

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

MOTIF FOODWORKS, INC.,

Petitioner,

v.

IMPOSSIBLE FOODS INC.,
Patent Owner.

Case No.: IPR2023-00322
U.S. Patent No. 10,273,492

**DECLARATION OF DR. CARL BATT IN SUPPORT OF
PETITION FOR *INTER PARTES* REVIEW of U.S. PATENT NO. 10,273,492
UNDER 35 U.S.C. §§ 311-319 AND 37 C.F.R. §§ 42.100 *et seq.***

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TABLE OF EXHIBITS

Exhibit	Description
EX-1001	U.S. Patent No. 10,273,492 (“the ’492 patent”)
EX-1005	Vogl et al., <i>Regulation of Pichia pastoris promoters and its consequences for protein production</i> , NEW BIOTECHNOLOGY, 30(4), 385-404 (2013) (“Vogl”)
EX-1006	Lin-Cereghino et al., <i>Regulator of the methanol utilization pathway and peroxisomal genes in Pichia pastoris</i> , MOLECULAR & CELLULAR BIOLOGY, 26(3), 883-897 (2006) (“Lin-Cereghino”)
EX-1007	U.S. Patent No. 8,143,023 B2 of Takagi et al., issued March 27, 2012 (“Takagi”)
EX-1008	U.S. Patent No. 5,824,511 of Mattoon et al., issued October 20, 1998 (“Mattoon”)
EX-1009	U.S. Patent No. 6,261,827 of Elrod et al., issued July 17, 2001 (“Elrod”)
EX-1010	U.S. Patent Publication No. 2011/0287467 A1, published November 24, 2011 (“Crane”)
EX-1011	Liu et al., <i>Balanced globin protein expression and heme biosynthesis improve production of human hemoglobin in Saccharomyces cerevisiae</i> , METABOLIC ENGINEERING, 21, 9-16 (2014) (“Liu”)
EX-1012	Vedvick et al., <i>High-level secretion of biologically active aprotinin from the yeast Pichia pastoris</i> , J. INDUSTRIAL MICROBIOLOGY, 7, 197-202 (1991) (“Vedvick”)
EX-1013	Engel et al., <i>Foods and food ingredients produced via recombinant DNA techniques</i> , GENETICALLY MODIFIED FOODS – ACS SYMPOSIUM SERIES, 605, Chapter 1, 1-10 (1995) (“Engel”)
EX-1014	Garrocho-Villegas et al., <i>Plant hemoglobin: What we know six decades after their discovery</i> , GENE, 398, 78-85 (2007) (“Garrocho”)
EX-1015	Lin-Cereghino & Cregg, <i>Heterologous protein expression in the methylotrophic yeast Pichia pastoris</i> , FEMS MICROBIOLOGY REVIEWS, 24, 45-66 (2000) (“Lin-Cereghino_2000”)
EX-1016	Hartner et al., <i>Regulation of methanol utilisation pathway genes in yeasts</i> , MICROBIAL CELL FACTORIES, 5, 39 (2006) (“Hartner”)
EX-1017	Hartner et al., <i>Promoter library designed for fine-tuned gene expression in Pichia pastoris</i> , NUCLEIC ACID RESEARCH, 36(12), e76 (2008) (“Hartner_2008”)

Exhibit	Description
EX-1018	De Schutter et al., <i>Genome sequence of the recombinant protein production host Pichia pastoris</i> , NATURE BIOTECHNOLOGY, 27(6), 561-566 (2009) (“De_Schutter”)
EX-1019	Kranthi et al., <i>Identification of key DNA elements involved in promoter recognition by Mxr1p, a master regulator of methanol utilization pathway in Pichia pastoris</i> , BIOCHIMICA ET BIOPHYSICA ACTA, 1789, 460-468 (2009) (“Kranthi”)
EX-1021	Sreekrishna, <i>Pichia, optimization of protein expression</i> , ENCYCLOPEDIA OF INDUSTRIAL BIOTECHNOLOGY: BIOPROCESS, BIOSEPARATION, AND CELL TECHNOLOGY, 1-16 (Flickinger ed. 2010) (“Sreekrishna”)
EX-1022	Zhang et al., <i>Heterologous protein expression in yeasts and filamentous fungi</i> , MANUAL OF INDUSTRIAL MICROBIOLOGY & BIOTECHNOLOGY, 145-156 (Bull et al. ed. 2010) (“Zhang”)
EX-1023	Kranthi et al., <i>Identification of Mxr1p-binding sites in the promoters of genes encoding dihydroxyacetone synthase and peroxin 8 of the methylotrophic yeast Pichia pastoris</i> , YEAST, 27, 705-711 (2010) (“Kranthi_2010”)
EX-1024	U.S. Patent Publication No. 2011/0129874 A1, issued June 2, 2011 (“Tsutsumi”)
EX-1026	Parua et al., <i>Pichia pastoris 14-3-3 regulates transcriptional activity of the methanol inducible transcription factor Mxr1 by direct interaction</i> , MOLECULAR MICROBIOLOGY, 85(2), 282-298 (2012) (“Parua”)
EX-1027	Rabert et al., <i>Recombinants proteins for industrial uses: utilization of Pichia pastoris expression system</i> , BRAZILIAN J. MICROBIOLOGY, 44(2), 351-356 (2013) (“Rabert”)
EX-1028	Stryjewska et al., <i>Biotechnology and genetic engineering in the new drug development. Part I. DNA technology and recombinant proteins</i> , PHARMACOLOGICAL REPORTS, 65, 1075-1085 (2013) (“Stryjewska”)
EX-1029	Ahmad et al., <i>Protein expression in Pichia pastoris: recent achievements and perspectives for heterologous protein production</i> , APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, 98, 5301-5317 (2014) (“Ahmad”)
EX-1030	Haon et al., <i>Recombinant protein production facility for fungal biomass-degrading enzymes using the yeast Pichia pastoris</i> , FRONTIERS IN MICROBIOLOGY, 6, 1002 (2015) (“Haon”)
EX-1031	Krainer et al., <i>Optimizing cofactor availability for the production of recombinant heme peroxidase in Pichia pastoris</i> , MICROBIAL CELL FACTORIES, 14(4), 1-9 (2015) (“Krainer”)

Exhibit	Description
EX-1036	Bawa et al., <i>Functional recombinant protein is present in the pre-induction phases of Pichia pastoris cultures when grown in bioreactors, but not shake-flasks</i> , MICROBIAL CELL FACTORIES 13(127), 1-13 (2014) (“Bawa”)
EX-1037	Ferreira, <i>Heme synthesis</i> , ENCYCLOPEDIA OF BIOLOGICAL CHEMISTRY, 539-42 (2013) (“Ferreira”)
EX-1039	Prosecution History of U.S. Patent No. 10,273,492
EX-1042	Alberts et al., MOLECULAR BIOLOGY OF THE CELL, 305-312, 334-336, 538-540 (4th ed. 2002) (“Alberts”)
EX-1043	Freeman, BIOLOGICAL SCIENCES, 338-400 (2d ed. 2005) (“Freeman”)
EX-1044	Nicola & Leach, <i>Structural Rearrangements Due to Ligand Binding and Haem Replacement in Myoglobin and Leghaemoglobins</i> , EUROPEAN J. BIOCHEM., 78, 133-140 (1977) (“Nicola”)
EX-1045	Hargrove et al., <i>Characterization of Recombinant Soybean Leghemoglobin a and Apolar Distal Histidine Mutants</i> , J. MOLECULAR BIOLOGY, 266, 1032-1042 (1997) (“Hargrove”)
EX-1046	GenPept and Revision History Summaries for GenBank Accession No. AAA33980: leghemoglobin, NAT’L LIBR. MED.: NAT’L CTR. FOR BIOTECH. INFORMATION, <i>available at</i> https://www.ncbi.nlm.nih.gov/protein/AAA33980.1
EX-1047	GenPept and Revision History Summaries for GenBank Accession No. BAA24685: leghemoglobin [Pisum sativum], NAT’L LIBR. MED.: NAT’L CTR. FOR BIOTECH. INFORMATION, <i>available at</i> https://www.ncbi.nlm.nih.gov/protein/BAA24685.1
EX-1048	GenBank Accession No. NF102272: Protein Family Model PF00042 (heme-binding globins), NAT’L LIBR. MED.: NAT’L CTR. FOR BIOTECH. INFORMATION, <i>available at</i> https://www.ncbi.nlm.nih.gov/genome/annotation_prok/evidence/NF012272
EX-1049	GenPept and Revision History Summaries for GenBank Accession No. XP_002493846: Delta-aminolevulinate dehydratase, a homo-octameric enzyme [Komagataella phaffi GS115], NAT’L LIBR. MED.: NAT’L CTR. FOR BIOTECH. INFORMATION, <i>available at</i> https://www.ncbi.nlm.nih.gov/protein/XP_002493846.1
EX-1050	GenPept and Revision History Summaries for GenBank Accession No. XP_002492481: Ferrochelatase [Komagataella phaffi GS115], NAT’L LIBR. MED.: NAT’L CTR. FOR BIOTECH. INFORMATION, <i>available at</i> https://www.ncbi.nlm.nih.gov/protein/XP_002492481.1

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EX-1053	<i>GenBank Overview</i> , NAT'L LIBR. MED.: NAT'L CTR. FOR BIOTECH. INFORMATION, <i>available at</i> https://www.ncbi.nlm.nih.gov/genbank
EX-1054	Benson et al., <i>GenBank</i> , 42 NUCLEIC ACIDS RESEARCH: DATABASE ISSUE, D32-D37 (2014)
EX-1055	<i>Curriculum Vitae</i> of Dr. Carl Batt
EX-1061	NCBI Resource Coordinators, <i>Database resources of the National Center for Biotechnology Information</i> , 44 NUCLEIC ACIDS RESEARCH: DATABASE ISSUE, D7-D19 (2015)

I, Dr. Carl Batt, Ph.D., declare as follows:

1. I have been retained by Quinn Emanuel Urquhart & Sullivan LLP on behalf of Motif FoodWorks, Inc. (“Petitioner” or “Motif”) as an independent expert in this *inter partes* review (“IPR”) proceeding pending before the Patent Trial and Appeal Board (“the Board”) of the United States Patent and Trademark Office (the “Proceeding”). I understand that, in this Proceeding, Motif is challenging the validity of claims 1-14 (the “Challenged Claims”) of U.S. Patent No. 10,273,492 (the “’492 patent”). Based on the first page of the ’492 patent, I understand it to be assigned to Impossible Foods Inc. (“Patent Owner”).

2. I have been asked by Motif to provide my opinions regarding the validity of the ’492 patent claims in this declaration (“Declaration”).

3. I am informed that the ’492 patent has been asserted by Patent Owner against Motif in the co-pending litigation *Impossible Foods Inc. v. Motif Foodworks Inc.*, Case No. 22-cv-311 (D. Del.) (“Litigation”). I understand that the Litigation is proceeding in the District Court for the District of Delaware (“District Court”).

4. I understand that, in the Litigation, neither party has exchanged any contentions relating to infringement (or non-infringement) or invalidity (or validity), and that neither party has yet proposed any claim constructions. I further understand that the District Court has not yet been asked to construe any claims of the ’492 patent and that no claim construction order has been issued. I also understand that

Patent Owner has not filed any motions seeking injunctive relief and the District Court likely will not order claim construction briefing or a hearing until a later point in the Litigation. Lastly, I understand that the '492 patent has not previously been asserted in any litigation nor subject to any prior post-grant proceeding to review its validity against the prior art. I reserve the right to amend or supplement my opinions in this Declaration in response to any positions that Patent Owner may take in the Litigation, or in response to any order of the District Court.

I. BACKGROUND

5. EX-1055 accompanying this Declaration is a true and correct copy of my current CV. It describes in greater detail my education, publications, employment and research history, and professional activities and awards.

6. I have over 35 years of experience in microbiology, molecular biology, protein engineering, cell culture and food science. In 1975, I was awarded Bachelor of Science Degree in Microbiology from Kansas State University in Manhattan, Kansas. In 1981, I was awarded a Doctor of Philosophy Degree in Food Science from Rutgers University in New Brunswick, New Jersey. I was a postdoctoral research associate from 1982-1984 in the Department of Nutrition and Food Science and a research scientist from 1984-1985 in the Department of Applied Biological Sciences at Massachusetts Institute of Technology.

7. In 1985, I joined the faculty of Cornell University as an Assistant Professor in the Department of Food Science, where I was promoted to Associate Professor in 1991 and Professor in 1998.

8. While at Cornell University, I founded the Cornell University/Ludwig Institute for Cancer Research Partnership and served as its Director from 1999-2011. As Director, I managed the design, construction and operation of a Good Manufacturing Practice (“GMP”) facility on the Cornell University Ithaca campus, which resulted in the production of recombinant proteins using microbial expression systems, including *Pichia pastoris*, that have been used in clinical trials.

9. I have conducted research in several areas that span the life and physical sciences. My work includes pioneering efforts in the fields of microbiology, molecular biology, and nanotechnology. One area of research that I have focused on is the use of protein engineering and expression systems for producing recombinant anti-cancer therapeutics and proteins in general. For the past 35, years, my laboratory has been engaged in protein expression using a variety of host systems including bacteria, yeast, fungi, and higher eukaryotic cells. Our work in *P. pastoris* began in the early 1990’s and is documented in publications expressing various proteins using this versatile expression system.

10. The major area of research currently underway in my laboratory concerns exploring parallel routes for the expression of therapeutic agents and

constructing rational approaches to scale-up of these systems. Our major focus is on the development and optimization of microbial-based expression systems for tumor antigens and tumor-specific antibody fragments. Our Biologics Production Facility enables us to carry out large-scale production of highly-purified immunotherapeutics suitable for early-phase human clinical trials.

11. Over the course of my career, I have authored or co-authored more than 300 peer-reviewed articles, book chapters and reviews. I have given more than 100 invited lectures worldwide. I am a named inventor on three United States patents.

