid. In some embodiments, the vector is a low-copy plasmid. In some embodiments, a yeast cell comprises
25 one or more plasmids comprising the one or more isolated nucleic acids. For example, a plasmid may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 isolated nucleic acids. In some embodiments, a plasmid comprises 1, 2, or 3 isolated nucleic acids.

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Methods

In some embodiments, the disclosure relates to methods of producing recombinant OxOx protein in a host cell as described by the disclosure. In some embodiments, the host cell is a bacterial cell, mammalian cell, insect cell, or yeast cell. In some embodiments, the host cell is a yeast cell. Yeast cell-based recombinant protein expression systems are known in the art and include, for example *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Yarrowia lipolytica*, *Arxula adeninivorans*, *Kluyveromyces lactis*, and *Schizosaccharomyces pombe* expression systems, for example as described by Baghban et al. (2019) *Mol Biotechnol* 61(5):365-384.

A yeast cell-based expression system can be a methylotrophic yeast expression system. In some embodiments, the methylotrophic yeast expression system is a *Pichia pastoris* (also referred to as *Komagataella pastoris*) expression system. *Pichia* expression systems are known in the art and are described, for example, by Ahmad et al. (2014) *Appl Microbiol Biotechnol* 98(12):5301-5317. In some embodiments, a *Pichia* yeast cell is selected from the following strains: CBS7435, CBS704, X-33, GS115, KM71, KM71H, BG09, GS190, GS200, JC220, JC220, JC254, JC227, JC300, JC301, JC202, JC303, JC304, JC305, JC306, JC307, JC308, YJN165, CBS7435*his4a*, SMD1163, BG21, etc. In some embodiments, a yeast cell is a *Saccharomyces cerevisiae* cell.

Typically, methods described by the disclosure comprise the steps: transforming a yeast cell with an isolated nucleic acid engineered to express a recombinant OxOx protein; and culturing (*e.g.*, growing) the yeast cell. Methods of introducing vectors into microorganisms, such as yeast, are well known in the art and described, for example, in Current Protocols in Molecular Biology, Ausubel et al. (Eds), John Wiley and Sons, New York, 2007. In some embodiments of methods described by the disclosure, a yeast cell is transformed with one or more isolated nucleic acids comprising the sequence set forth in any one of SEQ ID NOs: 1, 2, 7-9, and 14-16.

The skilled artisan recognizes that the conditions (*e.g.*, temperature, CO₂ concentration, humidity, presence or absence of inducers, such as methanol, etc.) under which a recombinant OxOx protein is expressed by the yeast cell as described herein is maintained may affect the production and/or stability and/or function of the recombinant OxOx protein by the yeast cell(s). In some embodiments, a yeast cell transformed with an isolated nucleic acid as described herein is

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incubated at temperatures between 10 to 30 °C (*e.g.*, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, or 30°C).

The length of time a yeast cell culture is grown after transformation with an isolated nucleic acid as described herein can vary. In some embodiments, a yeast cell is cultured for between about
5 10 hours and 120 (*e.g.*, 10, 12, 24, 48, 72, 84, 96, etc.) hours after being transformed with an isolated nucleic acid as described herein. In some embodiments, the yeast cell is cultured in the presence of methanol for a portion (or all) of the culture period. In some embodiments, a yeast cell is cultured without methanol in complete medium to expand the yeast culture. In some
10 embodiments, the yeast culture is expanded 10-fold every 24 hours. The length of this non-methanol culture depends on vessel volume. After attaining the desired growth, the culture volume is reduced. In some embodiments, the culture volume is reduced between the ranges of about 2:1 to about 10:1. For example, a 500ml culture is reduced to 250ml, or as low as 50ml.

In some embodiments, the yeast culture is induced (*e.g.*, induced by addition of methanol). In some embodiments, induction media is a minimal media with 0.5% methanol. In some
15 embodiments, induction media is added to the culture on a daily basis. In some embodiments, inducer (*e.g.*, methanol) is continuously infused into a yeast culture. In some embodiments, methanol induction ranges from about 1 to 10 days.

The disclosure is based, in part, on the recognition that recombinant OxOx protein expressed in yeast cells surprisingly accumulates in the cell membrane of the yeast cells. Thus, methods
20 described by the disclosure may further comprise a step of extracting, isolating, and/or purifying recombinant OxOx protein from the yeast cell by which it was produced. Recombinant OxOx protein can be separated or purified from yeast cells by any suitable methodology, for example physical separation or chemical separation. Examples of suitable separation methodologies include but are not limited to chromatography (affinity chromatography, high pressure liquid
25 chromatography (HPLC), anion exchange chromatography, etc.), mass spectrometry (MS), electrophoresis (*e.g.*, 2D or 3D gel electrophoresis, capillary electrophoresis, etc.), etc.

Cells (*e.g.*, yeast cells) are typically lysed prior to isolation or purification of recombinant proteins (*e.g.*, recombinant OxOx protein). In some embodiments, methods described by the disclosure further comprise the step of lysing yeast cells that have been transformed with an isolated
30 nucleic acid encoding a recombinant OxOx protein. In some embodiments, the cells are lysed using

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a lysis buffer selected from CellLytic Y, YeastBuster Protein Extraction Reagent, Y-PER Yeast Protein Extraction Reagent, etc. In some embodiments, the cell lysate is separated from an insoluble fraction, which typically comprises cellular membrane components. In some embodiments, an insoluble fraction comprises recombinant OxOx proteins. In some embodiments, an insoluble
5 fraction is treated with a detergent (e.g., sodium dodecylsulfate, SDS) prior to a purification step.

In some embodiments, a recombinant OxOx protein (e.g., a cell lysate or insoluble fraction comprising a recombinant OxOx protein) is treated by anion exchange chromatography. Anion exchange chromatography is a process that separates molecules (e.g., proteins) from a sample based on their electro-chemical charges by contacting the sample to a positively-charged solid substrate
10 (e.g., a resin or beads). Examples of anion exchange chromatography solid substrates include Diethylaminoethyl (DEAE), which is typically characterized as a “weak” anion exchanger, and quaternary ammonium (e.g., Q Sepharose), which is typically characterized as a “strong” anion exchanger. The disclosure is based, in part, on the recognition that recombinant OxOx proteins are characterized by several hydrophobic regions and that performing chromatography using “strong”
15 anion exchangers provides improved purity of recombinant OxOx protein samples relative to “weak” anion exchangers. In some embodiments, a cell lysate or insoluble fraction comprising recombinant OxOx protein is first subjected to anion exchange chromatography using a “weak” anion exchanger, and subsequently subjected to anion exchange chromatography using a “strong” anion exchanger. In some embodiments, a recombinant OxOx protein is bound to an anion
20 exchange solid substrate using a buffer having a pH ranged from 8.0-10.0 (e.g., 8, 8.5, 9, 9.5, or 10). In some embodiments, the buffer is pH 9.0. In some embodiments, recombinant OxOx protein is eluted from a substrate using a high salt” buffer, for example 1M NaCl. In some embodiments, a buffer comprises Tris buffer, phosphate buffer, sodium chloride buffer, glycine buffer, etc.

EXAMPLE

This example describes an expression system for recombinant oxalate oxidase (OxOx) protein using a yeast expression system.

A codon optimized synthetic OxOx gene derived from *Hordeum vulgare* (barley) was generated and cloned into the EcoRI/XbaI sites of the pPICZαA expression vector downstream of
30 the N-terminal alpha mating factor secretion signal peptide sequence and used for expression in

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Pichia pastoris X-33 strain (FIG. 1A). This expression system is capable of generating disulfide bonds and addition of glycans required for the functional expression of the OxOx enzyme. FIG. 1B shows confirmatory sequencing of the OxOx gene insert. FIG. 1C shows data from a NCBI nucleotide BLAST® alignment used to confirm the integrity of the inserted OxOx gene by
5 comparing the recombinant OxOx sequence to Wild-type *Hordeum vulgare* oxalate oxidase nucleic acid sequence.

P. pastoris was transformed with the expression plasmid and induced to express active enzyme. Purification was carried out as follows: following protein expression, yeast cultures are centrifuged. Cell-free culture media is collected and dialyzed overnight against distilled water.
10 Because the isoelectric point of the enzyme is approximately 5.5, an alkaline Tris buffer was used to induce a strong negative charge, allowing the recombinant OxOx enzyme to bind to a strong anion exchange column. The bound recombinant OxOx protein is eluted using 1M NaCl.

FIG. 2A shows presence of recombinant OxOx in the insoluble fraction of yeast lysate, indicating accumulation of recombinant protein in yeast cellular membranes. FIG. 2B shows *P. pastoris* culture media sample western blotting using an anti-OxOx antibody. Data indicate the
15 production of recombinant OxOx protein in the yeast system. Culture media was subsequently dialyzed overnight against distilled water. One advantage of using this yeast secretion system is that it functions as a first step in the process of protein purification. Since the isoelectric point (pI) of OxOx enzyme is approximately 5.5, anion exchange chromatography was used for purification (Q-sepharose: equilibration/binding with tris buffer pH 9.0 and elution with 1M NaCl). FIG. 2C
20 shows purification of recombinant OxOx using the Q-sepharose anion exchange column. FIG. 2D shows the eluted fraction separated by SDS-PAGE. Total protein was visualized with Coomassie stain to determine purity. A composition having a purity of over 90% recombinant OxOx was produced.

25 FIG. 3 shows Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Analysis. Following trypsin digestion, LC-MS/MS was used to confirm the identity of the major band from the Coomassie gel in FIG. 2C. A peptide that is unique to the barley-derived oxalate oxidase (OxOx) protein and that is not present in the *P. pastoris* proteome, "AGETFVIPR" (SEQ ID NO: 20), was identified with 100% accuracy.