12. I have been fortunate to receive a number of distinctions during my career. In 1990, I was awarded the Samuel Cate Prescott Award from the Institute of Food Technologists which is given to the outstanding scientist in my field under the age of 35. In 2000, I was named a Liberty Hyde Bailey Professor in the College of Agriculture and Life Sciences, a named chair created to honor the most outstanding faculty in the college. It is an honor that I hold for the time I am on the Cornell faculty. Today that distinction is held by only five professors among the 1,629 faculty at Cornell University.

13. I am co-founder of the Nanobiotechnology Center (“NBTC”) at Cornell University, a National Science Foundation supported science and technology center, and was co-Director of NBTC from 1999-2004. I am also co-founder of Main Street Science, a not-for-profit corporation established to develop and distribute K-12

science learning materials, and the founder of Nanooze, a science magazine for children.

14. In 2007, I was an NSF Discovery Corps Fellow in recognition of both my extraordinary research accomplishments and my efforts to communicate advances in science and technology to the general public. In addition, I have been the recipient of a number of awards from Cornell University for teaching (Teaching Excellence Award, 1990), service (Faculty Fellows in Service, 2001; Kaplan Family Distinguished Faculty Fellowship in Service-Learning, 2003), and diversity (CALS Kathy Berggren Diversity and Inclusion Award, 2008).

15. In addition to my teaching responsibilities, I have mentored over 50 graduate students, including 45 PhD students and over 100 undergraduates in my laboratory, many of whom now hold significant positions in academia, government and the private sector, both in the United States and internationally.

16. I served as Chief Editor for Food Microbiology (1987-2000), a peer-reviewed journal. I also served as Editor for the Encyclopedia of Food Microbiology (1996-1999) and Chief Editor of its 2nd Edition (2009-2015). I served as Editor of Elsevier's Food Microbiology, Food Science Reference Module (2015-2016). I have also been on the Editorial Boards of the peer-reviewed journals: Food Biotechnology (1987-1992), Journal of Food Science (2001-2008), and Protein Expression and Purification (2001-2004).

17. As a champion of making scientific concepts understandable for the general public, especially young students, I lead a team that developed a series of public exhibitions including “It’s a Nanoworld,” which was on a five-year tour in the United States after a six-month residency at EPCOT in Walt Disney World, and “Too Small To See,” which is also on tour after opening in EPCOT in 2007. Subsequently I designed and oversaw the construction of two additional exhibits, “Take a Nanooze Break” (2010-2020) and “Nanooze Labs” (2011-2014) that were on exhibit at Walt Disney World and Disneyland respectively.

II. SCOPE OF ASSIGNMENT

18. In forming my opinions regarding the validity of the ’492 patent, I have considered the ’492 patent’s specification, including the Abstract and the figures. I have also considered the language of the patent’s claims, as those terms would have been understood by a person having ordinary skill in the relevant field at least at the time of the ’492 patent’s earliest possible priority date. I have also reviewed the prosecution (the “file history”) of the ’492 patent.

19. In forming my opinions, I have also considered the Exhibits that are listed in the Petition and any additional materials and information cited in this Declaration. I have also searched for and reviewed technical literature relevant to the field of the ’492 patent.

20. I have relied on my personal knowledge, education, training, and professional experience in order to form my opinions concerning the '492 patent, including my experience in researching, studying, developing genetic expression systems.

21. This Declaration explains, based on facts and information currently available to me, the subject matter and opinions related to this Proceeding. As such, I am prepared to provide expert testimony regarding opinions formed resulting from my analysis of the issues and materials considered in this declaration, if asked to do so by the Board or by the parties' attorneys.

22. Additionally, I may discuss my own work, teachings, and knowledge of the state of the art in the relevant time period. I may rely on handbooks, textbooks, technical literature, and the like to demonstrate the state of the art in the relevant period and the evolution of relevant technologies.

23. Throughout this Declaration, I refer to specific pages of the '492 patent and other documents. These citations are intended to be exemplary and are not intended to convey that the citations are the only source of evidence to support the propositions for which they are cited. I reserve my right to amend or alter my analysis and opinions in response to positions Patent Owner may take or upon the discovery of new information.

24. I am being compensated at my customary consulting rate of \$375 per hour for the time I spend on this matter. My compensation is in no way contingent on the nature of my opinions or the outcome of this or any other proceeding. I have no other interest in this proceeding. All of the opinions expressed in this Declaration are my own.

III. LEGAL UNDERSTANDING

25. In this section I describe my understanding of certain legal standards that I have relied upon in forming my opinions set forth in this Declaration. I have been informed of these legal standards by Motif’s attorneys. I am not an attorney and I have not thoroughly researched the relevant patent law. I am relying only on instructions from Motif’s attorneys for these legal standards.

A. Person of Ordinary Skill in the Art

26. I am informed that patent validity is determined from the perspective of a person of ordinary skill in the pertinent art at the time of the invention (“POSITA”). I understand that a POSITA is a hypothetical person who is presumed to be aware of all pertinent art, thinks along conventional wisdom in the art, and is a person of ordinary creativity—not an automaton.

27. In forming my opinions, I have relied on my personal knowledge and professional experience in researching, studying, developing, or using recombinant

protein expression technology, and on the documents and information referenced in this Declaration.

28. In deciding the level of ordinary skill in the art, I am informed that the following factors may be considered:

- the levels of education and experience of persons working in the field;
- the types of problems encountered in the field; and
- the sophistication of the technology.

B. Claim Construction

29. I am informed that a patent may include two types of claims, independent claims and dependent claims. An independent claim stands alone and includes only the limitations it recites. A dependent claim can depend from an independent claim or another dependent claim. I am informed that a dependent claim includes all the limitations that it recites, in addition to the limitations recited in the claim from which it depends.

30. I understand that patent claim terms can be interpreted, or “construed.” I understand that, in construing a claim, the words of patent claims are given their plain meaning, as a POSITA would understand them at the time of the invention, view of the patent and file history, unless their meaning is defined or disclaimed. I am informed that dictionaries, treatises, and other “extrinsic” sources of evidence

may assist in determining the plain meaning of a term, but they cannot override a meaning that is otherwise unambiguous based on the intrinsic evidence.

31. I understand that in construing claims in an IPR proceeding, words in a claim are given their plain meaning, which is the meaning understood by a person of ordinary skill in the art in view of the patent and file history, unless specifically defined in the patent or prosecution history. I also understand that dictionaries or other extrinsic sources may assist in determining the plain and ordinary meaning but cannot override a meaning that is unambiguous from the intrinsic evidence.

32. I have been informed by Motif's counsel that claim construction is a matter of law for the arbiter of law to decide. I am informed that, in an *inter partes* review, the Patent Trial and Appeal Board interprets claims in the same way as a district court would. Likewise, I understand that a POSITA interprets claims in the same way in both types of proceedings.

33. I reserve my right to amend or alter my analysis and opinions in view of any claim constructions adopted by the arbiter of law.

C. Obviousness

34. I am informed that a patent claim is unpatentable and invalid if the subject matter of the claim as a whole would have been obvious to a person of ordinary skill in the art of the claimed subject matter as of the time of the inventions at issue. I am informed that the following factors must be evaluated to determine

whether the claimed subject matter is obvious: (1) the scope and content of the prior art; (2) the difference or differences, if any, between each claim of the patent and the prior art; and (3) the level of ordinary skill in the art at the time the patent was filed. Unlike anticipation, which allows consideration of only one item of prior art, I am informed that obviousness may be shown by considering more than one item of prior art. Moreover, I understand that objective indicia of non-obviousness, also known as “secondary considerations,” like the following also may be considered when assessing obviousness: (1) commercial success; (2) long-felt but unresolved needs; (3) copying of the invention by others in the field; (4) initial expressions of disbelief by experts in the field; (5) failure of others to solve the problem that the inventor solved; and (6) unexpected results. I also understand that evidence of objective indicia of non-obviousness must have a nexus with the claimed subject matter.

IV. THE '492 PATENT

A. Level of Ordinary Skill in the Art

35. The '492 patent is directed to the art of recombinant protein expression systems. A POSITA would have a Bachelor of Science degree in biochemistry, chemistry, biological sciences, or an equivalent area of study. A POSITA also would have at least three years of graduate study or professional experience with recombinant techniques for gene expression, protein production, or general genetic modification experience.

B. Claims

36. The '492 patent contains 14 claims, 1 independent claim and 13 dependent claims.

37. Claim 1 is representative and reproduced below.

A methylotrophic Pichia yeast cell comprising:

a nucleic acid molecule encoding a heme-containing protein operably linked to a promoter element from P. pastoris and a Mxr1 transcriptional activator sequence from P. pastoris;

and a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis operably linked to a promoter element from P. pastoris and a Mxr1 transcriptional activator sequence from P. pastoris.

38. The dependent claims are generally directed to a product comprising:

- a heme-containing protein that is Leghemoglobin (“LegH”) (claim 2);
- the LegH with the sequence shown in SEQ ID NO:6 (claim 3);
- and at least one polypeptide involved in heme biosynthesis is selected from the group consisting of ALA synthase, ALA dehydratase, porphogilinogen [sic] deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and/or ferrochelatase (claim 4);

- the promoter element from *P. pastoris* is a constitutive promoter element from *P. pastoris* or a methanol-inducible promoter element from *P. pastoris* (claim 5);
- the constitutive promoter element from *P. pastoris* is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene (claim 6);
- the methanol-inducible promoter element from *P. pastoris* is an alcohol oxidase I (AOX1) promoter element (claim 7);
- the promoter element from *P. pastoris* comprises a constitutive promoter element from *P. pastoris* and a methanol-inducible promoter element from *P. pastoris* (claim 8);
- the constitutive promoter element from *P. pastoris* is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene (claim 9);
- the methanol-inducible promoter element from *P. pastoris* is an alcohol oxidase I (AOX1) promoter element (claim 10);
- the nucleic acid molecule encoding the heme-containing protein is present in the yeast cell in at least two copies (claim 11);
- the nucleic acid molecule encoding the heme-containing protein operably linked to the promoter element from *P. pastoris* and the

Mxr1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence (claim 12);

- the nucleic acid encoding the at least one polypeptide involved in heme biosynthesis operably linked to the promoter element from *P. pastoris* and the Mxr1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence (claim 13); and
- a nucleic acid molecule encoding a Mxr1 transcriptional activator operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris* (claim 14).

C. Specification

39. The '492 patent describes “[m]ethods and materials for genetically engineering methylotrophic yeast.” EX-1001, Abstract. The '492 patent discloses a methylotrophic *P. pastoris* expression system that produces proteins by operably linking the promoter sequence “in such a way as to direct or regulate expression of the [exogenous] nucleic acid.” EX-1001, 4:50-51. The '492 patent discloses that nucleic acids can be introduced into the methylotrophic host cell through methods that are “known in the art, and include without limitation, transduction, electroporation, biolistic particle delivery, and chemical transformation.” EX-1001, 7:11-14.

40. Many aspects of the '492 patent are admittedly disclosed in the prior art. The '492 patent discloses that transcriptional activator Mxr1, a key regulator of the methanol utilization pathway, is “known in the art.” EX-1001, 4:25. The '492 patent teaches that the Mxr1 sequence should be placed “under control of a promoter that is inducible” for Mxr1 co-expression. EX-1001, 4:44-46. In addition, the '492 patent discloses “methanol inducible promoters that are known in the art,” such as the “commonly used methanol-inducible promoter from *P. pastoris*.” EX-1001, 4:56-59.

41. The '492 patent discloses several promoter elements that can be constructed into the claimed expression system. For example, the '491 patent discloses that the AOX1 promoter can be used to direct gene expression in methylotrophic *P. pastoris*. EX-1001, 4:56-59. The '492 patent discloses that the AOX1 promoter “is strongly transcribed in response to methanol.” The expression system of the '492 patent claims a construct “wherein the methanol-inducible element from *P. pastoris* is an alcohol oxidase I (AOX1).” EX-1001, 78:55-57.

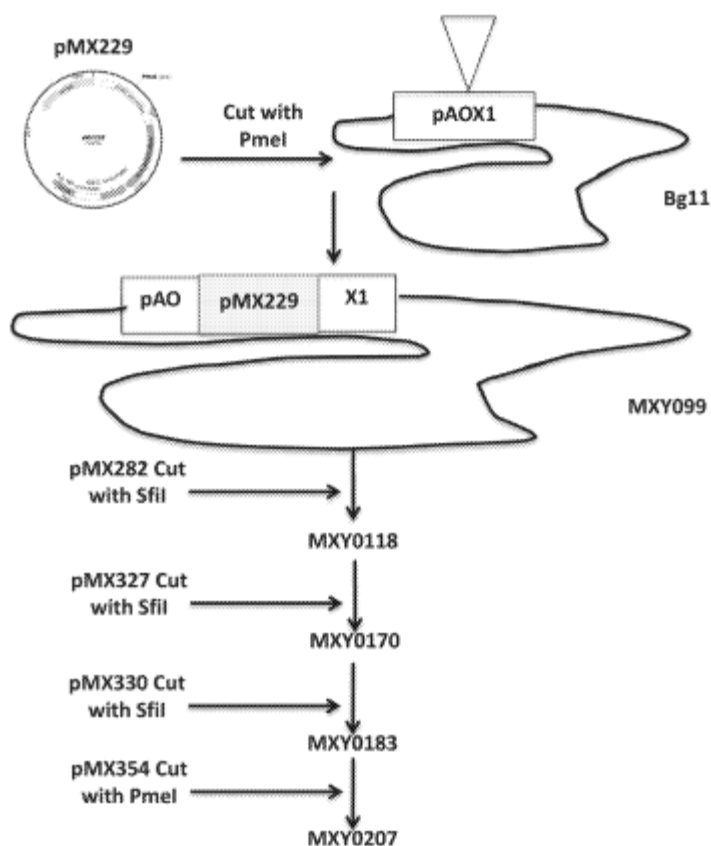


Figure 3

42. In addition, the patent discloses other “commonly used” promoters such as transcriptional elongation factor EF-1a (TEF1), “which is strongly transcribed in a constitutive manner,” and glyceraldehyde-3-phosphate dehydrogenase (“GAP”). EX-1001, 6:37-44; 78:52-54.

43. In order to fully incorporate heme-containing proteins, the ’492 patent recognizes that certain heme biosynthetic enzymes must be coexpressed with heme-containing proteins. For example, a heme-containing protein claimed by the ’492

patent is legH. EX-1001, 77:62-67. The '492 patent claims co-expression of "at least one polypeptide involved in heme biosynthesis" from the following group: ALA synthase, ALA dehydratase, porphogilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and/or ferrochelatase.

44. The heme biosynthetic enzyme is encoded within an expression vector that is operably linked to the promoter and MXR1. EX-1001, 78:52-54 .

D. Prosecution History

45. I have reviewed the prosecution history of the '492 patent (EX-1001). The Examiner did not render any rejections based on any substantive consideration of prior art. Rather the claims were rejected for failing to comply with the written description requirement, being indefinite, and double-patenting.

46. Based on my review of the first page of the '492 patent (EX-1001), I see that U.S. Application No. 15/678,342 (the "'342 application") was filed on August 16, 2017, and issued as the '492 patent on April 30, 2019. EX-1001. I understand that the '492 patent is a continuation of International Application No. PCT/US2016/031,797 (the "'797 application") filed on May 11, 2016, which claims the benefit of priority of U.S. Provisional Application No. 62/313, 49, filed March 25, 2016; U.S. Provisional Application No. 62/236,506, filed Oct. 2, 2015; U.S. Provisional Application No. 62/222,388, filed Sep. 23, 2015; U.S. Provisional Application No. 62/220,366, filed Sep. 18, 2015; U.S. Provisional Application No.

62/203,052, filed Aug. 10, 2015; U.S. Provisional Application No. 62/185,921, filed Jun. 29, 2015; U.S. Provisional Application No. 62/183,074, filed Jun. 22, 2015; and U.S. Provisional Application No. 62/159,899, filed May 11, 2015. For the purposes of my analysis in this Declaration, I have assumed May 11, 2015 as the priority date for the Challenged Claims.

47. I have reviewed the prosecution history of the '492 patent. The '698 application was filed on August 16, 2017. EX-1039, 1-7. I see that the claims were issued a non-final rejection on December 4, 2017. EX-1039, 937-944. The Examiner rejected claims 1, 4-7, and 10-13. EX-1039, 937. Claims 2-3 and 8-9 were objected to as depending from a rejected base claim. EX-1039, 938. In addition, claims 1-14 were provisionally rejected on non-statutory double patenting grounds over co-pending Application No. 15/678,891. EX-1039, 939.

48. The Examiner rejected claims 1, 4-7, 10 and 13 as unpatentable under 35 U.S.C. 112(a) for failing to comply with the written description requirement. EX-1039, 939-940. The Examiner reasoned that the claims were drawn to a genus of *Pichia* yeast cell comprising a nucleic acid molecule encoding a heme-containing protein. EX-1039, 940. However, only the expression of a globin was described in the specification without any significant description of expression of any other polypeptides which bind heme. EX-1039, 940-941. Thus, the Examiner concluded

that one of skill would not have envisioned the full scope of the claimed invention.
EX-1039, 942.

49. The Examiner further rejected claims 11 and 12 under 35 U.S.C. 112(b) as being indefinite. EX-1039, 942-43. The Examiner reasoned that term “leghemoglobin” lacks antecedent basis in claim 1 which claim 11 and 12 depend. EX-1039, 942-43.

50. On March 1, 2018, the applicant amended the claims in response to the Examiner’s Non-Final Rejection. EX-1039, 958-64. The applicant agreed to submit a Terminal Disclaimer should the claims be allowed in response to the Examiner’s Double Patenting Rejection. EX-1039, 962.

51. With respect to the written description rejection for claims 1, 4-7, 10 and 13, the applicant specified the specific portions of the specification that it contends disclose members of the globin family of proteins, specifically Leghemoglobin. EX-1039, 962.

52. Further, the applicant amended Claim 1 in response to the Examiner’s rejection of Claims 11 and 12. EX-1039, 963. The claims were amended to replace “leghemoglobin” with “heme-containing protein,” as shown below. EX-1039, 963.

Claim 11: The yeast cell of claim 1, wherein the nucleic acid molecule encoding the heme-containing protein ~~leghemoglobin~~ is present in the yeast cell in at least two copies.

Claim 12: The yeast cell of claim 1, wherein the nucleic acid molecule encoding the heme-containing protein leghemoglobin operably linked to the promoter element from *P. pastoris* and the MXR1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence.

53. On June 13, 2018, The Examiner issued a Final Rejection on the basis of statutory double-patenting—requiring the applicant to file a Terminal Disclaimer before allowance. EX-1039, 969-73. The applicant submitted a Terminal Disclaimer on August 7, 2018. EX-1039, 990-93. Thereafter, the Examiner issued a Notice of Allowance on August 15, 2018. EX-1039, 1005.

V. CHALLENGED CLAIMS

54. I have analyzed claims 1-14 of the '492 patent in connection with Motif's Petition for *inter partes* review that accompanies this Declaration. I understand Motif is challenging the validity of all 14 claims ("Challenged Claims"). The Challenged Claims are reproduced below for reference.

Claim 1	
[1 pre]	A methylotrophic <i>Pichia</i> yeast cell comprising:
[1a]	a nucleic acid molecule encoding a heme-containing protein operably linked to a promoter element from <i>P. pastoris</i> and a Mxr1 transcriptional activator sequence from <i>P. pastoris</i> ; and

[1b]	a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis operably linked to a promoter element from <i>P. pastoris</i> and a Mxr1 transcriptional activator sequence from <i>P. pastoris</i> .
Claim 2	
The yeast cell of claim 1, wherein the heme-containing protein is Leghemoglobin (LegH).	
Claim 3	
The yeast cell of claim 2, wherein the LegH has the sequence shown in SEQ ID NO:6.	
Claim 4	
The yeast cell of claim 1, wherein the at least one polypeptide involved in heme biosynthesis is selected from the group consisting of ALA synthase, ALA dehydratase, porphogilinogen [sic] deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and/or ferrochelatase.	
Claim 5	
The yeast cell of claim 1, wherein the promoter element from <i>P. pastoris</i> is a constitutive promoter element from <i>P. pastoris</i> or a methanol-inducible promoter element from <i>P. pastoris</i> .	
Claim 6	
The yeast cell of claim 5, wherein the constitutive promoter element from <i>P. pastoris</i> is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene.	
Claim 7	
The yeast cell of claim 5, wherein the methanol-inducible promoter element from <i>P. pastoris</i> is an alcohol oxidaseI (AOX1) promoter element.	
Claim 8	
The yeast cell of claim 1, wherein the promoter element from <i>P. pastoris</i> comprises a constitutive promoter element from <i>P. pastoris</i> and a methanol-inducible promoter	

element from *P. pastoris* a second wireless communication link between the transmitter and the mobile unit.

Claim 9

The yeast cell of claim 8, wherein the constitutive promoter element from *P.pastoris* is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene.

Claim 10

The yeast cell of claim 8, wherein the methanol inducible promoter element from *P. pastoris* is an alcohol oxidase I (AOX1) promoter element.

Claim 11

The yeast cell of claim 1, wherein the nucleic acid molecule encoding the heme-containing protein is present in the yeast cell in at least two copies.

Claim 12

The yeast cell of claim 1 wherein the nucleic acid molecule encoding the heme-containing protein operably linked to the promoter element from *P. pastoris* and the Mxr1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence.

Claim 13

The yeast cell of claim 1, wherein the wherein the nucleic acid molecule encoding the at least one polypeptide involved in the heme biosynthesis operably linked to the promoter element from *P. pastoris* and the Mxr1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence.

Claim 14

The yest cell of claim 1, further comprising a nucleic acid molecule encoding a Mxr1 transcriptional activator operably linked to promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.

VI. STATE OF THE ART

A. Recombinant DNA Technology

55. Proteins are made in cells starting from the sequence information encoded in a nucleic acid, such as deoxyribonucleic acid (“DNA”). The DNA is transcribed into a messenger-ribonucleic acid (“mRNA”), which transfers the information encoded for in the DNA sequence and ultimately results in an amino acid sequence. mRNA is translated into the amino acid sequence, which is either a peptide or a protein. A gene is a discrete unit of information encoded for in the DNA that results in a protein. For example, the gene coding for leghemoglobin is transcribed and translated to form the protein leghemoglobin.

56. Recombinant DNA (“rDNA”) technology emerged between 1971–1973, marking what POSITAs have hailed as a “a great scientific turning point.” EX-1028(Stryjewska), 1075-76. rDNA is the result of genetic engineering in which DNA from a heterologous source can be combined and used to express a protein of interest in a different host. Recombinant proteins initially derived from a different cell type or a different species than the host in which it is being expressed are referred to as “heterologous” proteins. rDNA technology to produce such heterologous proteins is a cornerstone of the biotechnology industry as we know it and has been widely utilized in the agriculture, food and medical sectors. EX-1028(Stryjewska), 1076; EX-1013(Engel), 1-6.

57. rDNA technology allows DNA to be artificially combined to create sequences that are not naturally found in nature. EX-1028(Stryjewska), 1076-79. The procedure has been used to alter DNA in many different types of hosts including bacteria, yeast, animals and plants. EX-1028(Stryjewska), 1076-79. rDNA has been applied, for example, in the medical industry to make monoclonal antibodies and vaccines. EX-1028(Stryjewska), 1076-79. rDNA also has become a pillar in the food industry in order to make food ingredients or to genetically modify plants and animals to improve aroma, taste, shelf-life and nutritional value. EX-1013(Engel), 1-6.

58. The multifaceted nature of rDNA lies in the ability to produce “a wide range of peptides, proteins, and biochemicals from naturally nonproducing cells.” EX-1028(Stryjewska), 1075. rDNA modification is precise and sequence-specific. EX-1028(Stryjewska), 1076-79. The DNA sequence for a protein of interest can be identified, for example, by searching the NIH: National Center for Biotechnology Information’s (NCBI) GenBank database. *See generally* EX-1053(NCBI); EX-1054(Benson). The GenBank database “is a comprehensive public database of nucleotide sequences” (EX-1054(Benson), D32) that “is designed to provide and encourage access within the scientific community to the most up-to-date and comprehensive DNA sequence information.” EX-1053(NCBI). GenBank data is exchanged on a daily basis with GenBank’s partners in the International Nucleotide

Sequence Database Collaboration (INSDC)¹ “to ensure that that a uniform and comprehensive collection of sequence information is available worldwide.” EX-1054(Benson), D32. It also obtains sequences disclosed in issued patents directly from the USPTO. EX-1054(Benson), D32.

59. A POSITA interested in producing a recombinant protein or peptide would be able to search the GenBank database to obtain “the most up-to-date and comprehensive DNA sequence information” for it. EX-1053(NCBI). For example, to produce a recombinant heme-containing protein or polypeptide, one early step could be to search the GenBank database for the gene sequence encoding the protein of interest. By 2015 the sequences for a number of heme-containing proteins and heme biosynthesis proteins were available. *See, e.g.*, EX-1046 (GenBank entry for “leghemoglobin [Glycine max],” available since April 27, 1993); EX-1047 (GenBank entry for “leghemoglobin [Pisum sativum],” available since February 6, 1998); EX-1048 (GenBank entry for protein family PF00042, heme-binding globins); EX-1049 (GenBank entry for ALA-dehydratase in *P. pastoris*, available

¹ The NCBI’s partners in the INSDC include the EMBL Nucleotide Sequence Database, which is “part of the European Nucleotide Archive,” and the DNA Data Bank of Japan. EX-1054(Benson), D32.

since July 22, 2009); EX-1050 (GenBank entry for ferrochelatase in *P. pastoris*, available since July 22, 2009).²

60. To express a protein of interest, the gene encoding the protein is inserted, or “cloned,” into an expression vector, which is a DNA fragment that can be replicated in the host cell. This cloning process can be accomplished in different ways. One method involves using restriction endonucleases to excise the gene of interest and cleave the vector and then use ligases to join the fragments together, thereby inserting the gene into the vector. EX-1028(Stryjewska), 1076. This

² When a coding genetic sequence is submitted to the GenBank database, NCBI produces a protein sequence translation of that sequence, which it calls a “GenPept sequence[]” and stores in its “Protein database.” EX-1061(NCBI), D15. These protein (GenPept) sequences are available through a unique GenBank Accession number, just like the gene sequences from which they were translated. For simplicity, I refer to the GenBank and Protein databases collectively as the GenBank/GenPept database. Every entry in the GenBank/GenPept database contains multiple tabs that provide different information about the sequence, its origin, and its history on GenBank. The “GenBank” (or “GenPept” for proteins) and “Revision History” summaries in Petitioner’s GenBank exhibits are two of the tabs available for each sequence.

gene/expression vector combination is one form of rDNA that is routinely used by persons of skill in the art.

61. In order to produce a protein of interest using rDNA, the gene of interest inserted in the vector must be introduced, or transformed, into a host cell. To transform yeast, the yeast cell membrane is perturbed to allow exogenous rDNA to enter the cell. EX-1028(Stryjewska), 1078. Methods of transformation have been well-established since the late 1970's. *See, e.g.*, EX-1028(Stryjewska), 1076-79.

62. If a host cell is successfully transformed with rDNA, the gene of interest can be expressed to form proteins. EX-1028(Stryjewska), 1078-79. The host RNA polymerase first transcribes the DNA into mRNA. EX-1042(Alberts), 305; EX-1043(Freeman), 338-39, 342-45. Host cell ribosomes then translate the mRNA into the recombinant protein. EX-1042(Alberts), 305; EX-1043(Freeman), 338, 345-55.