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Kinetic assays were performed at optimal enzyme conditions as noted in FIGs. 4A-4B. Purified OxOx enzyme displayed standard Michaelis-Menten kinetics at substrate concentrations up to 400 μ M (after which substrate inhibition occurs) and had an estimated K_m value of 256 μ M based on linear regression analysis using a Lineweaver-Burk plot.

One challenge of recombinant OxOx expression in yeast is that the expressed heterologous protein is often not secreted properly. For example, recombinant OxOx expressed in yeast cells often is trapped in subcellular compartments (e.g., endoplasmic reticulum, Golgi apparatus, etc.) instead of being secreted from the cells. Thus, recombinant OxOx expression vectors comprising alternative yeast secretion signals were tested. FIG. 5 shows a schematic representing expression vectors comprising yeast secretion signals. The top construct comprises a wild-type yeast α -factor secretion signal containing a “pre” region and a “pro” region upstream of a codon-optimized OxOx nucleic acid sequence. The α -factor secretion signal shown in the top vector comprises the sequence set forth in SEQ ID NO: 12. The bottom construct comprises a chimeric secretion signal containing a yeast OST “pre” region (SEQ ID NO: 21) and a yeast pro α pp-8 “pro” region (SEQ ID NO: 23) upstream of a codon-optimized OxOx nucleic acid sequence. The entire secretion signal sequence of the bottom construct is represented by SEQ ID NO: 25. Yeast cells were transformed with the OxOx expression constructs and protein secretion was measured by Western blot. Surprisingly, the expression construct comprising the chimeric secretion signal resulted in increased secretion of recombinant OxOx protein relative to the wild-type α -factor secretion signal (FIG. 6).

SEQUENCES

>Codon-optimized *Hordeum vulgare* OxOx nucleic acid sequence 1 no leader (SEQ ID NO:1)

TCTGACCCAGATCCATTGCAGGATTTCTGTGTTGCTGACTTGGACGGTAAGGCCGTTTC
 TGTTAACGGTCACACTTGTAAGCCAATGTCTGAAGCTGGTGACGACTTCCTGTTCTCCT
 CAAAGTTGACTAAGGCTGGTAACACCTCCACTCCAAACGGTTCTGCTGTTACCGAATTG
 GACGTTGCTGAATGGCCTGGAACCTTGGGTGTCTCCATGAACAGAGTTGACTT
 TGCTCCAGGTGGTACAAACCCACCACATATTCATCCAAGAGCTACCGAGATCGGTATG
 GTCATGAAGGGTGAGTTGTTGGTCGGTATCTTGGGTCTTTGGACTCCGGTAACAAGCT
 GTACTCCAGAGTTGTTAGAGCCGGTGAGACTTTCGTTATCCCAAGAGGTTTGATGCACT

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TCCAGTTCAACGTTGGTAAGACCGAGGCCTACATGGTTGTGTCCTTCAACTCTCAAAAC
CCCGGTATCGTTTTTCGTCCCATTTGACTTTGTTCGGTTCTGACCCACCAATTCCAACCTCCA
GTTTTGACCAAGGCTTTGAGAGTCGAGGCTGGTGTGTTGAATTGCTGAAGTCTAAGTT
CGCCGGTGGTTCC

5

>Codon-optimized *Hordeum vulgare* OxOx nucleic acid sequence 2 with leader (SEQ ID NO: 2)
AGTGACCCTGACCCACTTCAGGACTTCTGTGTGGCTGACCTGGACGGAAAAGCTGTGTC
TGTAACCGGCCATACGTGCAAACCTATGTCAGAGGCTGGTGATGACTTCCTGTTCTCTT
CTAAACTAACTAAGGCAGGTAACACCAGTACCCCCAATGGAAGTGCCGTAACAGAATT
GGATGTAGCTGAATGGCCTGGAACAAACACGTTGGGCGTTAGTATGAATAGGGTAGAC
TTCGCACCTGGTGGAAACCAATCCCCCTCATATACACCCACGTGCAACGGAAATTGGCAT
GGTGATGAAGGGCGAGCTGCTTGTAGGCATACTTGAAGTCTGGACTCTGGCAATAAA
CTATACTCCAGAGTCGTCAGAGCAGGAGAACTTTCGTCATTCCCAGAGGACTTATGC
ACTTCCAGTTCAACGTAGGCAAGACGGAAGCATATATGGTCGTCAGTTTCAACAGTCA
GAACCCTGGCATTGTATTTCGTTCCATTAACCTCTGTTTGGCAGTGATCCCCCAATCCCAA
CCCCTGTTTTTAACAAAAGCCTTGAGAGTAGAAGCAGGCGTCGTGGAATTATTGAAGAG
TAAATTCGCAGGTGGCTCA

10

15

>Wild-type *Hordeum vulgare* oxalate oxidase nucleic acid sequence no leader (SEQ ID NO:3)

20

25

30

TCCGACCCAGACCCACTCCAGGACTTCTGCGTCGCGGACCTCGATGGCAAGGCGGTCT
CGGTGAACGGGCATACGTGTAAGCCCATGTCGGAGGCCGGCGACGACTTCCTCTTCTC
GTCCAAGCTGACCAAGGCCGGCAACACGTCCACCCCGAACGGCTCGGCCGTGACGGAG
CTCGACGTGGCCGAGTGGCCCGGTACGAACACGCTGGGCGTGTCCATGAACCGTGTGG
ACTTCGCGCCGGGGGGCACCAACCCGCCGCACATCCACCCGCGTGCAACCGAGATCGG
CATGGTGATGAAAGGTGAGCTCCTCGTTGGAATCCTCGGCAGCCTTGACTCCGGAAAC
AAGCTCTACTCCAGGGTGGTGCGTGCCGGAGAGACTTTCGTCATCCCGCGCGGCCTCAT
GCACTTCCAGTTCAACGTTGGTAAGACGGAAGCCTACATGGTTGTGTCCTTCAACAGCC
AGAACCCTGGCATCGTCTTCGTGCCGCTCACACTCTTCGGCTCCGACCCTCCCATCCCC
ACGCCCCGTGCTACCAAGGCTCTCCGGGTGGAGGCCGGAGTCGTGGAATTCTCAAGT
CCAAGTTCGCCGGTGGGTCT

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>Wild-type *Hordeum vulgare* oxalate oxidase nucleic acid sequence with leader (SEQ ID NO: 4)

ATGGGTACTCTAAAAACCTAGGGGCTGGCCTGTTACCATGCTGCTCCTTGCTCCGGC
 CATCATGGCTACCGACCCTGACCCTCTACAGGACTTCTGCGTCGCGGACCTCGATGGCA
 5 AGGCGGTCTCGGTGAACGGGCATACGTGTAAGCCCATGTCGGAGGCCGGCGACGACTT
 CCTCTTCTCGTCCAAGCTGACCAAGGCCGGCAACACGTCCACCCCGAACGGCTCGGCC
 GTGACGGAGCTCGACGTGGCCGAGTGGCCCGGTACGAACACGCTGGGCGTGTCCATGA
 ACCGTGTGGACTTCGCGCCGGGCGGCACCAACCCGCCGCACATCCACCCGCGTGCAAC
 CGAGATCGGCATGGTGATGAAAGGTGAGCTCCTCGTTGGAATCCTCGGCAGCTTTGAC
 10 TCCGGAAACAAGCTCTACTCCAGGGTGGTGCGTGCCGGAGAGACTTTCGTCATCCCGC
 GCGGCCTCATGCACTTCCAGTTCAACGTTGGTAAGACGGAAGCCTACATGGTTGTGTCC
 TTCAACAGCCAGAACCCTGGCATCGTCTTCGTGCCGCTCACACTCTTCGGTTCCAACCC
 GCCCATCCCCACACCGGTGCTCACCAAGGCTCTTCGGGTGGAGGCCGGGGTTCGTGGAA
 CTTCTCAAGTCCAAGTTCGCCGGTGGGTCT

15

>*H. vulgare* Oxalate Oxidase amino acid sequence no leader (SEQ ID NO: 5)

SDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFLFSSKLTKAGNTSTPNGSAVTELD
 VAEWPGTNTLGVSMNRVDFAPGGTNPPHIHPRATEIGMVMKGELLVGILGSLDSGNKLYS
 RVVRAGETFVIPRGLMHFQFNVGKTEAYMVVSFNSQNPQIVFVPLTLFGSDPPIPTPVLTKA
 20 LRVEAGVVELLKSKFAGGS

>*H. vulgare* Oxalate Oxidase amino acid sequence with leader (SEQ ID NO: 6)

MGYSKNLGAGLFTMLLLAPAIMATDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDF
 LFSSKLTKAGNTSTPNGSAVTELDVAEWPGTNTLGVSMNRVDFAPGGTNPPHIHPRATEIG
 25 MVMKGELLVGILGSFDSGNKLYSRVVRAGETFVIPRGLMHFQFNVGKTEAYMVVSFNSQN
 PGIVFVPLTLFGSNPPIPTPVLTKALRVEAGVVELLKSKFAGGS

>Wild-type *Triticum aestivum* oxalate oxidase nucleic acid sequence no leader (SEQ ID NO: 7)

GTCTTGGCCACCGACCCAGACCCTCTCCAGGACTTCTGTGTCGCCGACCTCGACGGCAA
 30 GGCGGTCTCGGTGAACGGGCACACGTGCAAGCCCATGTCGGAGGCCGGCGACGACTTC