63. Not every host cell takes up the rDNA. EX1018(De Schutter), 1078. To select for cells that have successfully been transformed, rDNA vectors typically include "selection markers," such as genes that confer resistance to antibiotics like zeocin or blasticidin. EX-1028(Stryjewska), 1078-79; EX-1015(Lin-Cereghino 2000), 48-50; EX-1029(Ahmad), 5304, 5310-11. When an antibiotic resistance gene is used as a selection marker, the transformed host cells can grow in medium containing the antibiotic. EX-1028(Stryjewska), 1078-79. Only the host cells with

the rDNA (including the antibiotic-resistant gene) will replicate and produce the protein of interest when grown in a medium containing that antibiotic. *Id.*

B. Protein Expression Systems

64. In the almost 50 years since the inception of rDNA technology, certain microbial gene expression systems have become particularly popular. For example, *Escherichia coli* (*E. coli*) was an early favorite and is still used for many applications. EX-1028(Stryjewska), 1084. Other scientists have described *P. pastoris* as having “become the premier example of yeast species used for the production of recombinant proteins.” EX-1030(Haon), 1-2.³

65. By the early 2000s, reports in the literature had described *P. pastoris* as one of the “most commonly used yeast species in the production of recombinant proteins.” EX-1018(De Schutter), 561. To add to this, the FDA has approved recombinant proteins expressed by *P. pastoris* as safe for use in humans and animals

³ In my Declaration, I refer repeatedly to *Pichia pastoris*. I am aware that this organism, as referred to in the art, has undergone name changes including *Komagataella pastoris* and *Komagataella phaffii* that reflect evolving taxonomical descriptions. In my experience, and as a POSITA would understand, these terms are synonymous (including as this term is used in the '656 patent) and, consequently, the use of alternative names does not affect my analysis herein.

in 2015. EX-1029(Ahmad), 5301-02; EX-1018(De Schutter), 561, 565; EX-1021(Sreekrishna), 1-2; EX-1022(Zhang), 148. The industry recognizes that *P. pastoris* expression systems have certain “advantages over other eukaryotic and prokaryotic expression systems,” including:

(i) rapid growth rate, coupled with ease of high cell-density fermentation; (ii) high levels of productivity in an almost protein-free medium; (iii) elimination of endotoxin and bacteriophage contamination; (iv) ease of genetic manipulation of well-characterized yeast expression vectors; (v) absence of known human pathogenicity in the spectrum of lytic viruses that prey on *P. pastoris*; (vi) diverse post-translational modifications that include polypeptide folding, glycosylation, methylation, acylation, proteolytic adjustment, and targeting to subcellular compartments; and (vii) the ability to engineer secreted proteins that can be purified from growth medium without harvesting the yeast cells themselves.

EX-1027(Rabert), 351-52; *see also* EX-1005(Vogl), 385-86; EX-1017(Hartner 2008), 1; EX-1022(Zhang), 149; EX-1015(Lin-Cereghino 2000), 52-53; EX-1018(De Schutter), 561; EX-1021(Sreekrishna), 6.

66. By the early 2000s, POSITAs understood that *P. pastoris*—as well as other “methyloptrophic” yeast species—could “use methanol as a sole carbon source.” EX-1021(Sreekrishna), 1. This “provides tightly controlled methanol-inducible transgene expression” and allows the system to “efficiently secrete[] heterologous proteins in defined media.” EX-1018(De Schutter), 561.

67. In addition, *P. pastoris* expression systems are capable of “industrial scale production of proteins via recombinant DNA technology.” EX-1012(Vedvick), 197. *P. pastoris* has been “employed in laboratories around the world to produce proteins for basic research and medical applications.” EX-1018(De Schutter), 561. By 2008, “[s]everal hundred proteins from almost all kingdoms of life ha[d] been expressed in this yeast.” EX-1017(Hartner 2008), 1.

C. Gene Expression in Methylophilic Yeast

68. Transcription of genes is highly regulated in eukaryotes such as yeast (including *P. pastoris*). EX-1031(Krainer), 1. Proteins that regulate gene expression include transcription factors—the “most basic set of proteins needed to activate gene transcription.” EX-1031(Krainer), 1.

69. A promoter is the non-coding region of DNA upstream of the gene’s coding region. EX-1042(Alberts), 306. Elements of the cell’s transcription machinery—transcription factors—bind promoters and regulate transcription, largely determining the timing of gene expression and amount of protein that will be produced. EX-1042(Alberts), 306-07. Transcription factors can activate or repress gene transcription. EX-1042(Alberts), 310-11; EX-1005(Vogl), 386. They do so by binding the promoter in ways that either allow transcription to occur (e.g., they are “transcriptional activators”), or cause it not to occur (“repressors”). EX-1042(Alberts), 310-11; EX-1005(Vogl), 386. Transcription factors control when,

where, and how efficiently a gene is transcribed. EX-1042(Alberts), 306-35. To initiate gene expression, transcription factors typically bind directly to “promoter” regions of DNA, which lie upstream of the coding region in a gene. EX-1042(Alberts), 306-35.⁴

70. Numerous promoters that regulate transcription in methylotrophic *P. pastoris* were well-characterized by 2015. For example, the literature reported that *P. pastoris* provides an array of “strong and tightly regulated promoters.” EX-1017(Hartner 2008), 13. In yeast species such as *P. pastoris*, native promoters can be constitutive or inducible. EX-1005(Vogl), 393; EX-1029(Ahmad), 5302-04. Constitutive promoters are “on” regardless of external stimuli (i.e., constitutively active); transcription factors bind to them, leading to transcription under most

⁴ “Upstream” refers to a relative location on the DNA strand. DNA is a double-stranded molecule with each strand oriented in opposite directions. One end of the strand is known as the 5’ end, and it generally contains a phosphate group attached to the 5’ carbon of the deoxyribose ring in the backbone of the nucleoside at that end. At the other side of the strand is the 3’ end, which generally contains a hydroxyl group at the 3’ carbon of the deoxyribose ring. Because DNA is transcribed in the 5’ to 3’ direction, DNA “upstream” to the gene of interest refers to DNA toward the 5’ end with respect to that coding region.

conditions. EX-1005(Vogl), 386, 392; EX-1029(Ahmad), 5304. Inducible promoters can be made more or less active in response to external stimuli, such as temperature, oxygen, or chemicals. EX-1005(Vogl), 386, 392; EX-1029(Ahmad), 5304, 5302-03. It was known that proteins can be expressed in *P. pastoris* using constitutive and inducible promoters, including methanol-inducible promoters. EX-1017(Hartner 2008), 13 (“several promoters, either constitutive or inducible, are available for this yeast [PAOX1 (8), PAOX2 (9), PFLD1 (10), PDAS (8), PPEX8 (PPER3) (11), PYPT1 (12), PGAP (13), PTEF1 (14), PPGK1 (15), PICL1 (16)]”). By 2015, various *P. pastoris* expression systems for rDNA expression had been constructed that included constitutive and/or inducible promoters to control heterologous protein expression. EX-1017(Hartner 2008), 1; EX-1005(Vogl), 385-87, 392-95; EX-1015(Lin-Cereghino 2000), 46-48; EX-1016(Hartner), 2; EX-1021(Sreekrishna), 7; EX-1029(Ahmad), 5302-04. Examples of routinely used promoters alcohol oxidase I gene promoter (“PAOX1”), the dihydroxyacetone synthase promoter (“PDAS”), and the peroxisomal matrix protein promoter (“PPEX8”), among others. EX-1005(Vogl), 385-87, 392-95; EX-1015(Lin-Cereghino 2000), 46-48; EX-1021(Sreekrishna), 7; EX-1029(Vogl), 5302-04.

71. One *P. pastoris* promoter that has been “well-characterized” is the alcohol oxidase I (“AOX1”) gene promoter. EX-1017(Hartner 2008), 1. In the early 2000s, the AOX1 promoter was “employed in most studies and applications”

relating to recombinant protein production using *P. pastoris* expression systems. EX-1017(Hartner 2008), 1.

72. One of the recognized advantages of using the AOX1 promoter in *P. pastoris* systems is the ability to turn on gene expression by adding methanol in the growth medium. Transcription initiated from the AOX1 promoter “produces extremely high levels of these transcripts upon switching the carbon source to methanol.” EX-1018(De Schutter), 564.

73. The AOX1 promoter can be used to drive the expression of genes to which it is operably linked only in the presence of methanol. Accordingly, POSITAs understood that one could engineer a gene of interest to be under the control of the AOX1 promoter in a recombinant *P. pastoris* expression system, and essentially turn on expression by adding methanol to the growth medium. Even in the absence of methanol, however, the AOX1 promoter is not fully shut “off”. EX-1036(Bawa), 1 (describing “leaky expression” from the AOX1 promoter); EX-1006(Lin-Cereghino), 893.

74. The Mxr1 transcription factor binds to the AOX1 promoter, as well as one or more other transcription regulatory elements, to begin transcription. EX-1017(Hartner 2008), 13 (“Mxr1p not only binds the AOX1 promoter but also can regulate at least one additional *P. pastoris* TF protein additionally involved in regulation of the AOX1 promoter”). Mxr1 is crucial to inducing transcription

controlled by the AOX1 promoter—in fact, “[c]ells lacking MXR1 function cannot induce the AOX1 promoter at all” and thereby cannot express any gene operably linked to the AOX1 promoter. EX-1017(Hartner 2008), 13.

75. Other promoters such as “the constitutively active GAP (glyceraldehyde-3- phosphate dehydrogenase) promoter” are also effective for recombinant protein production. EX-1021(Sreekrishna), 7. The GAP promoter “can be produced more straightforwardly” and “the promoter strength is quite high.” EX-1021(Sreekrishna), 7. I discuss other known promoters below with respect to the Vogl review.

76. The vector illustrated below shows an exemplary construct from *P. pastoris* as reported in the literature, which includes an AOX1 promoter (“PROMOTER”) that controls the expression of the AOX1 gene, engineered for insertion and expression of a gene of interest (shown below as “YFG”):

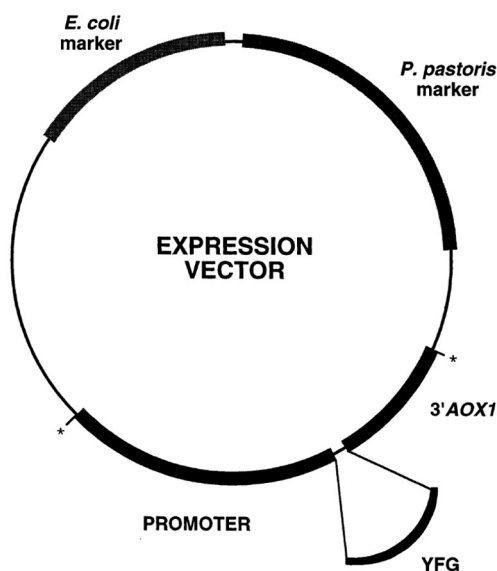


Fig. 3. General diagram of a *P. pastoris* expression vector. YFG, 'Your Favorite Gene'; * sites for cassette amplification.

EX-1015(Lin-Cereghino 2000), 47-48;

77. Downstream of the promoter a gene coding for a protein of interest can be inserted. The vector is then transformed into *P. pastoris* cells. This results in an AOX1 promoter operably linked to expression of the gene of interest, such that the AOX1 promoter controls transcription of the gene. EX-1015(Lin-Cereghino 2000), 47-48.

D. Carbon Source-Mediated Protein Expression

78. In methylotrophic yeast such as *P. pastoris*, the transcription of “methanol-inducible” genes is regulated by the type of carbon source, *e.g.*, glucose, glycerol, and methanol, in the growth medium. EX-1005(Vogl), 386-87; EX-1015(Lin-Cereghino 2000), 46-47; EX-1029(Ahmad), 5302. Vogl explains, for example, that

[c]ertain genes of the methanol utilization (MUT) pathway are completely repressed under growth on repressing carbon sources (e.g. glucose), but are strongly induced on methanol, constituting up to 20–30% of total intracellular protein [15,16]. This tight regulation is exerted on the transcriptional level, making MUT promoters ideal tools for heterologous protein production [17]. Therefore also most of the published knowledge about the regulation of *P. pastoris* promoters is focused on the most commonly used classic *P. pastoris* promoters, the promoters of the MUT pathway gene alcohol oxidase 1 (PAOX1), its rarely used homolog PAOX2, and also the glycolysis gene glyceraldehyde 3-phosphate dehydrogenase (PGAP), which was described as a constitutive promoter in the early days of development. EX-1005(Vogl), 386. In wild-type *P. pastoris* cells with methanol as the only carbon source in the medium, for example, the methanol-inducible AOX1 promoter “drive[s] the production of enzymes” needed for methanol metabolism by “produc[ing] extremely high levels” of the transcripts of those enzymes. EX-1018(De Schutter), 564. However, glucose or glycerol “repress[es] transcription” (*i.e.*, minimal or no transcription) from the AOX1 promoter, with “derepression” upon “carbon starvation” (*i.e.*, slightly higher, but still low levels of transcription). EX-1017(Hartner 2008), 1; EX-1005(Vogl), 387; EX-1015(Lin-Cereghino), 47, 51-52; EX-1029(Ahmad), 5302.

79. When the AOX1 promoter is used in *P. pastoris*, for example, the expression of a gene of interest is subject to “tight regulation”, making it an “ideal

tool[] for heterologous protein production” in *P. pastoris*. EX-1005(Vogl), 386. Some recombinant proteins, however, can inhibit the growth of *P. pastoris*. To counter this problem, one common approach involves expressing the recombinant protein under the control of the AOX1 promoter and selective carbon sources, taking advantage of the fact that the AOX1 promoter is a “strong promoter” whose activity is “directly controllable by simply changing the inexpensive carbon source.” EX-1005(Vogl), 386-87; EX-1015(Lin-Cereghino), 51-52; EX-1029(Ahmad), 5302.

80. Methanol-inducible promoters can promote high levels of recombinant protein expression. EX-1005(Vogl), 386-87; 50-51. Several factors impact the yield of the recombinant protein. *Id.* POSITAs must also take into account the total number of the host cells. EX-1021(Sreekrishna), 6-8; EX-1022(Zhang), 148-49. For example, high expression of certain recombinant proteins may interfere with the growth of the host cells. EX-1021(Sreekrishna), 6-8; EX-1022(Zhang), 148. To take advantage of the strength and inducible nature of methanol-inducible promoters, and produce large amounts of a recombinant protein without interfering with growth of the host cells, POSITAs select the carbon sources in the growth medium. Once the cells have grown to significant numbers, methanol-inducible promoters can be turned on by exhausting the glucose or glycerol and adding methanol to the medium, resulting in high levels of recombinant protein expression from a large number of cells. For example, though the AOX1 promoter is known to be leaky, it can in large

part be turned “on” by the addition of methanol to the growth medium. EX-1021(Sreekrishna), 7-8; EX-1022(Zhang), 149; EX-1005(Vogl), 386-87. In the absence of methanol, but the presence of glucose or glycerol, the AOX1 promoter is turned “off.” This allows *P. pastoris* to grow to high cell densities, with minimal or no recombinant protein production that would otherwise inhibit growth. EX-1021(Sreekrishna), 7-8; EX-1022(Zhang), 149; EX-1005(Vogl), 386-87. After the cells have grown to the desired cell density, the AOX1 promoter is turned “on” by depleting the glucose or glycerol and then adding methanol to the medium. This will greatly induce AOX1 promoter-driven recombinant protein expression from the high cell density that amassed in glucose- or glycerol-based medium. EX-1021(Sreekrishna), 7-8; EX-1022(Zhang), 149, EX-1005(Vogl), 386-87.

81. In this way, POSITAs can use different carbon sources to regulate transcription and to control recombinant protein expression in methylotrophic yeast. For example, using carbon sources to essentially turn the AOX1 promoter “on” or “off” has allowed POSITAs to control the timing and yield of recombinant protein expression. EX-1005(Vogl), 386-87; EX-1015(Lin Cereghino), 51; EX-1029(Ahmad), 5302. Even recombinant proteins that are “toxic” to *P. pastoris* have been produced using the AOX1 promoter and a two-stage growth-induction approach. EX-1029(Ahmad), 5302; EX-1021(Sreekrishna), 7.

82. The AOX1 promoter is regarded as the “the most commonly used promoter for heterologous protein expression in *P. pastoris*.” EX-1005(Vogl), 386. The AOX1 promoter has been “well-characterized,” is available in commercial kits, and is “employed in most studies and applications” of the *P. pastoris* expression system. EX-1017(Hartner 2008), 1.

E. Mxr1p Regulation of AOX1 Promoter

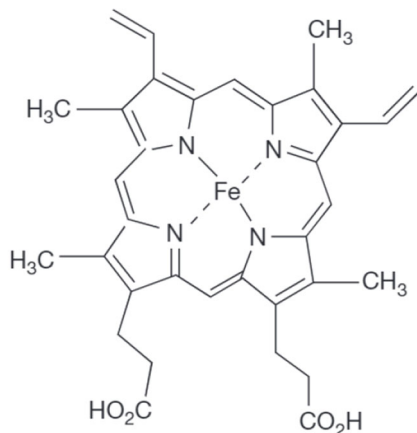
83. The AOX1 promoter is regulated by a transcriptional activator called methanol expression regulator 1 (“Mxr1”), sometimes also referred to as Mxr1 protein (“Mxr1p”). Mxr1 is “an important transcription factor” and “global regulator” of genes involved in methanol utilization in *P. pastoris*. EX-1026(Parua), 282, 288; EX-1019(Kranthi), 460; EX-1016(Hartner), 13. Mxr1 binds to the AOX1 promoter to activate transcription, and in fact “[c]ells lacking *MXR1* function cannot induce the *AOX1* promoter.” EX-1017(Hartner 2008), 2, 13; EX-1026(Parua), 282; EX-1016(Hartner), 13.

84. In wild-type cells, Mxr1 is produced constitutively at a low levels. Its activity, however, is regulated by the carbon source present in the growth medium. EX-1016(Hartner), 13; EX-1026(Parua), 282-284, 287-88. For example, Vogl explains that “MXR1 seemed to be regulated post-translationally by the subcellular localization, as it was constitutively transcribed at low levels and found to localize to the cytosol in cells grown on glucose but to migrate to the nucleus upon shift to

methanol or oleic acid. However, also when cells were grown on glycerol or ethanol Mxr1p was localized in the nucleus, while AOX1 expression was still repressed on these carbon sources. Therefore regulation of MXR1 cannot solely rely on the subcellular localization of the Mxr1p [28].” EX-1005(Vogl), 390. Increasing expression of Mxr1p in *P. pastoris* by, for example, “controlling the expression of Mxr1 [by a strong promoter] or by increasing the copy number of the gene encoding Mxr1,” has also resulted in “improved expression” of proteins under a promoter that is activated by Mxr1. EX-1024(Tsutsumi), [0075], [0137]-[0145].

F. Expression of Heme-Containing Proteins

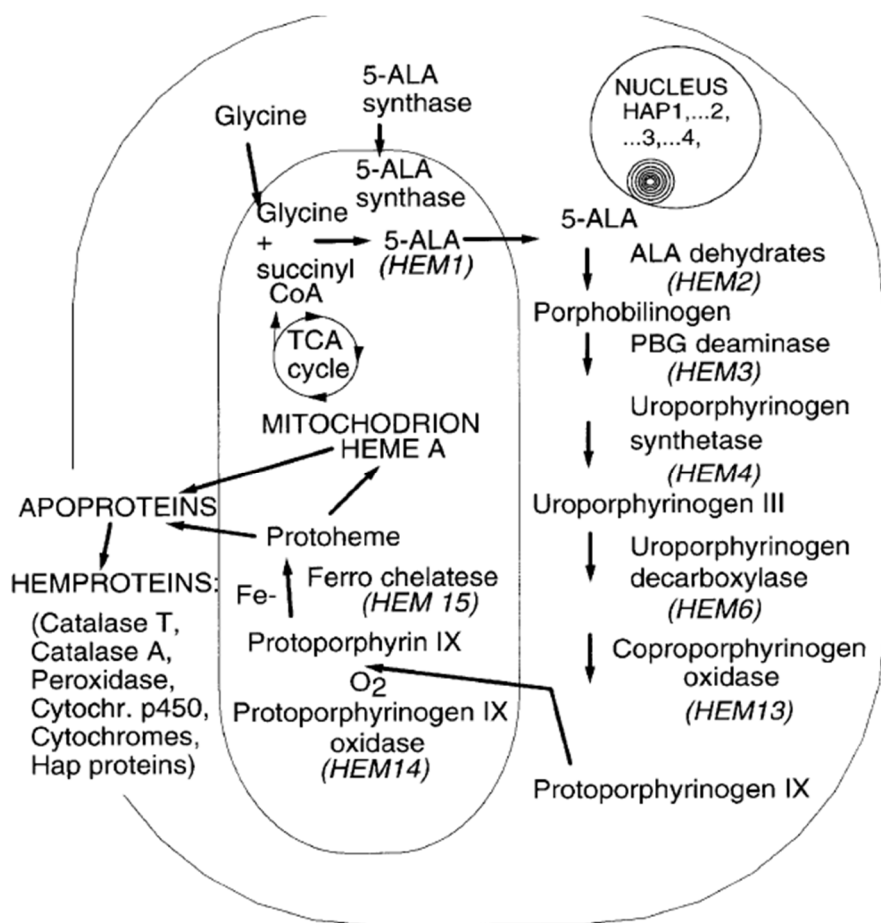
85. Microbial rDNA gene expression systems are employed routinely to produce an important class of proteins known as heme-containing proteins, or hemoproteins. EX-1008(Mattoon); EX-1009(Elrod); EX-1010(Crane); EX-1011(Liu); EX-1031(Krainer). Heme-containing proteins are a group of proteins that contain heme, which is an organic compound (a porphyrin ring) that binds iron, as shown below. EX-1008(Mattoon), at 3-4.



86. Heme plays multiple roles in cellular processes including oxygen transport and storage, electron transfer, and drug metabolism. EX-1008(Mattoon), 1:39-48; EX-1010(Crane), [0021]; EX-1011(Liu), 9; EX-1014(Garrocho), 78-79; EX-1037(Ferreira), 539-40. Heme-containing proteins include hemoglobin, myoglobin, leghemoglobin (a plant hemoglobin), catalases, peroxidases, and cytochromes. EX-1010(Crane), [0021]; EX-1014(Garrocho), 78-79.

87. Typically, cells make heme through a multi-step biosynthetic pathway, involving enzymes that catalyze individual steps. In this pathway, a series of biosynthetic enzymes are needed to process heme into a form that can be incorporated into heme-containing proteins in a functional manner. The enzymes involved in heme biosynthesis are conventionally known as: HEM1 (ALA synthase); HEM2 (ALA dehydratase); HEM3 (porphobilinogen, or PBG, deaminase); HEM4 (uroporphyrinogen, or UPG, synthase); HEM6 or HEM12 (uroporphyrinogen, or UPG, decarboxylase); HEM13 (coproporphyrinogen, or CPG, oxidase); HEM14

(protoporphyrinogen, or PPG, oxidase); and HEM15 (ferrochelatase). EX-1008(Mattoon), 2:65-67, Fig. 1; EX-1009(Elrod), 8:1-9:4; EX-1011(Liu), 10-11; EX-1037(Ferreira), 539-40. The below figure illustrates an exemplary heme biosynthetic pathway:

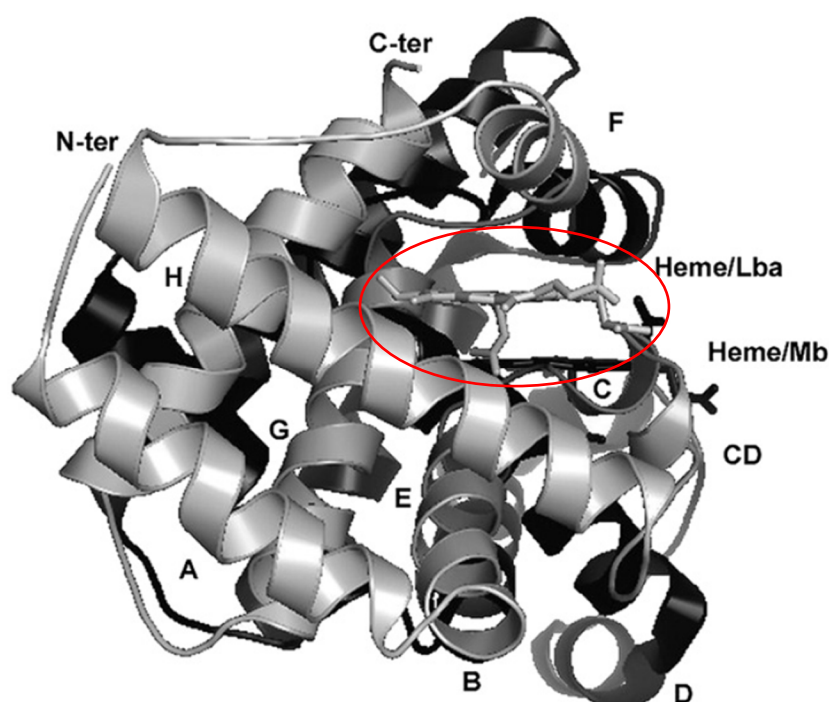


EX-1008(Mattoon), Fig. 1; *accord* EX-1011(Liu), 11.

88. This is a multi-step biosynthetic pathway, involving multiple enzymes, each of which catalyzes a step of heme biosynthesis. EX-1037(Ferreira), 539-40. Before 2015, one strategy for hemoprotein production was co-express hemoprotein and enzymes involved in heme biosynthesis in host cells. EX-1008(Mattoon), 2:17-

26; EX-1010(Crane), [0020], EX-1011(Liu), 9. This strategy ensures that the appropriate amount of heme is produced by the host cells to bind the recombinant heme-binding apoprotein produced resulting in the hemoprotein.

89. An exemplary 3-D heme-containing protein structure (with overlays of the soybean (leghemoglobin Lba) and sperm whale (Mb)) is shown below. The heme molecule is highlighted by a red circle. The letters refer to different segments of the protein. Also annotated are the beginning (N-ter) and end (C-ter) of the protein. EX-1014(Garraocho), 78-79.



VII. SUMMARY OF THE PRIOR ART

A. Mattoon

90. Mattoon teaches “[a] method for increasing the production” of hemoproteins. EX-1008(Mattoon), Abstract. Mattoon explains that there was a considerable interest in producing “cell-free hemoglobin as a substitute” for blood, and “[m]any commercial ventures [we]re working to enhance the biosynthesis of heme protein[s]” in “new strains” of yeast. EX-1008(Mattoon), 1:14-30.

91. Mattoon explains that “[f]or efficient industrial production of hemoproteins, appropriate ratios of heme and apoproteins must be simultaneously produced in a host cell.” EX-1008(Mattoon), 3:21-23. To accomplish this, Mattoon teaches “a method to produce hemoproteins by transforming a host cell with a nucleic acid sequence encoding a rate-limiting *enzyme in a heme biosynthetic pathway*” (e.g., *HEM2*), “coupled with the expression of a gene encoding a *heme-binding agent*” (e.g., heme-binding apoprotein), to “prevent[] undesired feedback inhibition that would otherwise be caused by excess concentrations of free heme.” EX-1008(Mattoon), 2:9-30. Mattoon also teaches that “[i]t is preferable to have an adequate supply of heme present while a cell is expressing a particular apoprotein, so as to provide an adequate amount of the constituents required to form desired hemoproteins, thereby ending up with purer materials.” EX-1008(Mattoon), 6:3-8, 6:27-38.

92. According to Mattoon, “[p]referred host cells are eukaryotic,” “[i]n particular, yeast of the genus *Pichia*, and *even more preferably, Pichia pastoris*.” EX-1008(Mattoon), 4:29-40. Mattoon teaches that vectors used to transform host cells are “controllable by *inducible or constitutive promoters*” that are “operatively linked” to the nucleic acid sequences encoding the proteins of interest. EX-1008(Mattoon), 2:41-44, 5:51-59, 6:9-63. As an example, Mattoon discloses increased production of catalase, a hemoprotein, by co-expressing the catalase apoprotein and HEM2. EX-1008(Mattoon), 7:26-8:10.