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CTCTTCTCGTCCAAGTTGGCCAAGGCCGGCAACACGTCCACCCCGAACGGCTCCGCCGT
GACGGAGCTCGACGTGGCCGAGTGGCCCGGTACCAACACGCTGGGTGTGTCCATGAAC
CGCGTGGACTTTGCTCCCGGAGGCACCAACCCACCACACATCCACCCGCGTGCCACCG
AGATCGGCATCGTGATGAAAGGTGAGCTTCTCGTGGGAATCCTTGGCAGCCTCGACTC
5 CGGGAACAAGCTCTACTCGAGGGTGGTGC GCGCCGGAGAGACGTTTCCTCATCCCACGG
GGCCTCATGCACTTCCAGTTCAACGTCGGTAAGACCGAGGCCTCCATGGTCGTCTCCTT
CAACAGCCAGAACCCCGGCATTGTCTTCGTGCCCCCTCACGCTCTTCGGCTCCAACCCGC
CCATCCCAACGCCGGTGCTCACCAAGGCACTCCGGGTGGAGGCCAGGGTCGTGGAAC
TCTCAAGTCCAAGTTTGCCGCTGGGTTT

10

>Wild-type *Triticum aestivum* oxalate oxidase nucleic acid sequence with leader (SEQ ID NO: 8)
ATGGGGTACTCCAAAACCCTAGTAGCTGGCCTGTTGCAATGCTGTTACTAGCTCCGGC
CGTCTTGGCCACCGACCCAGACCCTCTCCAGGACTTCTGTGTCGCCGACCTCGACGGCA
AGGCGGTCTCGGTGAACGGGCACACGTGCAAGCCCATGTTCGGAGGCCGGCGACGACTT
15 CCTCTTCTCGTCCAAGTTGGCCAAGGCCGGCAACACGTCCACCCCGAACGGCTCCGCCG
TGACGGAGCTCGACGTGGCCGAGTGGCCCGGTACCAACACGCTGGGTGTGTCCATGAA
CCGCGTGGACTTTGCTCCCGGAGGCACCAACCCACCACACATCCACCCGCGTGCCACC
GAGATCGGCATCGTGATGAAAGGTGAGCTTCTCGTGGGAATCCTTGGCAGCCTCGACT
CCGGGAACAAGCTCTACTCGAGGGTGGTGC GCGCCGGAGAGACGTTTCCTCATCCCACG
20 GGCCTCATGCACTTCCAGTTCAACGTCGGTAAGACCGAGGCCTCCATGGTCGTCTCCT
TCAACAGCCAGAACCCCGGCATTGTCTTCGTGCCCCCTCACGCTCTTCGGCTCCAACCCG
CCCATCCCAACGCCGGTGCTCACCAAGGCACTCCGGGTGGAGGCCAGGGTCGTGGAAC
TTCTCAAGTCCAAGTTTGCCGCTGGGTTT

25 >Wild-type *Triticum aestivum* oxalate oxidase codon optimized nucleic acid sequence (SEQ ID
NO: 9)

ACCGACCCTGATCCACTTCAAGACTTCTGTGTGGCAGACTTGGATGGTAAGGCCGTGTC
AGTAAATGGTCACACTTGCAAACCAATGAGTGAAGCTGGAGATGATTTCTTATTTAGTA
GTAAGTTGGCTAAAGCTGGCAACACCTCTACACCTAACGGTTCCGCAGTCACCGAGCT
30 AGACGTAGCAGAATGGCCAGGAACAAATACGTTAGGCGTCTCAATGAACAGGGTGGA

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TTTCGCCCCCTGGAGGTACGAACCCCCCACATATCCACCCTCGTGCTACCGAAATTGGAA
 TCGTGATGAAGGGCGAACTACTGGTCGGAATCCTAGGCAGTTTGGATTCCGGTAACAA
 ACTTTATTCTCGTGTCGTTAGAGCAGGTGAAACCTTCTTAATTCCAAGGGGTTTAATGC
 ACTTTCAATTCAATGTCGGTAAGACGGAAGCCTCAATGGTGGTCTCTTTCAACTCTCAG
 5 AATCCAGGTATCGTTTTTGTTCCTCTGACATTATTCGGTTCCAACCCTCCTATACCCACG
 CCTGTGCTTACAAAAGCCTTGAGGGTAGAAGCCCGTGTAGTGGAGCTATTAAAAAGTA
 AGTTTGCCGCAGGTTTC

> *T. aestivum* Oxalate Oxidase amino acid sequence no leader (SEQ ID NO: 10)

10 TDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFLFSSKLAKAGNTSTPNGSAVTELD
 VAEWPGTNTLGVSMNRVDFAPGGTNPPPHIHPRATEIGIVMKGELLVGILGSLDSGNKLYSR
 VVRAGETFLIPRGLMHFQFNVGKTEASMVVSFNSQNP GIVFVPLTLFGSNPPIPTPVLTKAL
 RVEARVVELLKSKFAAGF

15 > *T. aestivum* Oxalate Oxidase amino acid sequence with leader (SEQ ID NO: 11)

MGYSKTLVAGLFAMLLAPAVLATDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDF
 LFSSKLAKAGNTSTPNGSAVTELDVAEWPGTNTLGVSMNRVDFAPGGTNPPPHIHPRATEIGI
 VMKGELLVGILGSLDSGNKLYSRVVRAGETFLIPRGLMHFQFNVGKTEASMVVSFNSQNP
 GIVFVPLTLFGSNPPIPTPVLTKALRVEARVVELLKSKFAAGF

20

> *S. cerevisiae* wild-type α -factor signal nucleic acid sequence; pre region is underlined and pro
 region is bolded (SEQ ID NO: 12)

ATGAGATTTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCT
CCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCA
 25 **TCGGTTACTCAGATTTAGAAGGGGATTTTCGATGTTGCTGTTTTGCCATTTTCCAAC**
AGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAA
AGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCT

>AOX1 promoter nucleic acid sequence (SEQ ID NO: 13)

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GATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACA
GGTCCATTCTCACACATAAGTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGAC
CGTTGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAACACCCACTTTTGCCATCGAAA
AACCAGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGGC
5 TACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCCCCCTGGCGAGGTTTCATGTTT
GTTTATTTCCGAATGCAACAAGCTCCGCATTACACCCGAACATCACTCCAGATGAGGGC
TTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCCAAATGGCCCCAAACTGACAGTTT
AAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACTAAG
TTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAACTTCCAAAAGTCGGC
10 ATACCGTTTGTCTTGTTTGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAAATGCT
TAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTGCACCTGTGCCGAAACGCAAATGGG
GAAACACCCGCTTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAAGATTCT
GGTGGGAATACTGCTGATAGCCTAACGTTTCATGATCAAAATTTAACTGTTCTAACCCCT
ACTTGACAGCAATATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTATC
15 ATCATTATTAGCTTACTTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTA
ACGACTTTTAACGACAACCTTGAGAAGATCAAAAAACAACCTAATTATTCGAAACG

> *H. vulgare* OxOx Expression Construct 1 nucleic acid sequence (SEQ ID NO: 14)

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCAC
20 AGGTCCATTCTCACACATAAGTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGA
CCGTTGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAACACCCACTTTTGCCATCGAA
AAACCAGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGG
CTACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCCCCCTGGCGAGGTTTCATGTT
TGTTTATTTCCGAATGCAACAAGCTCCGCATTACACCCGAACATCACTCCAGATGAGGG
25 CTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCCAAATGGCCCCAAACTGACAGTT
TAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACTAA
GTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAACTTCCAAAAGTCGG
CATACCGTTTGTCTTGTTTGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAAATG
CTTAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTGCACCTGTGCCGAAACGCAAATG
30 GGGAAACACCCGCTTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAAGAT

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TCTGGTGGGAATACTGCTGATAGCCTAACGTTTCATGATCAAAATTTAACTGTTCTAACC
CCTACTTGACAGCAATATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTT
ATCATCATTATTAGCTTACTTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATT
TTAACGACTTTTAACGACAACCTTGAGAAGATCAAAAAACAATAATTATTCGAAACGA
5 TGAGATTTTCCTTCAATTTTTACTGCTGTTTTATTTCGCAGCATCCTCCGCATTAGCTGCTC
CAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGG
TTACTCAGATTTAGAAGGGGATTTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAA
ATAACGGGTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGG
GTATCTCTCGAGAAAAGAGAGGCTGAAGCTGAATTCAGTGACCCTGACCCACTTCAGG
10 ACTTCTGTGTGGCTGACCTGGACGGAAAAGCTGTGTCTGTAAACGGCCATACGTGCAA
ACCTATGTCAGAGGCTGGTGATGACTTCCTGTTCTTCTAACTAACTAAGGCAGGTA
ACACCAGTACCCCAATGGAAGTGCCGTAACAGAATTGGATGTAGCTGAATGGCCTGG
AACAAACACGTTGGGCGTTAGTATGAATAGGGTAGACTTCGCACCTGGTGGAACCAAT
CCCCCTCATATACCCACGTGCAACGGAAATTGGCATGGTGATGAAGGGCGAGCTGC
15 TTGTAGGCATACTTGGAAGTCTGGACTCTGGCAATAAACTATACTCCAGAGTCGTCAGA
GCAGGAGAACTTTTCGTCATTCCCAGAGGACTTATGCACTTCCAGTTCAACGTAGGCA
AGACGGAAGCATATATGGTCGTCAGTTTCAACAGTCAGAACCCCTGGCATTGTATTCGTT
CCATTAACCTCTGTTTGGCAGTGATCCCCCAATCCCAACCCCTGTTTTAACAAAAGCCTT
GAGAGTAGAAGCAGGCGTCGTGGAATTATTGAAGAGTAAATTTCGCAGGTGGCTCATGA
20 TCTAGAACAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCAT
CATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCTCAGTTCAAGTTGGGCACTT
ACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGCCT
GAGAGATGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTT
TTTTTGTCATTTTGTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCTG
25 ATGAATATCTTGTGGTAGGGGTTTGGGAAAATCATTCGAGTTTGATGTTTTTCTTGGTA
TTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACCTTCGTTTGTGCGGATCCC
CCACACACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGA
CTCCGCGCATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATTTTCCCTC
TTTCTTCCTCTAGGGTGTCGTTAATTACCCGTAATAAGGTTTGGAAAAGAAAAAAGAG
30 ACCGCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTATCACGTTTCTTT

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TTCTTGAAATTTTTTTTTTTTAGTTTTTTTCTCTTTCAGTGACCTCCATTGATATTTAAGTT
AATAAACGGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCTTGTTCTATTACAACCTTT
TTTACTTCTTGTTTCATTAGAAAGAAAGCATAGCAATCTAATCTAAGGGGCGGTGTTGAC
AATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACAAGGTGAGGAACTAAA
5 CCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGC
GGTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTC
GCCGGTGTGGTCCGGGACGACGTGACCCTGTTTCATCAGCGCGGTCCAGGACCAGGTGG
TGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGA
GTGGTCGGAGGTCGTGTCCACGAACCTCCGGGACGCCTCCGGGGCCGGCCATGACCGAG
10 ATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCG
TGCACTTCGTGGCCGAGGAGCAGGACTGACACGTCCGACGGCGGCCACGGGTCCCAG
GCCTCGGAGATCCGTCCCCCTTTTCCTTTGTGCGATATCATGTAATTAGTTATGTCACGCT
TACATTCACGCCCTCCCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTAGACAACC
TGAAGTCTAGGTCCCTATTTATTTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTTAT
15 ATTTCAAATTTTTCTTTTTTTTTCTGTACAGACGCGTGTACGCATGTAACATTATACTGAA
AACCTTGCTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCAAGCTGGAGACC
AACATGTGAGCAAAAAGGCCAGCAAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTG
GCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCA
GAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCC
20 CTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCT
TCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGT
CGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCT
TATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCA
GCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCT
25 TGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTG
CTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCA
CCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGG
ATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAAC
CACGTTAAGGGATTTTGGTCATGAGATC

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> *H. vulgare* OxOx Expression Construct 2 nucleic acid sequence (SEQ ID NO: 15)

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCAC
AGGTCCATTCTCACACATAAGTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGA
CCGTTGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAACACCCACTTTTGCCATCGAA
5 AAACCAGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGG
CTACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCCCCCTGGCGAGGTTTCATGTT
TGTTTATTTCCGAATGCAACAAGCTCCGCATTACACCCGAACATCACTCCAGATGAGGG
CTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCCAAATGGCCCCAAACTGACAGTT
TAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACTAA
10 GTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAACTTCCAAAAGTCGG
CATACCGTTTGTCTTGTTTGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAAATG
CTTAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTGCACCTGTGCCGAAACGCAAATG
GGGAAACACCCGCTTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAAGAT
TCTGGTGGGAATACTGCTGATAGCCTAACGTTTCATGATCAAAATTTAACTGTTCTAACC
15 CCTACTTGACAGCAATATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTT
ATCATCATTATTAGCTTACTTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATT
TTAACGACTTTTAACGACAACCTTGAGAAGATCAAAAAACAATAATTATTCGAAACGA
TGAGATTTTCTTCAATTTTTACTGCTGTTTTATTTCGCAGCATCCTCCGCATTAGCTGCTC
CAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGG
20 TTAATCAGATTTAGAAGGGGATTTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAA
ATAACGGGTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGG
GTATCTCTCGAGAAAAGAGAGGCTGAAGCTGAATTCTCTGACCCAGATCCATTGCAGG
ATTTCTGTGTTGCTGACTTGGACGGTAAGGCCGTTTCTGTTAACGGTCACACTTGTAAG
CCAATGTCTGAAGCTGGTGACGACTTCCTGTTCTCCTCAAAGTTGACTAAGGCTGGTAA
25 CACCTCCACTCCAAACGGTTCTGCTGTTACCGAATTGGACGTTGCTGAATGGCCTGGAA
CTAACACTTTGGGTGTCTCCATGAACAGAGTTGACTTTTGCTCCAGGTGGTACAAACCCA
CCACATATTCATCCAAGAGCTACCGAGATCGGTATGGTCATGAAGGGTGAGTTGTTGG
TCGGTATCTTGGGTCTTTTGGACTCCGGTAACAAGCTGTACTCCAGAGTTGTTAGAGCC
GGTGAGACTTTCGTTATCCCAAGAGGTTTGATGCACTTCCAGTTCAACGTTGGTAAGAC
30 CGAGGCCTACATGGTTGTGTCCTTCAACTCTCAAAACCCCGGTATCGTTTTTCGTCCCAT

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GACTTTGTTTCGGTTCTGACCCACCAATTCCAACCTCCAGTTTTGACCAAGGCTTTGAGAG
TCGAGGCTGGTGTGTTGAATTGCTGAAGTCTAAGTTCGCCGGTGGTTCCTAATCTAGA
ACAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCAT
CATTGAGTTTGTAGCCTTAGACATGACTGTTCTCAGTTCAAGTTGGGCACTTACGAGA
5 AGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGCCTGAGAGA
TGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTTGT
CATTTTGTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATA
TCTTGTGGTAGGGGTTTGGGAAAATCATTCGAGTTTGATGTTTTTCTTGGTATTTCCCAC
TCCTCTTCAGAGTACAGAAGATTAAGTGAGACCTTCGTTTGTGCGGATCCCCACACAC
10 CATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGC
ATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAATTTCCCTCTTTCTTCC
TCTAGGGTGTCTTAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACCGCCT
CGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAAATTTTTATCACGTTTCTTTTTCTTGA
AATTTTTTTTTTTAGTTTTTTTCTCTTTCAGTGACCTCCATTGATATTTAAGTTAATAAAC
15 GGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCTTGTTCATTACAACCTTTTTTTACTT
CTTGTTCAATTAGAAAGAAAGCATAGCAATCTAATCTAAGGGGCGGTGTTGACAATTAA
TCATCGGCATAGTATATCGGCATAGTATAATACGACAAGGTGAGGAACTAAACCATGG
CCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCTGA
GTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTG
20 TGGTCCGGGACGACGTGACCCTGTTTCATCAGCGCGGTCCAGGACCAGGTGGTGCCGGA
CAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGAGTGGTCG
GAGGTCGTGTCCACGAACCTCCGGGACGCCTCCGGGGCCGGCCATGACCGAGATCGGCG
AGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTT
CGTGGCCGAGGAGCAGGACTGACACGTCCGACGGCGGCCACGGGTCCCAGGCCTCGG
25 AGATCCGTCCCCCTTTTCCTTTGTCGATATCATGTAATTAGTTATGTCACGCTTACATTC
ACGCCCTCCCCCACATCCGCTCTAACCAGAAAAGGAAGGAGTTAGACAACCTGAAGTC
TAGGTCCTATTTATTTTTTTATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAA
ATTTTTCTTTTTTTTTCTGTACAGACGCGTGTACGCATGTAACATTATACTGAAAACCTTG
CTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCAAGCTGGAGACCAACATGT
30 GAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTT

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CCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGG
CGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGC
GCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAA
GCGTGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTTCGC
5 TCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTACAGCCCGACCGCTGCGCCTTATCCGG
TAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCC
ACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGT
GGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAA
GCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCT
10 GGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTC
AAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACCTCACGT
TAAGGGATTTTGGTCATGAGATC

> *H. vulgare* OxOx Expression Construct 3 nucleic acid sequence (SEQ ID NO: 16)

15 AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCAC
AGGTCCATTCTCACACATAAGTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGA
CCGTTGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAACACCCACTTTTGCCATCGAA
AAACCAGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGG
CTACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCCCCCTGGCGAGGTTTCATGTT
20 TGTTTATTTCCGAATGCAACAAGCTCCGCATTACACCCGAACATCACTCCAGATGAGGG
CTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCCAAATGGCCCCAAAACCTGACAGTT
TAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACTAA
GTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAACTTCCAAAAGTCGG
CATACCGTTTGTCTTGTTTGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAATG
25 CTTAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTGCACCTGTGCCGAAACGCAAATG
GGGAAACACCCGCTTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAAGAT
TCTGGTGGGAATACTGCTGATAGCCTAACGTTTCATGATCAAAATTTAACTGTTCTAACC
CCTACTTGACAGCAATATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTT
ATCATCATTATTAGCTTACTTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATT
30 TTAACGACTTTTAACGACAACCTTGAGAAGATCAAAAAACAACCTAATTATTCGAAACGA