B. Vogl

93. I have reviewed an article authored by Vogl and Glieder, titled *Regulation of Pichia pastoris promoters and its consequences for protein production*, published in volume 30 of New Biotechnology (pages 385-404) in May 2013 (“Vogl”) (EX-1005). As a person of ordinary skill in the art, I am familiar with the journal “New Biotechnology”, which is a highly-reputable scientific journal reporting research in the field of biotechnology and recombinant protein expression systems, as well as its publisher, Elsevier. Prior to 2015, a POSITA would have considered the publication date printed on the article itself to be accurate. Based on the date appearing in the article, it is my opinion that the Vogl reference was published in and available to a POSITA exercising reasonable diligence on the date of publication, if not before. Accordingly, I understand that Vogl is considered prior

art to the '492 patent under (post-AIA) 35 U.S.C. § 102(a)(1) as it was published in 2013.

94. Vogl reviews prior publications on *P. pastoris* promoters and transcription factors, and “their application to improve heterologous protein production.” EX-1005(Vogl), 386. This includes reviewing the promoter of the *AOXI* gene—“the most commonly used” promoter—and its regulation. EX-1005(Vogl), 386-92.

95. Vogl teaches the effect of carbon source on the *AOXI* promoter, explaining that the *AOXI* promoter is “repressed” when *P. pastoris* is grown on a “repressing carbon source,” such as glucose or glycerol, and strongly induced when cells are grown on methanol. EX-1005(Vogl), 386. Vogl explains that this is a useful tool “for heterologous protein expression in *P. pastoris*,” as it allows “cell growth to high densities on a repressing carbon source without heterologous protein expression hampering the cellular metabolism.” EX-1005(Vogl), 386. Vogl further explains that heterologous protein expression under the control of the *AOXI* promoter often involves “three regulatory states”:

- repression in the presence of a repressing carbon source such as glucose or glycerol, which allows the growth of yeast cells to higher densities;

- derepression when the repressing carbon source is depleted or at low concentrations, with the *AOX1* promoter's activity at **2-4%** of the induced levels; and
- induction when methanol is added to the derepressed system leading to strong transcription driven by the *AOX1* promoter and heterologous protein expression.

EX-1005(Vogl), 387.

96. Vogl also provides an overview of other promoters, including constitutive promoters of the *GAP* and *TEF* genes. EX-1005(Vogl), 392-95. Vogl explains that promoters have different strengths. EX-1005(Vogl), 392-95. Vogl further explains that the *GAP* and *AOX1* promoters are both strong that “provide similar expression levels under the respective optimal growth conditions.” EX-1005(Vogl), 392. Vogl explains, however, that “the strong expression from the promoters of the *GAP* and *AOX1* genes is not always the most favorable condition for heterologous protein production”; rather, “a weaker expression level can be more desirable” in some cases. EX-1005(Vogl), 392. Weaker promoters such as the promoters of the *PEX8* gene (inducible) and *YPT1* gene (constitutive) are “alternatives in case [AOX1 or GAP promoter] driven expression is too strong.” EX-1005(Vogl), 393. In Table 1, Vogl lists over 20 promoters that can be used to

provide varying levels of protein expression in *P. pastoris* as desired by POSITAs. EX-1005(Vogl), 393.

97. Vogl explains that methanol-inducible promoters are regulated by the transcriptional activator Mxr1p, and that Mxr1p binds to specific sites of methanol-inducible promoters (such as the *AOX1*, *DAS*, and *PEX8* promoters) to activate transcription. EX-1005(Vogl), 390-91 (citing EX-1019(Kranthi) and EX-1023(Kranthi 2010)).

98. Vogl reviews several prior studies involving increased expression of Mxr1p in *P. pastoris*. EX-1005(Vogl), 391, 394. In one study, Takagi co-expressed (1) Mxr1p under the strong constitutive *GAP* promoter and (2) a phytase under the *AOX1* promoter regulated by Mxr1 in *P. pastoris* cells grown on glucose, which “resulted in a **7.6-fold** increased activity of the phytase expressed from P_{AOX1} compared to the non *MXR1* coexpressing strain.” EX-1005(Vogl), 391 (citing EX-1007(Takagi)); EX-1007(Takagi), 12:43-44, 14:40-41, 17:30-18:40. Vogl notes that this “7.6-fold increase might still account only for a low absolute expression level, as P_{AOX1} is increased ~1000-fold from glucose to methanol.” EX-1005(Vogl), 391. However, Takagi is described as a method for producing exogenous protein with “no addition of methanol.” EX-1007(Takagi), Abstract, 2:4-5. Vogl observes that the results of Takagi “oppose previous findings by Lin-Cereghino *et al.* [EX-1006], where overexpression of *MXR1* under control of P_{AOX1} caused a growth defect [of *P.*

pastoris cells grown] on methanol.” EX-1005(Vogl), 391 (citing EX-1006(Lin-Cereghino)).

99. Vogl also reviews Tsutsumi, another study of Mxr1, which I discuss above. EX-1005(Vogl), 394 (citing EX-1024(Tsutsumi)).

C. Crane

100. I have reviewed U.S. Patent Application Publication No. 2011/0287467, which was published on November 24, 2011 and names as inventors Brian Crane and Jawahar Sudhamsu (“Crane”). EX-1010(Crane). I understand that Crane is considered prior art to the ’492 patent under (post-AIA) 35 U.S.C. § 102(a)(1) as it was published in 2011.

101. Crane teaches “methods of producing recombinant functional heme-binding proteins with complete heme incorporation” by “co-expressing a recombinant *heme-binding protein* and [a] recombinant *ferrochelatase protein*,” a heme biosynthetic enzyme. EX-1010(Crane), Abstract, [0009]. Crane teaches that heme-containing proteins, which Crane calls heme-binding proteins, include “hemoglobin, myoglobin, [and] *legghemoglobin*.” EX-1010(Crane), [0021]. Crane notes that “[y]east strains and yeast-derived vectors are used commonly” for heterologous protein expression and “*Pichia pastoris* expression systems[] may be used to co-express a recombinant heme-binding protein of interest with ferrochelatase.” EX-1010(Crane), [0043]-[0044].

102. Crane describes expression systems with “one or more expression constructs encoding a recombinant heme-binding protein and a recombinant ferrochelatase,” commonly known as HEM15. EX-1010(Crane), [0056]; *supra* §III.H. Crane teaches that “[t]he protein-encoding nucleic acid sequence is cloned into an expression vector,” which includes “*promoters*[] that can be *operably linked* to the desired protein-encoding nucleic acid molecule to cause the expression of such nucleic acid molecule in the host cell.” EX-1010(Crane), [0032]-[0033], [0047].

D. Lin-Cereghino

103. I have reviewed an article authored by Lin-Cereghino et al., titled *Mxr1p, A Key Regulator of the Methanol Utilization Pathway and Peroxisomal Genes in Pichia pastoris*, published in Volume 26, Number 3 of the journal *Molecular and Cellular Biology* (pages 883-97) in February 2006 (“Lin-Cereghino_2006”) EX-1006(Lin-Cereghino). As a person of ordinary skill in the art, I am familiar with the journal “Molecular and Cellular Biology”, which is a highly-reputable scientific journal reporting research in the field of biotechnology and recombinant protein expression systems, as well as its publisher, Taylor & Francis. Prior to 2015, a POSITA would have considered the publication date printed on the face of a journal or article itself to be the publication date. Based on the face of the article, it is my opinion that the Lin-Cereghino reference was published in and available to a POSITA exercising reasonable diligence on the date

of publication, if not before. Accordingly, I understand that Lin-Cereghino is considered prior art to the '492 patent under (post-AIA) 35 U.S.C. § 102(a)(1), as it was published in 2006.

104. Lin-Cereghino is one of the first reports that characterized Mxr1 (which it refers to as “Mxr1p”) as a transcriptional activator of methanol-inducible genes in *P. pastoris*. EX-1006(Lin-Cereghino), 883. For example, as shown in Lin-Cereghino, the *MXR1* gene was constitutively expressed at low levels in either glucose- or methanol-grown *P. pastoris* cells. EX-1006(Lin-Cereghino), 890. Lin-Cereghino also describes *P. pastoris* strains with increased expression of Mxr1p under control of either promoter of the *AOX1* gene (a strong methanol-inducible promoter) or a promoter of the *PEX8* gene (a weaker methanol-inducible promoter). EX-1006(Lin-Cereghino), 884-86.

105. Lin-Cereghino reported that the yeast strains that produced Mxr1p under the weaker methanol-inducible *PEX8* promoter (pGC217) were able to grow on methanol and produced functional Mxr1p. EX-1006(Lin-Cereghino), 893. Lin-Cereghino screened for pGC217 strains with higher copy numbers of the *P_{PEX8}-MXR1* construct incorporated in the yeast genome and identified a “multicopy strain that grew slowly on methanol” but “contained relatively high levels of the product.” EX-1006(Lin-Cereghino), 893.

VIII. SUMMARY OF OPINIONS

106. In my opinion, claims 1-14 are unpatentable based on the following grounds:

No.	Claims	Ground for Challenge
1A	1, 4-11	§103: Mattoon and Vogl
1B	2-3, 12-13	§103: Mattoon, Vogl, and Crane
1C	14	§103: Mattoon, Vogl, and Lin-Cereghino
2A	1-13	§103: Crane and Vogl
2B	14	§103: Crane, Vogl, and Lin-Cereghino

IX. CLAIM CONSTRUCTION

107. In my opinion, the '492 patent's claims need no construction for purposes of this proceeding. Therefore, they should be given their plain and ordinary meaning.

X. GROUNDS 1A-1C: MATTOON, VOGL, CRANE, LIN-CEREGHINO

A. Overview

108. In my opinion, Claims 1-14 are obvious over Mattoon in view of Vogl (Claims 1, 4-11), Mattoon in view of Vogl and Crane (Claims 2-3, 12-13), and Mattoon in view of Vogl and Lin-Cereghino (Claim 14).

109. As I discuss below, Mattoon, Vogl, Crane, and Lin-Cereghino are each analogous art to the '492 patent. The '492 patent describes its purported invention as follows:

This disclosure describes the use of *P. pastoris* strains that overexpress the transcriptional activator, Mxr1, from the AOX1 promoter to increase expression of transgenes that also are expressed from the AOX1 promoter, which significantly improves the recombinant production of one or more proteins. In addition, expression of Mxr1 from the AOX1 promoter creates a positive feedback loop that allows for expression of other transgenes from the AOX1 promoter in the absence of methanol, the normally obligate inducer, when repressing carbon sources are depleted. Expression of Mxr1 results in a significant increase in the amount of protein produced.

EX-1001, 1:37-48.

110. In its written description, the '492 patent explains that “it is desirable to place the exogenous nucleic acid (i.e., the transcriptional activator) under control of a promoter that is inducible.” EX-1001, 4:45-47. The '492 patent goes on to explain that “[m]ethanol inducible promoters are known in the art,” with the AOX1 gene from *P. pastoris*, or a part thereof, being one that is “commonly used.” EX-1001, 4:56-60.

111. Mattoon, as discussed below, discloses nearly all limitations of claim 1 of the '492 except “a Mxr1 transcriptional activator sequence from *P. pastoris*.” Mattoon explains that there was a considerable interest in producing “cell-free hemoglobin as a substitute” for blood, and “[m]any commercial ventures [we]re working to enhance the biosynthesis of heme protein[s]” in “new strains” of yeast. EX-1008(Mattoon), 1:14-30. Mattoon notes that production of heme-

containing proteins in yeast strains had “proven difficult due to the lack of simultaneous abundant amounts of both apoproteins and heme” in the cells. EX-1008(Mattoon), 1:34-38.

112. To solve that problem, Mattoon teaches “a method to produce heme-containing proteins by transforming a host cell with a nucleic acid sequence encoding a rate-limiting *enzyme in a heme biosynthetic pathway*” (e.g., HEM2), “coupled with the expression of a gene encoding a *heme-binding agent*” (e.g., heme-binding apoprotein), to “prevent[] undesired feedback inhibition that would otherwise be caused by excess concentrations of free heme.” EX-1008(Mattoon), 2:9-30. Mattoon also teaches that “[i]t is preferable to have an adequate supply of heme present while a cell is expressing a particular apoprotein, so as to provide an adequate amount of the constituents required to form desired heme-containing proteins, thereby ending up with purer materials.” EX-1008(Mattoon), 6:3-8, 6:27-38.

113. Vogl discloses commonly used methanol-inducible promoters from *P. pastoris*, such as the AOX1 promoter, and Mxr1 as a key transcriptional activator that regulates such methanol-inducible promoters. Vogl explains that methylotrophic *P. pastoris* is a “wildly used host cell for heterologous protein production” due to having “favorable properties such as growth to high cell density and high capacities for protein.” EX-1005(Vogl), 385. Vogl further discloses rDNA

constructs for “tailor-made production strains” of methylotrophic *P. pastoris* for protein production. EX-1005(Vogl), 385. Vogl reports that *P. pastoris* and its methanol-inducible promoter AOX1 have been “extensively studied” and compiles over 170 studies related to *P. pastoris*, its promoters, and its transcription factors. EX-1005(Vogl), 385.

114. Vogl teaches that Mxr1 is “crucial for methanol-dependent []AOX1 induction.” EX-1005(Vogl), 389. Notably, when Mxr1 is deleted there are “highly reduced protein and mRNA levels of AOX1 and other genes involved in MUT [methanol induction]” when methanol is added EX-1005(Vogl), 390.

115. In addition, Vogl discloses that there are six binding sites for Mxr1 within the AOX1 promoter region. According to Vogl, overexpression of Mxr1 is required to enable Mxr1 to bind to all binding sites within the AOX1 promoter, to activate any expression of a gene of interest under its control. EX-1005(Vogl), 389. In some of these studies, Vogl examined overexpression of Mxr1 through “constitutive expression of MXR1 under control of P_{GAP} [GAP promoter].” EX-1005(Vogl), 391. Vogl reviewed the report that when Mxr1 was overexpressed using the GAP promoter, it resulted in a significant increase in the expression of a protein of interest under the control of the AOX1 promoter. The GAP promoter is a strong promoter with protein expression levels similar to those of AOX1. EX-1005(Vogl), 393.

116. Vogl teaches that “strong and controllable promoters are crucial for efficient heterologous protein production.” EX-1005(Vogl), 386. In addition to disclosing that *P. pastoris* “provides a strong, methanol inducible promoter of the alcohol oxidase 1 (AOX1) gene,” Vogl teaches, in Table 1 below, that *P. pastoris* expression systems can be constructed with over 20 different promoters:

TABLE 1

Promoters of *P. pastoris* genes used for heterologous protein expression. The approximate expression levels of alternative promoters relative to the classic promoters of AOX1 and GAP (listed at the top of the table) are given if they were provided in the respective reference. Promoters characterized by Stadlmayr *et al.* [93] were tested under different cultivation conditions with multiple reporter proteins, therefore the estimated expression range from weakest to highest is given

Gene name	Gene product	Regulation	Expression level	Reference
AOX1	Alcohol oxidase 1	Induced by methanol	Strong (naturally ~5% of mRNA and ~30% of total protein)	[3,21,27,29]
GAP	Glyceraldehyde 3-phosphate dehydrogenase	Constitutive	Strong (similar to P _{AOX1})	[68]
AOD	Alternative oxidase	Expression on glucose but not on methanol or upon glucose depletion if integrated in natural locus	~40% of P _{GAP}	[71]
AOX2	Alcohol oxidase 2	Induced by methanol	~5–10% of P _{AOX1}	[22,23]
DAS	Dihydroxyacetone synthase	Induced by methanol	Strong (similar to P _{AOX1})	[21,27,29,85]
ENO1	Enolase	Constitutive	~20–70% of P _{GAP}	[93]
FLD1	Formaldehyde dehydrogenase	Induced by methanol and methylamine	Strong (similar to P _{AOX1})	[76]
GPM1	Phosphoglycerate mutase	Constitutive	~15–40% of P _{GAP}	[93]
HSP82	Cytoplasmic chaperone (Hsp90 family)	Constitutive	~10–40% of P _{GAP}	[93]
ICL1	Isocitrate lyase	Derepression and ethanol induction	Not compared to P _{AOX1} or P _{GAP}	[83]
ILV5	Acetohydroxy acid isomeroreductase	Constitutive	~15% of P _{GAP}	[108]
KAR2	ER resident chaperone (also termed Bip)	Constitutive	~10–70% of P _{GAP}	[93]
KEX2	Endopeptidase involved in the processing of secreted proteins	Constitutive	~10% of P _{GAP}	[108]
PET9	ADP/ATP carrier of the inner mitochondrial membrane	Constitutive	~10–1700% of P _{GAP}	[93]
PEX8	Peroxisomal matrix protein	Induced by methanol or oleate	Weak	[3,79]
PGK1	Phosphoglycerate kinase	Constitutive	~10% of P _{GAP}	[92,93]
PHO89 or NSP	Sodium-coupled phosphate symporter	Induced by phosphate limitation	Strong (similar to P _{GAP})	[103,104]
SSA4	Heat shock protein	Constitutive	~10–25% of P _{GAP}	[93]
TEF1	Translation elongation factor 1 alpha	Constitutive and strong growth association	Strong (similar to P _{GAP})	[93,99,100]
THI11	Protein involved in thiamine biosynthesis	Completely repressed by thiamin	~70% of P _{GAP} on medium lacking thiamin	[93,111]
TPI1	Triose phosphate isomerase	Constitutive	~10–80% of P _{GAP}	[93]
YPT1	GTPase involved in secretion	Constitutive	Weak	[3,80]

EX-1005(Vogl), 393. Vogl, for example at Table 1, further teaches that *P. pastoris* can be genetically modified to express varying levels of a recombinant protein depending on which promoter is selected. EX-1005(Vogl), 393.

117. Crane teaches that its “invention relates to a purified preparation of recombinant functional heme-binding proteins.” EX-1010(Crane), [0060]. The methods of the Crane invention involve “co-expressing a recombinant heme-binding protein and recombinant ferrochelatase protein, or polypeptide thereof, under conditions effective for complete heme incorporation into the recombinantly produced heme binding protein, thereby producing a functional heme-binding protein.” EX-1010(Crane), [0009]; *see generally* EX-1010(Crane), [0009]-[0014]. Accordingly, “[t]he [Crane] invention is a straightforward and inexpensive method for high fidelity incorporation of heme into recombinantly overexpressed heme proteins,” as “[c]o-expression of just one native protein, ferrochelatase (FC), in the presence of δ -[amino levulinic acid (ALA)] is sufficient to achieve 100% heme incorporation into three unrelated h[e]me proteins derived from different organisms.” EX-1010(Crane), [0014]. Crane teaches generally applicable methods for various classes of heme-binding proteins, including hemoglobins such as leghemoglobin. EX-1010(Crane) , [0021]. According to the specification, “these proteins are completely functional and are more suitable for research, clinical, and commercial applications.” EX-1010(Crane) *Id.*, [0024].

118. Lin-Cereghino describes Mxr1 as a transcriptional activator for methanol-inducible genes in *P. pastoris*. EX-1006(Lin-Cereghino), 883. Lin-Cereghino reports that in *P. pastoris* most foreign genes have been expressed using the AOX1 promoter in cells grown on methanol. EX-1006(Lin-Cereghino), 883. Lin-Cereghino notes that “derepressed (carbon-starved) cells display levels of *AOX1* transcriptional activity that are approximately 2% of that seen on methanol, whereas methanol-grown cells display activity that is more than 1,000-fold higher than that observed in fully repressed (glucose-grown) cells.” EX-1006(Lin-Cereghino), 884.

119. Lin-Cereghino describes experiments that characterized Mxr1. EX-1006(Lin-Cereghino), 884-94. For example, the *MXR1* gene is constitutively expressed at low levels in either glucose- or methanol-grown *P. pastoris* cells. EX-1006(Lin-Cereghino), 890. Mxr1 was “mostly cytoplasmic in glucose-grown cells, but it was mostly localized” in the nucleus in methanol-grown cells, and it was also localized to the nucleus in glycerol-grown cells. EX-1006(Lin-Cereghino), 893-94.

120. Lin-Cereghino also describes *P. pastoris* strains with increased expression of Mxr1 under the control of either the AOX1 promoter (a strong methanol-regulated promoter) or the PEX8 promoter (a weaker methanol-regulated promoter). EX-1006(Lin-Cereghino), 885. The yeast strains were tested for their ability to grow on a medium containing 0.5% methanol as the sole carbon source. The authors interpreted the data as showing that when Mxr1 is expressed at “high

levels” under the control of the strong methanol-regulated AOX1 promoter, the Mxr1 is “[a]pparently ... toxic” because the authors did not observe growth on medium containing methanol under the conditions described. In contrast, when Mxr1 is expressed under the control of a methanol-regulated PEX8 promoter, which is “not nearly as strongly induced or repressed as AOX1,” the cells were able to grow on methanol and produced functional Mxr1. EX-1006(Lin-Cereghino), 884, 886, 893.

B. Ground 1A: Mattoon and Vogl

1. Claim 1 (Mattoon and Vogl)

(a) Claim 1[pre]: A methylotrophic *Pichia* yeast cell comprising

121. In my opinion, this claim element is obvious over Mattoon.

122. Mattoon teaches that *P. pastoris* yeast cells are the most preferred host cells for expressing recombinant heme-containing proteins. EX-1008(Mattoon), 4:32-37, 9:20-10:4. Consistent with Mattoon’s teaching, Vogl also discloses that “methylotrophic yeast *Pichia pastoris* is a widely used host for heterologous protein production” because it has “favorable properties such as growth to high cell density and high capacities for protein.” EX-1005(Vogl), 385.

(b) Claim 1[a]: ... a nucleic acid molecule encoding a heme-containing protein operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris* ...

123. In my opinion, this claim element is obvious over Mattoon in view of Vogl.

124. Mattoon discloses “transforming yeast cells with CTT1 [the gene encoding catalase T, a cytoplasmic heme-containing protein] to produce catalase T” in yeast. EX-1008(Mattoon), 7:37-41, 7:56-60. A POSITA would have understood that the yeast cells transformed with CTT1 contain “a nucleic acid molecule encoding a heme-containing protein” because they would encode for cytoplasmic heme-containing protein. More generally, Mattoon teaches increasing “production of heme, *to increase the production of hemoproteins such as hemoglobin, myoglobin, various cytochromes and hemoprotein enzymes such as catalase,*” which, as a POSITA would recognize, would each be encoded by a nucleic acid molecule encoding a heme-containing protein. EX-1008(Mattoon), 6:45-63. As I discuss further below with respect to limitation 1[b], Mattoon teaches methods to improve the yield of the heme-containing protein of interest. EX-1008(Mattoon), 6:45-63.

125. Mattoon further motivates a POSITA to utilize a nucleic acid molecule encoding a heme-containing protein operably linked to a promoter element from *P. pastoris* by teaching the importance of producing heme-containing proteins (e.g., for

therapeutic purposes). For example, Mattoon teaches that “proteins carrying a heme prosthetic group” are “[a]n important class of ... heterologous proteins produced in genetically engineered cells,” EX-1008(Mattoon), 1:13-16, and that “[m]any commercial ventures are working to enhance the biosynthesis of the heme protein family ... in yeast.” EX-1008(Mattoon), 1:17-22; 1:23-30 (discussing the “considerable current interest in the potential of cell-free hemoglobin as a substitute for red blood cells”).

126. Although Mattoon describes catalase overexpression in *S. cerevisiae*, it also expressly teaches that “[p]referred host cells” are “yeast of the genus *Pichia*, **and even more preferably, *Pichia pastoris*.**” EX-1008(Mattoon), 2:31-33, 4:32-37. Mattoon explains that “[a]lthough the yeast *Saccharomyces cerevisiae* can be used,” “**preferred** yeast strains include ***Pichia pastoris***” due to its “exceptionally high yields.” EX-1008(Mattoon), 9:20-10:4. A POSITA would have understood from this that *P. pastoris* is the preferred host for producing recombinant heme-containing proteins, especially a POSITA looking to produce high yields of the recombinant heme-containing protein. Additionally, a POSITA would have used *P. pastoris* as the host cell because it was “a highly successful system” for recombinant protein production and regarded by some scientists as “the most commonly used yeast species.” EX-1018(De Schutter), 561; EX-1005(Vogl), 386; EX-1015(Lin-Cereghino 2000), 45.

127. Further, Mattoon teaches that the nucleic acid sequences encoding the proteins of interest are “operatively linked” to promoters. In other words, the levels of expression of the genes-of-interest are “controllable by inducible or constitutive *promoters*.” EX-1008(Mattoon), 2:41-44, 5:51-59, 6:9-63. A POSITA would have understood from Mattoon that the expression levels of the genes encoding proteins of interest can be under the control of a promoter using the preferable *P. pastoris* host cell as detailed in the paragraph above. Thus Mattoon teaches encoding a protein that is “operably linked to a promoter element from *P. pastoris*.”

128. Mattoon does not disclose “a Mxr1 transcriptional activator sequence from *P. pastoris*.” As discussed below, however, a POSITA would have been motivated to combine Mattoon with Vogl to engineer *P. pastoris* with “a nucleic acid molecule encoding a heme-containing protein operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.”

129. Vogl teaches that methylotrophic *P. pastoris* expression systems are constructed for the “[e]xpression of the respective gene of interest (GOI) for recombinant protein production.” EX-1005(Vogl), 386. Vogl explains that Mxr1 plays an important role in the expression of proteins of interest as a “positive regulator of methanol inducible genes” in methylotrophic *P. pastoris*. EX-1005(Vogl), 387. Vogl teaches that “MXR1 was shown to be crucial for the

activation of numerous genes involved in MUT [methanol utilization pathway].”

EX-1005(Vogl), 390.

130. Vogl teaches that methylotrophic *P. pastoris* expression systems must have a Mxr1 transcriptional activator sequence because, without Mxr1, the methanol utilization pathway will not be induced. EX-1005(Vogl), 390 (“Deletion of *MXR1* in the wild type strain resulted in abolished growth on the peroxisomal substrates methanol and oleate. In conclusion, the *mxr1* deletion strain showed under methanol induction highly reduced protein and mRNA levels”). Indeed, at the time of Vogl’s publication, Mxr1 was a well-characterized transcriptional activator for methanol-inducible promoters from *P. pastoris*. EX-1005(Vogl), 390-91, 400.