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TGAGATTTCTTCAATTTTTACTGCTGTTTTATTTCGCAGCATCCTCCGCATTAGCTGCTC
CAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGG
TTACTCAGATTTAGAAGGGGATTTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAA
ATAACGGGTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGG
5 GTATCTCTCGAGAAAAGAGAGGGCTGAAGCTGAATTCATGGGTACTCTAAAAACCTAG
GGGCTGGCCTGTTACCATGCTGCTCCTTGCTCCGGCCATCATGGCTACCGACCCTGAC
CCTCTACAGGACTTCTGCGTCGCGGACCTCGATGGCAAGGCGGTCTCGGTGAACGGGC
ATACGTGTAAGCCCATGTCGGAGGCCGGCGACGACTTCCTCTTCTCGTCCAAGCTGACC
AAGGCCGGCAACACGTCCACCCCGAACGGCTCGGCCGTGACGGAGCTCGACGTGGCCG
10 AGTGGCCCGGTACGAACACGCTGGGCGTGTCCATGAACCGTGTGGACTTCGCGCCGGG
CGGCACCAACCCGCCGCACATCCACCCGCGTGCAACCGAGATCGGCATGGTGATGAAA
GGTGAGCTCCTCGTTGGAATCCTCGGCAGCTTTGACTCCGGAAACAAGCTCTACTCCAG
GGTGGTGCGTGCCGGAGAGACTTTCGTCATCCCGCGCGGCCTCATGCACTTCCAGTTCA
ACGTTGGTAAGACGGAAGCCTACATGGTTGTGTCCTTCAACAGCCAGAACCCTGGCAT
15 CGTCTTCGTGCCGCTCACACTCTTCGGTTCCAACCCGCCCATCCCCACACCGGTGCTCA
CCAAGGCTCTTCGGGTGGAGGCCGGGGTCGTGGAACCTTCTCAAGTCCAAGTTCGCCGG
TGGGTCTTAATCTAGAACAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGA
CCATCATCATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCTCAGTTCAAGT
TGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGC
20 CATTTGCCTGAGAGATGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGT
ATAGGATTTTTTTTGTGCTTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCT
CGCAGCTGATGAATATCTTGTGGTAGGGGTTTGGGAAAATCATTGAGTTTGATGTTTT
TCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACCTTCGTTTGTG
CGGATCCCCCACACACCATAGCTTCAAAATGTTTCTACTCCTTTTTTACTCTTCCAGATT
25 TTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAAAT
TTTCCCTCTTTCTTCTCTAGGGTGTGCTTAATTACCCGTACTAAAGGTTTGGAAAAGAA
AAAAGAGACCGCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTATCAC
GTTTCTTTTTCTTGAAATTTTTTTTTTTAGTTTTTTCTCTTTCAGTGACCTCCATTGATAT
TTAAGTTAATAAACGGTCTTCAATTTCTCAAGTTTCAGTTTCATTTTTCTTGTTCTATTAC
30 AACTTTTTTTACTTCTTGTTTATTAGAAAGAAAGCATAGCAATCTAATCTAAGGGGCGG

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TGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACAAGGTGAGG
AACTAAACCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCG
CCGGAGCGGTTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGA
CGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTTCATCAGCGCGGTCCAGGAC
5 CAGGTGGTGCCGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGT
ACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGGCCGGCCAT
GACCGAGATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGG
CAACTGCGTGCACCTTCGTGGCCGAGGAGCAGGACTGACACGTCCGACGGCGGCCACG
GGTCCCAGGCCTCGGAGATCCGTCCCCCTTTTCCTTTGTTCGATATCATGTAATTAGTTAT
10 GTCACGCTTACATTCACGCCCTCCCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTA
GACAACCTGAAGTCTAGGTCCCTATTTATTTTTTTATAGTTATGTTAGTATTAAGAACGT
TATTTATATTTCAAATTTTTCTTTTTTTCTGTACAGACGCGTGTACGCATGTAACATTA
TACTGAAAACCTTGCTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCAAGCTG
GAGACCAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCG
15 TTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAATCGACGCTC
AAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGA
AGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTT
CTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGT
GTAGGTCGTTGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCT
20 GCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCA
CTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAG
AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGC
GCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAAC
AAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAA
25 AAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACG
AAAACCTCACGTTAAGGGATTTTGGTCATGAGATC

> *H. vulgare* OxOx signal peptide sequence (SEQ ID NO: 17)

MGYSKNLGAGLFTMLLLAPAIMA

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> *T. aestivum* OxOx signal peptide sequence (SEQ ID NO: 18)

MGYSKTLVAGLFAMLLAPAVLA

>Bacterial Tat pathway signal peptide sequence (SEQ ID NO: 19)

5 SRRQFLK

>OxOx peptide sequence (SEQ ID NO: 20)

AGETFVIPR

10 >*S. cerevisiae* OST1 pre-region encoding nucleic acid sequence (SEQ ID NO: 21)

ATGAGGCAGGTTTGGTTCTCTTGGATTGTGGGATTGTTCCCTATGTTTTTTCAACGTGTCT
TCT

>*S. cerevisiae* OST1 pre-region signal peptide sequence (SEQ ID NO: 22)

15 MRQVWFSWIVGLFLCFFNVSS

>*S. cerevisiae* mutant α -factor pro-region ("proapp-8") encoding nucleic acid sequence (SEQ ID NO: 23)

GCTGCTCCAGCTAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTG
20 TCATCGATTACTCAGATTTAGAAGGGGATTTTCGATGATGCTGATTTGCCATTGTCCAAC
AGCACAAATAACGGGTATCGTCTACAAATACTACTATTGCCAGCATTGCTGCTAAAG
AAGAAGGGGTATCTCTCGAGTCTAGAGAGGCTGAAGCT

> *S. cerevisiae* mutant α -factor pro-region signal peptide sequence (SEQ ID NO: 24)

25 AAPANTTTTEDETAQIPAEVIDYSDLEGDFDDADLPLSNSTNNGLSSTNTTIIASIAAKEEGVS
LESREAEA

>Chimeric signal peptide encoding nucleic acid sequence (SEQ ID NO: 25)

ATGAGGCAGGTTTGGTTCTCTTGGATTGTGGGATTGTTCCCTATGTTTTTTCAACGTGTCT
30 TCTGCTGCTCCAGCTAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAG

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CTGTCATCGATTACTCAGATTTAGAAGGGGATTTTCGATGATGCTGATTTGCCATTGTCC
AACAGCACAAATAACGGGTTATCGTCTACAAATACTACTATTGCCAGCATTGCTGCTAA
AGAAGAAGGGGTATCTCTCGAGTCTAGAGAGGCTGAAGCT

5 >Chimeric signal peptide sequence (SEQ ID NO: 26)

MRQVWFSWIVGLFLCFFNVSSAAPANTTTTEDETAQIPAEAVIDYSDLEGDFDDADLPLSNS
TNNGLSSTNTTIIASIAAKEEGVSLESREAEA

>*S. cerevisiae* wild-type α -factor secretion signal peptide (SEQ ID NO: 27)

10 MRFPSIFTAVLFAASSALAAPVNTTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNG
LLFINTTIIASIAAKEEGVSLEKREAEA

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CLAIMS

What is claimed is:

1. An isolated nucleic acid comprising an expression construct comprising the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9.

5

2. The isolated nucleic acid of claim 1, wherein the isolated nucleic acid encodes a protein comprising the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11.

10

3. The isolated nucleic acid of claim 1 or 2, wherein the expression construct further comprises an alpha factor (α -factor) signal sequence operably linked to the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9.

15

4. The isolated nucleic acid of claim 3, wherein the α -factor signal sequence is positioned 5' relative to the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9.

5. The isolated nucleic acid of claim 3 or 4, wherein the α -factor signal sequence comprises the nucleic acid sequence set forth in SEQ ID NO: 12.

20

6. The isolated nucleic acid of any one of claims 1 to 5 further comprising a non-yeast signal sequence, optionally wherein the non-yeast signal sequence encodes a peptide comprising the sequence set forth in any one of SEQ ID NOs: 17-19.

25

7. The isolated nucleic acid of any one of claims 1 to 6, wherein the expression construct further comprises one or more purification tag sequences positioned 3' relative to the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9.

8. The isolated nucleic acid of any one of claims 1 to 7, wherein the expression construct further comprises a promoter operably linked to the α -factor signal sequence or the nucleic

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acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9, optionally wherein the promoter is an AOX1 promoter.

9. The isolated nucleic acid of any one of claims 1 to 8, wherein the expression
5 construct further comprises a transcription terminator sequence, optionally wherein the transcription terminator sequence is an AOX1 transcription terminator sequence.

10. A plasmid comprising the isolated nucleic acid of any one of claims 1 to 9.

10 11. The plasmid of claim 10, wherein the plasmid comprises the nucleic acid sequence set forth in any one of SEQ ID NOs: 14-16.

12. A host cell comprising the isolated nucleic acid of any one of claims 1 to 9, or the
plasmid of claim 10 or 11.

15 13. The host cell of claim 12, wherein the host cell is a yeast cell.

14. The host cell of claim 13, wherein the yeast cell is a *Pichia pastoris* cell.

20 15. A container housing a population of yeast cells, wherein at least one yeast cell comprises the isolated nucleic acid of any one of claims 1 to 9 or the plasmid of claim 10 or 11.

16. An isolated nucleic acid comprising an expression construct comprising a nucleic
acid sequence encoding an oxalate oxidase (OxOx) protein operably linked to a non-yeast protein
25 transduction domain sequence.

17. The isolated nucleic acid of claim 16, wherein the OxOx protein comprises the amino
acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11.

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18. The isolated nucleic acid of claim 16 or 17, wherein the nucleic acid sequence encoding a protein comprises the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9.

5 19. The isolated nucleic acid of any one of claims 16 to 18, wherein the protein transduction domain sequence comprises bacterial transduction domain sequence, optionally a twin-arginine translocation (Tat) pathway signal sequence.

10 20. The isolated nucleic acid of claim 19, wherein the Tat pathway signal sequence comprises the nucleic acid sequence set forth in SEQ ID NO: 19.

21. The isolated nucleic acid of any one of claims 16 to 20, wherein the expression construct further comprises a promoter operably linked to the nucleic acid sequence encoding a protein, optionally an AOX1 promoter.

15 22. A plasmid comprising the isolated nucleic acid of any one of claims 16 to 21.

23. The plasmid of claim 22 comprising the nucleic acid sequence set forth in any one of SEQ ID NOs: 14-16.

20 24. A host cell comprising the isolated nucleic acid of any one of claims 16 to 21, or the plasmid of claim 22 or 23.

25 25. The host cell of claim 24, wherein the host cell is a yeast cell.

26. The host cell of claim 25, wherein the yeast cell is a *Pichia pastoris* cell.

27. A container housing a population of yeast cells, wherein at least one yeast cell comprises the isolated nucleic acid of any one of claims 16 to 21 or the plasmid of claim 22 or 23.

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28. A method for producing a recombinant protein, the method comprising introducing into a cell the isolated nucleic acid of any one of claims 1 to 9 or 16 to 21 under conditions under which the cell expresses a recombinant oxalate oxidase (OxOx) protein.

5 29. The method of claim 28, wherein the cell is a yeast cell, optionally wherein the yeast cell is a *Pichia pastoris* cell.

30. The method of claim 28 or 29, wherein the recombinant OxOx protein comprises the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11, or is encoded by the
10 nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2 and 7-9.

31. The method of any one of claims 28 to 30 further comprising purifying the recombinant OxOx protein from the cell.

15 32. The method of claim 31, wherein the purifying comprises lysing the cell to produce cell lysate and an insoluble fraction.

33. The method of claim 32, wherein the purifying comprises contacting the cell lysate or insoluble fraction with an anion exchange media, optionally wherein the anion exchange media
20 comprises a Q-sepharose anion exchange column under conditions under which the recombinant OxOx protein binds to the column.

34. The method of claim 33, wherein the method further comprises eluting the bound recombinant OxOx protein from the column to produce a purified protein fraction.

25 35. The method of claim 34, wherein the purified protein fraction comprises >90% recombinant OxOx protein.

36. An isolated nucleic acid comprising a nucleic acid sequence encoding a protein
30 comprising a chimeric secretion signal having the amino acid sequence set forth in SEQ ID NO: 26.

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37. The isolated nucleic acid of claim 36, wherein the chimeric secretion signal comprises a pre region encoded by the nucleic acid sequence set forth in SEQ ID NO: 21.

5 38. The isolated nucleic acid of claim 36 or 37, wherein the chimeric secretion signal comprises a pro region encoded by the nucleic acid sequence set forth in SEQ ID NO: 23.

39. The isolated nucleic acid of any one of claims 36 to 38, wherein the protein comprises an oxalate oxidase (OxOx) protein.

10

40. The isolated nucleic acid of claim 39, wherein the OxOx protein is a barley OxOx protein.

41. The isolated nucleic acid of any one of claims 36 to 40, wherein the protein
15 comprises the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, and 11.

42. The isolated nucleic acid of any one of claims 36 to 41 further comprising a promoter operably linked to the nucleic acid sequence encoding the protein.

20 43. The isolated nucleic acid of claim 42, wherein the promoter comprises an AOX1 promoter.

44. The isolated nucleic acid of any one of claims 36 to 43 further comprising a transcription terminator sequence, optionally wherein the transcription terminator sequence is an
25 AOX1 transcription terminator sequence.

45. A protein comprising a chimeric secretion signal having the amino acid sequence set forth in SEQ ID NO: 26.

30 46. The protein of claim 45, wherein the protein comprises an oxalate oxidase protein.

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47. The protein of claim 45 or 46, wherein the protein comprises the amino acid sequence set forth in any one of SEQ ID NOs: 5, 6, 10, or 11.

5 48. The protein of any one of claims 45 to 47, wherein the protein further comprises a purification tag.

49. A cell comprising the isolated nucleic acid of any one of claims 36 to 44 or the protein of any one of claims 45 to 48.