131. The Vogl reference reviews a variety of *P. pastoris* promoters that were available to a POSITA for use in a recombinant nucleic acid molecule. I excerpt below Table 1 from Vogl, which describes “Promoters of *P. pastoris* genes used for heterologous protein expression” and their relative level of promoter strength. (“[A]pproximate expression levels of alternative promoters relative to the classic promoters of *AOX1* and *GAP* (listed at the top of the table) are given if they were provided in the respective reference.”). EX-1005(Vogl), 393. In Table 1, Vogl discloses the particular promoters available for *P. pastoris* expression systems and the expected recombinant protein secretion levels from each promoter:

TABLE 1

Promoters of *P. pastoris* genes used for heterologous protein expression. The approximate expression levels of alternative promoters relative to the classic promoters of *AOX1* and *GAP* (listed at the top of the table) are given if they were provided in the respective reference. Promoters characterized by Stadlmayr *et al.* [93] were tested under different cultivation conditions with multiple reporter proteins, therefore the estimated expression range from weakest to highest is given

Gene name	Gene product	Regulation	Expression level	Reference
<i>AOX1</i>	Alcohol oxidase 1	Induced by methanol	Strong (naturally ~5% of mRNA and ~30% of total protein)	[3,21,27,29]
<i>GAP</i>	Glyceraldehyde 3-phosphate dehydrogenase	Constitutive	Strong (similar to P_{AOX1})	[68]
<i>AOD</i>	Alternative oxidase	Expression on glucose but not on methanol or upon glucose depletion if integrated in natural locus	~40% of P_{GAP}	[71]
<i>AOX2</i>	Alcohol oxidase 2	Induced by methanol	~5–10% of P_{AOX1}	[22,23]
<i>DAS</i>	Dihydroxyacetone synthase	Induced by methanol	Strong (similar to P_{AOX1})	[21,27,29,85]
<i>ENO1</i>	Enolase	Constitutive	~20–70% of P_{GAP}	[93]
<i>FLD1</i>	Formaldehyde dehydrogenase	Induced by methanol and methylamine	Strong (similar to P_{AOX1})	[76]
<i>GPM1</i>	Phosphoglycerate mutase	Constitutive	~15–40% of P_{GAP}	[93]
<i>HSP82</i>	Cytoplasmic chaperone (Hsp90 family)	Constitutive	~10–40% of P_{GAP}	[93]
<i>ICL1</i>	Isocitrate lyase	Derepression and ethanol induction	Not compared to P_{AOX1} or P_{GAP}	[83]
<i>ILV5</i>	Acetohydroxy acid isomeroreductase	Constitutive	~15% of P_{GAP}	[108]
<i>KAR2</i>	ER resident chaperone (also termed Bip)	Constitutive	~10–70% of P_{GAP}	[93]
<i>KEX2</i>	Endopeptidase involved in the processing of secreted proteins	Constitutive	~10% of P_{GAP}	[108]
<i>PET9</i>	ADP/ATP carrier of the inner mitochondrial membrane	Constitutive	~10–1700% of P_{GAP}	[93]
<i>PEX8</i>	Peroxisomal matrix protein	Induced by methanol or oleate	Weak	[3,79]
<i>PGK1</i>	Phosphoglycerate kinase	Constitutive	~10% of P_{GAP}	[92,93]
<i>PHO89 or NSP</i>	Sodium-coupled phosphate symporter	Induced by phosphate limitation	Strong (similar to P_{GAP})	[103,104]
<i>SSA4</i>	Heat shock protein	Constitutive	~10–25% of P_{GAP}	[93]
<i>TEF1</i>	Translation elongation factor 1 alpha	Constitutive and strong growth association	Strong (similar to P_{GAP})	[93,99,100]
<i>THI11</i>	Protein involved in thiamine biosynthesis	Completely repressed by thiamin	~70% of P_{GAP} on medium lacking thiamin	[93,111]
<i>TPI1</i>	Triose phosphate isomerase	Constitutive	~10–80% of P_{GAP}	[93]
<i>YPT1</i>	GTPase involved in secretion	Constitutive	Weak	[3,80]

132. Prior to 2015, as taught in Vogl, the promoters for the *DAS* and *AOX1* genes (sometimes abbreviated as “ P_{AOX1} ” and “ P_{DAS} ” in the literature) were well known in the art as strong methanol-inducible promoters that result in high levels of recombinant protein expression upon methanol induction.

P_{AOX1} is a *remarkably strong* promoter and at the same time *directly controllable by simply changing the inexpensive carbon source*, it is the *most commonly used promoter* for heterologous protein expression in *P. pastoris*. The highest expression levels of heterologous proteins

in *P. pastoris* have been achieved with P_{AOX1} (up to 22 g/l intracellular and 15 g/l secreted protein) [25,26]. Concerning controlled bioreactor cultivation, the tight regulation of P_{AOX1} is used to enable cell growth to high densities on a repressing carbon source without heterologous protein expression hampering the cellular metabolism.

EX-1005(Vogl), 386.

P_{DAS} is a promoter from the MUT pathway showing a similar regulatory pattern and expression level as P_{AOX1} [27].

EX-1005(Vogl), 393. In contrast, use of the methanol-inducible promoter for the *PEX8* gene was observed to result in lower levels of expression of genes of interest.

The Vogl review explains, for example, that “ ***P_{PEX8} and P_{YPT1} are weak promoters and considered alternatives in case P_{AOX1} or P_{GAP} driven expression is too strong.***”

EX-1005(Vogl), 393. A POSITA would be motivated to choose a particular promoter among the various available options, for example, in order to achieve higher or lower levels of the heterologous gene sequence of interest (and the levels of protein it encodes).

133. Vogl reviews several prior studies involving increased expression of Mxr1p in *P. pastoris*. EX-1005(Vogl), 391, 394. In one study, Takagi co-expressed (1) Mxr1p under the strong constitutive *GAP* promoter and (2) a phytase under the *AOX1* promoter regulated by Mxr1 in *P. pastoris* cells grown on glucose, which “resulted in a **7.6-fold** increased activity of the phytase expressed from P_{AOX1}

compared to the non *MXR1* coexpressing strain.” EX-1005(Vogl), 391 (citing EX-1007(Takagi)); EX-1007(Takagi), 12:43-44, 14:40-41, 17:30-18:40.

134. **Motivation to Combine.** In my opinion, a POSITA designing *P. pastoris* to express a heme-containing protein would have naturally looked to Vogl’s teaching “to improve heterologous protein production” through constructs comprising promoters from *P. pastoris* and the transcriptional activator Mxr1. EX-1005(Vogl), 385-87; EX-1008(Mattoon), 2:41-44, 5:51-59, 6:9-63.

135. Mattoon teaches that vectors used to transform host cells are “**controllable by inducible or constitutive promoters**,” or “operatively linked” to “a suitable promoter.” EX-1008(Mattoon), 2:41-49, 4:65-5:10, 5:51-64, Fig. 4; *id.*, 6:18-23. Mattoon further teaches that “[i]t is **preferred to have promoters**, whether inducible or constitutive,” to control expression of genes encoding heme-containing protein **and** heme biosynthetic enzymes, so as to regulate and adjust the amounts of heme-binding apoprotein and heme produced by yeast cells. EX-1008(Mattoon), 8:27-32.

136. Vogl summarizes over twenty promoters that were reported in *P. pastoris* to regulate heterologous protein expression before 2013. EX-1005(Vogl), 393 (Table 1). For each promoter, Vogl also discloses their category (inducible or constitutive) and strength (e.g., strong or weak). EX-1005(Vogl), 393. As listed in Table 1, for example, Vogl teaches that strong constitutive promoters such as the

TEF1 and GAP promoters, or strong methanol-inducible promoters such as the AOX1 and DAS promoters, have been used in *P. pastoris* expression systems to provide “strong” levels of protein expression. EX-1005(Vogl), 392-93. Vogl explains that GAP and AOX1 promoters “provide similar expression levels under the respective optimal growth conditions.” EX-1005(Vogl), 392.

137. Vogl explains that methanol-inducible promoters are regulated by the transcriptional activator Mxr1 (Mxr1p), and that Mxr1 binds to specific sites of methanol-inducible promoters (such as the *AOX1*, *DAS*, and *PEX8* promoters) to activate transcription. EX-1005(Vogl), 390-91 (citing EX-1019(Kranthi) and EX-1023(Kranthi_2010)). Vogl teaches that, for example, “systematic analysis for Mxr1p binding sites” prior to 2015 revealed Mxr1 binding sites in, among others, “the promoters of dihydroxyacetone synthase (*DAS*) and peroxin 8 (*PEX8*).” EX-1005(Vogl), 390. Vogl also teaches that Mxr1 binds to six places in the *AOX1* promoter. EX-1005(Vogl), 390. Indeed, Vogl teaches that “[o]ne of the most favorable properties of *P. pastoris*” is its “methanol inducible promoters.” EX-1005(Vogl), 386. Vogl explains that because “P_{AOX1} is a remarkably strong promoter and at the same time directly controllable by simply changing the inexpensive carbon source, it is the **most commonly used promoter** for heterologous protein expression in *P. pastoris*.” EX-1005(Vogl), 386. A POSITA would understand that Mxr1 activates other promoters as well.

138. Accordingly, in view of Vogl, a POSITA would have incorporated Mxr1 in the nucleic acid construct of Mattoon to express a heme-containing protein in *P. pastoris*, for example, by expressing Mxr1 under the GAP promoter, to regulate AOX1 promoter-controlled co-expression of a heme-containing protein and heme biosynthetic enzymes. In particular, a POSITA would have been motivated to (1) select a promoter from *P. pastoris* such as the AOX1 promoter to control the expression of a heme-containing protein, thus providing “*a nucleic acid molecule encoding a heme-containing protein operably linked to a promoter element from P. pastoris*” and (2) include in the rDNA construct “*a Mxr1 transcriptional activator sequence from P. pastoris*” that is expressed under the control of a promoter such as GAP, where the expressed Mxr1 further regulates a methanol-inducible promoter such as that of the AOX1 promoter in *P. pastoris*. EX-1005(Vogl), 391.

139. ***Reasonable Expectation of Success.*** A POSITA would have reasonably expected success in combining Mattoon and Vogl to produce “a nucleic acid molecule encoding a heme-containing protein operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*” into methylotrophic *Pichia*. As discussed above, Vogl provides a comprehensive review of prior studies using promoters and transcription factors from *P. pastoris* to regulate recombinant protein production. EX-1005(Vogl), 385-

86. A POSITA would have reasonably expected success in following the well-known techniques disclosed in these prior studies to construct similar *P. pastoris* expression systems. Moreover, as expressly admitted by the '492 patent, incorporating the claimed nucleic acid molecule into *P. pastoris* yeast cells only requires routine techniques, known methods, and readily available materials for genetic engineering and recombinant protein production. *See supra* § IV.C.

(c) **Claim 1[b]: ... a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.**

140. In my opinion, the combination of Mattoon and Vogl renders this limitation obvious for largely the same reasons discussed above in Section X.B.1.b.

141. Mattoon teaches that to form stable heme-containing proteins, and to improve the yield and purity, yeast strains must co-express: (1) a gene encoding recombinant heme-containing protein; and (2) one or more genes encoding heme biosynthetic enzymes. EX-1008(Mattoon), 1:14-2:38. For example, Mattoon discloses the “simultaneous production of heme and heme binding agents (e.g., apoprotein)” in yeast cells. EX-1008(Mattoon), 2:22-26. Mattoon teaches balancing the amount of apoprotein produced by yeast cells with the amount of heme synthesized in the same cells, so that the apoprotein would readily bind heme to form stable heme-containing protein, thereby improving yield and purity. EX-

1008(Mattoon), 1:14-2:38. To achieve this end, Mattoon teaches yeast strains that co-express (1) a gene encoding recombinant apoprotein and (2) one or more genes encoding heme biosynthetic enzymes. EX-1008(Mattoon), 1:14-2:38.

142. Mattoon further discloses “transforming a host cell with a nucleic acid sequence encoding *a rate-limiting enzyme in a heme biosynthetic pathway*,” such as *HEM2*, which encodes an enzyme (*i.e.*, a polypeptide) involved in heme biosynthesis. EX-1008(Mattoon), 2:9-12, 2:27-30. Mattoon teaches the identity of these enzymes, and that they can be “manipulated to affect heme production”:

It is known that identified *enzymes in the heme biosynthetic pathway can be manipulated to affect heme production*, such enzymes including ALA synthase (encoded by *HEM1*), ALA dehydratase (encoded by *HEM2*), and PBG deaminase (encoded by *HEM3*). EX-1008(Mattoon), 1:54-58; 6:9-12 (“[T]he present invention therefore involves the use of yeast transformed with particular genes encoding enzymes that are involved in the production of ... heme and porphyrin.”). The cellular pathways involving these enzymes is also summarized in Figure 1 of Mattoon:

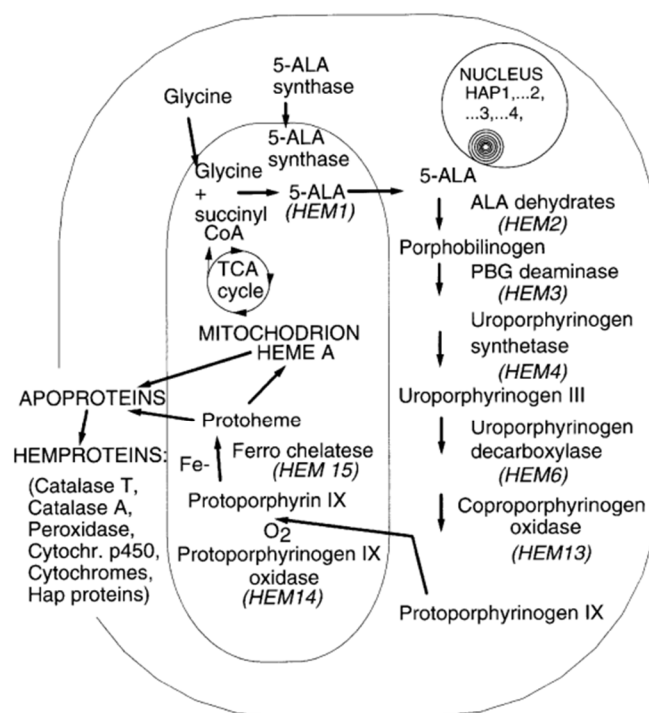


Fig. 1

143. Mattoon also discloses “double transformant yeast cells simultaneously harboring the multicopy plasmid pCCT1 and pHEM2,” and “double transformant yeast cultures simultaneously harboring the multicopy plasmid pCCT1 and one of the following multicopy plasmids: pHEM2, pHEM3, pHEM13, and pHEM15.” EX-1008(Mattoon), 7:63-65. A POSITA would have understood that the yeast cells transformed with HEM2, HEM3, HEM13, or HEM15 would comprise “a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis.” Additionally, Mattoon would motivate a POSITA to utilize a nucleic acid encoding at least one heterologous polypeptide involved in biosynthesis of an iron co-factor in the claimed recombinant nucleic acid molecule by teaching that “[t]he