10

50. The cell of claim 49, wherein the cell is a yeast cell, optionally a *Pichia pastoris* cell.

OxOx Plasmid Construction

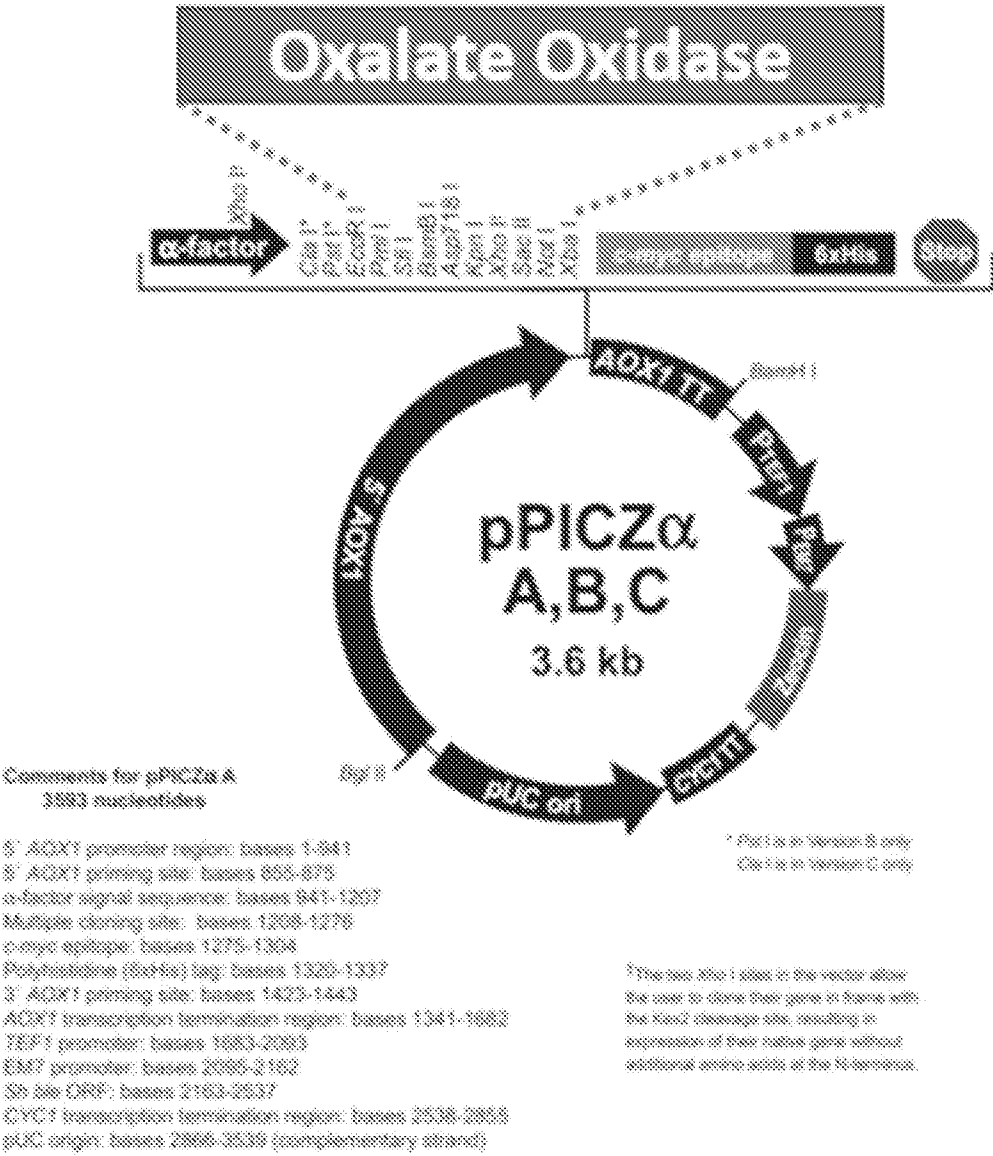


FIG. 1A



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Nucleotide Blast Alignment

Score	Expect	Identities	Gaps	Strand
1120 bits(606)	0.0	606/606(100%)	0/606(0%)	Plus/Plus
Query 1		AGTGACCCCTGACCCCACTTCAGGACTTCTGTGTGGCTGACCTGGACGGAAAAAGCTGTGTCT		60
Sbjct 350		AGTGACCCCTGACCCCACTTCAGGACTTCTGTGTGGCTGACCTGGACGGAAAAAGCTGTGTCT		409
Query 61		GTAAACGGCCATACGTGCAAAACCTATGTTCAGAGGCTGGTGAAGACTTCCTGTTCTCTTCT		120
Sbjct 410		GTAAACGGCCATACGTGCAAAACCTATGTTCAGAGGCTGGTGAAGACTTCCTGTTCTCTTCT		469
Query 121		AAACTTAACCTAAGGCAGGTAAACACCACTAACCCCAATGGAACTGCCCTAACAGAAATTGGAT		180
Sbjct 470		AAACTTAACCTAAGGCAGGTAAACACCTAAGTACCCCAATGGAACTGCCCTAACAGAAATTGGAT		529
Query 181		GTAGCTGAATGGCCCTGGAAACAACACCGTTGGGCGTTAATATGAAATAGGGTAGACTTCGCA		240
Sbjct 530		GTAGCTGAATGGCCCTGGAAACAACACCGTTGGGCGTTAATATGAAATAGGGTAGACTTCGCA		589
Query 241		CCCTGGTGGAAACCAATCCCCCTCATATACACCCACCTGCAACGGAAATTTGGCATGGGGAATG		300
Sbjct 590		CCCTGGTGGAAACCAATCCCCCTCATATACACCCACCTGCAACGGAAATTTGGCATGGGGAATG		649
Query 301		AAGGSCHELCTCTCTTGTATAGGCATACCTTGGAAGTCTGGAAGCTCTGGCAATTAAGTATACTCC		360
Sbjct 650		AAGGSCHELCTCTCTTGTATAGGCATACCTTGGAAGTCTGGAAGCTCTGGCAATTAAGTATACTCC		709
Query 361		AGAGTCCTTCAGAGCAGGAGAAACCTTTCCTCATTCCCAGAGGACTTATGCACCTTCCAGTTC		420
Sbjct 710		AGAGTCCTTCAGAGCAGGAGAAACCTTTCCTCATTCCCAGAGGACTTATGCACCTTCCAGTTC		769
Query 421		AACGTAAAGCAAGACGGAAAGCATATATGGTCTCTCAGTTTCAACAGTCAAGAACCCCTGGCATT		480
Sbjct 770		AACGTAAAGCAAGACGGAAAGCATATATGGTCTCTCAGTTTCAACAGTCAAGAACCCCTGGCATT		829
Query 481		GTATTCCTTCCATTAACTCTGTGTTGGCAGTGAATCCCCCAATCCCACCCCTGTTTTAACA		540
Sbjct 830		GTATTCCTTCCATTAACTCTGTGTTGGCAGTGAATCCCCCAATCCCACCCCTGTTTTAACA		889
Query 541		AAAGCCTTGAGAGTAGAAGCAGGCGTCTGTGGAAATTATTGAAGAGTAAGATTCCGAGGTGGC		600
Sbjct 890		AAAGCCTTGAGAGTAGAAGCAGGCGTCTGTGGAAATTATTGAAGAGTAAGATTCCGAGGTGGC		949
Query 601	TCATGA 606	SEQ ID NO: 2		
Sbjct 950	TCATGA 955	SEQ ID NO: 2		

FIG. 1C

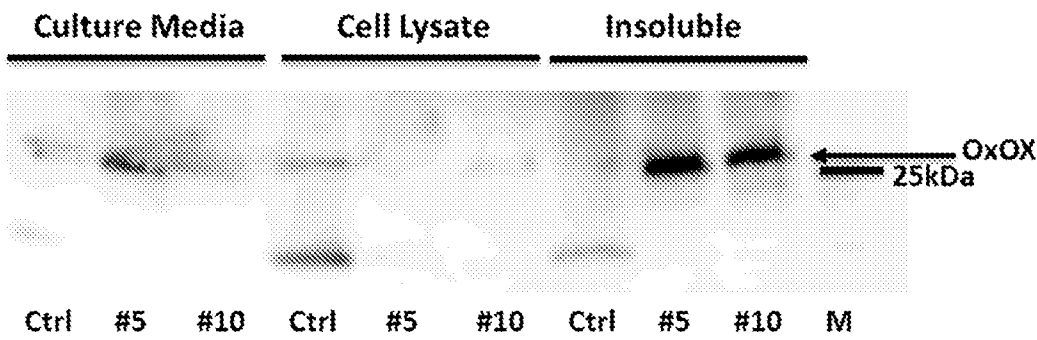


FIG. 2A

OxOx Expression Time-course
Hrs: - 24 48 72 96

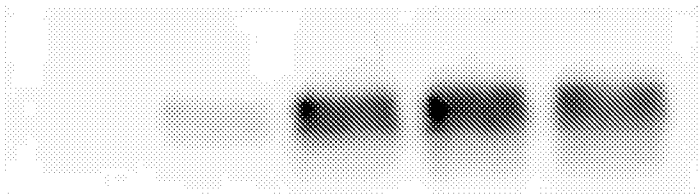


FIG. 2B

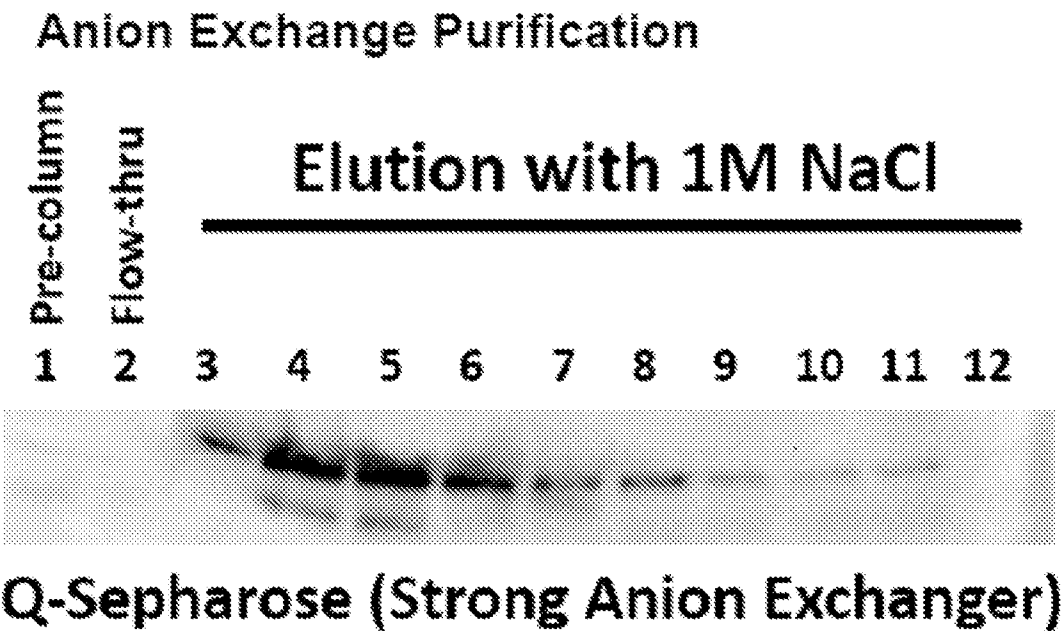


FIG. 2C

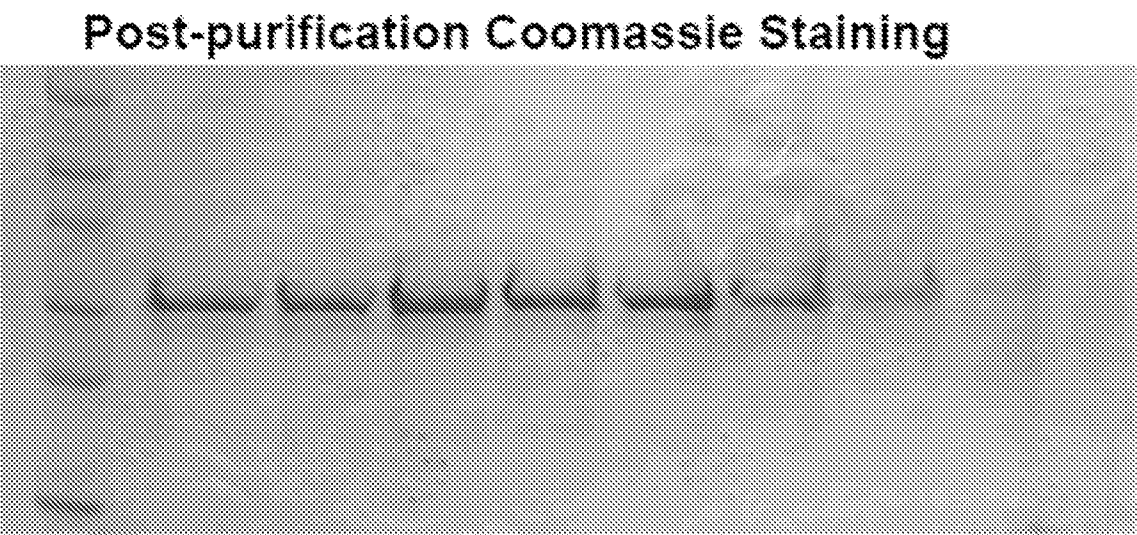


FIG. 2D

LC-MS/MS Analysis

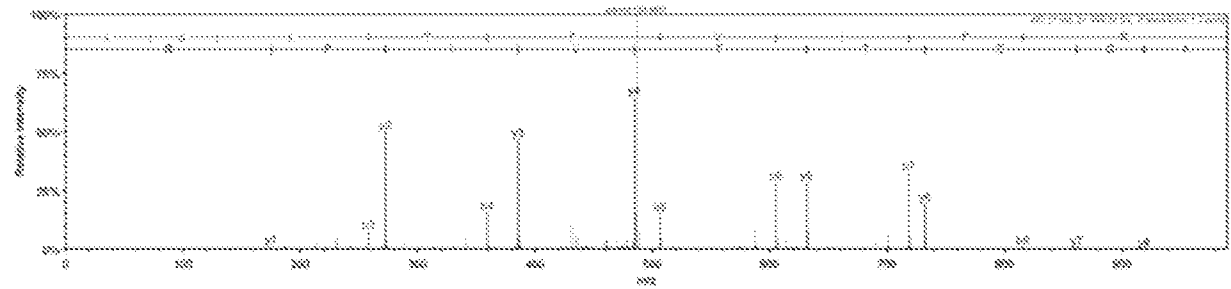
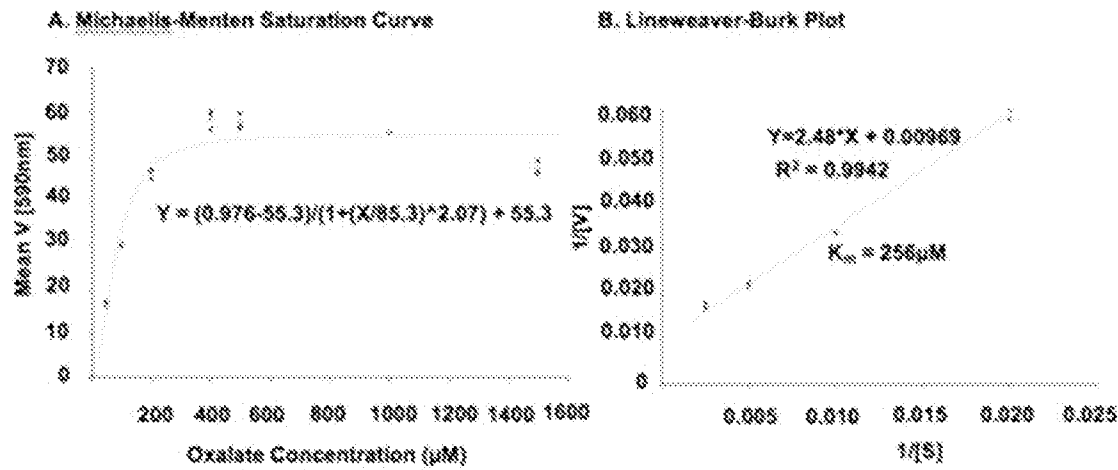


FIG. 3



FIGs. 4A-4B

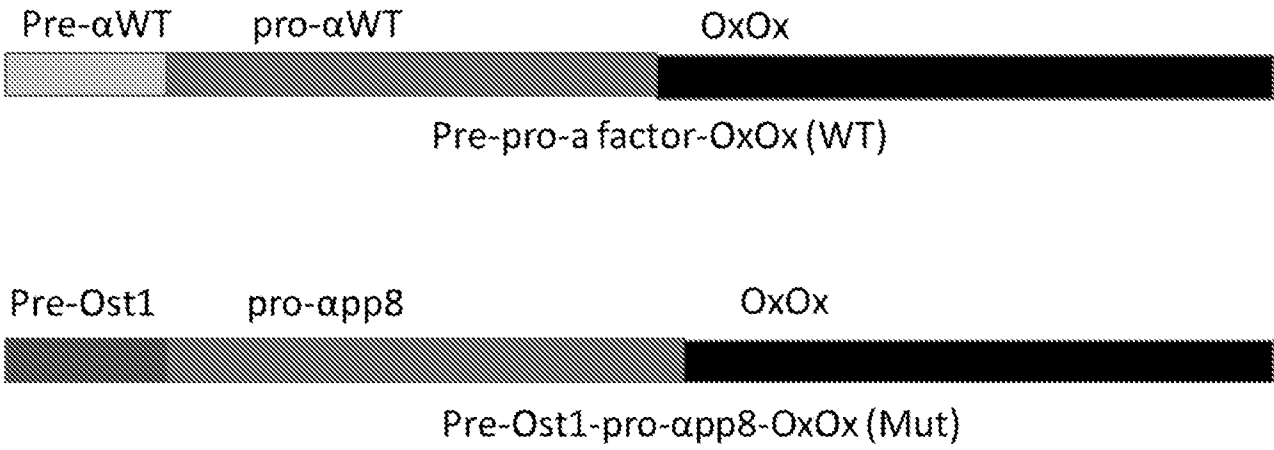


FIG. 5

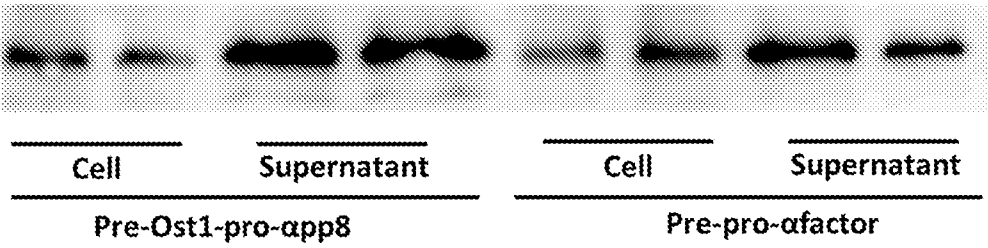


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/21990

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 1/00; C12N 9/04; C12P 21/06; C12N 15/00; C07H 21/04; A61K 36/00 (2021.01)

CPC - C12N 1/14; C12N 1/16; C12N 1/00; C12Y 102/03004; C12N 9/0006; C12N 9/0008; C12P 21/00; C07K 4/10; C12N 15/00; C12N 15/86; C12N 15/52; C07K 14/415; C07H 21/00; A61K 38/168

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 6,187,571 B1 (PIGNARD et al.) 13 February 2001 (13.02.2001), Abstract; col 3, ln 33-35; col 5, ln 3-5; col 6, ln 10-18; col 11, ln 27-31; col 12, ln 10-17; SEQ ID NO: 1; and SEQ ID NO: 2	1-2 ----- 3-4
Y	— FITZGERALD et al., Secretion of a foreign protein from budding yeasts is enhanced by cotranslational translocation and by suppression of vacuolar targeting. Microb Cell Fact. 2014, Vol. 13: 125. PDF File: pg 1-12. Abstract; pg 1, col 1; and pg 2, col 1, last para, col 2, top para, and Fig 1	3-4
A	US 2017/0037383 A1 (NEXTTOBE AB) 09 February 2017 (09.02.2017), para [0035], [0036], [0038], [0047], and [0105]	1-4
A	— MOUSSATCHE et al., Characterization of Ceriporiopsis subvermisporea Bicipin Oxalate Oxidase Expressed In Pichia pastoris. Arch Biochem Biophys. 2011, Vol. 509(1), p. 100-107. PDF File: pg 1-23. Entire documentation especially Abstract; and pg 6, para 2	1-4
A	US 2018/0265847 A1 (RUBIUS THERAPEUTICS, INC.) 20 September 2018 (20.09.2018), para [0055], [0641], [0836], and [1176]	1-4
A	— LANE et al., Germin, a Protein Marker of Early Plant Development, Is an Oxalate Oxidase. J Biol Chem. 1993, Vol. 268(17), p. 12239-42. Entire documentation especially Abstract; and pg 12240, Fig 1	1-4



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

"D" document cited by the applicant in the international application

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

07 May 2021

Date of mailing of the international search report

AUG 04 2021

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Lee Young

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/21990

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

3. ☒ Claims Nos.: 5-15, 19-35, 39-44, 48-50
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-4, directed to an isolated nucleic acid comprising an expression construct comprising the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9.

Group II, claims 16-18, directed to an isolated nucleic acid comprising an expression construct comprising a nucleic acid sequence encoding an oxalate oxidase (OxOx) protein operably linked to a non-yeast protein transduction domain sequence.

Group III, claims 36-38, 45-47, directed to an isolated nucleic acid comprising a nucleic acid sequence encoding a protein (claims 36-38) or a protein (claims 45-47) comprising a chimeric secretion signal having the amino acid sequence set forth in SEQ ID NO: 26.

*****Continued in the extra sheet*****

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

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

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

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

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/21990

Continuation of:
Box No III (unity of invention is lacking)

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Feature

Group I includes the special technical feature of the nucleic acid sequence set forth in any one of SEQ ID NOs: 1, 2, and 7-9, not required by Groups II-III.

Group II includes the special technical feature of a non-yeast protein transduction domain sequence, not required by Groups I and III.

Group III includes the special technical feature of a protein comprising a chimeric secretion signal having the amino acid sequence set forth in SEQ ID NO: 26, not required by Groups I-II.

Common Technical Features

The inventions of Groups I-II and part of Group III (claims 36-38) share the technical feature of an isolated nucleic acid sequence; –the inventions of Groups I-II further share the technical feature of an isolated nucleic acid comprising an expression construct comprising a nucleic acid sequence encoding an oxalate oxidase (OxOx) protein (part of claim 16; Group I, wherein each of SEQ ID NOs: 1, 2, and 7-9 encoding an oxalate oxidase (OxOx) protein, which is not expressly claimed); and –the inventions of group II and part of Group III (claims 36-38) further share the technical feature of an isolated nucleic acid comprising a nucleic acid sequence encoding a protein (as claimed).

Therefore, the common technical features shared by inventions of Groups I-III can be covered by:

An isolated nucleic acid comprising an expression construct comprising a nucleic acid sequence encoding an oxalate oxidase (OxOx) protein (part of claim 16).

However, these shared technical features do not represent a contribution over prior art as being anticipated by US 2017/0037383 A1 to NEXTTOBE AB (hereinafter 'NEXTTOBE') as follows:

NEXTTOBE discloses an isolated nucleic acid comprising an expression construct (para [0105] - 'Nucleic acid construct: ... means a nucleic acid molecule...which is isolated from a naturally occurring gene'; para [0047] - 'inserting ... a nucleic acid construct comprising the polynucleotide into an appropriate vector for expression') comprising a nucleic acid sequence encoding an oxalate oxidase (OxOx) protein (para [0035] - 'isolated polynucleotides encoding the oxalate oxidases'; para [0038] - 'nucleic acid constructs comprising a polynucleotide encoding an oxalate oxidase...operably linked to one or more control sequences that direct the expression').

Without a shared special technical feature, the inventions lack unity with one another.

Groups I-III therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Continuation of item 4: Claims 5-15, 19-35, 39-44, 48-50 are not drafted in accordance with the second and third sentences of Rule 6.4 (a). These claims are improper multiple dependent claims.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/21990

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments: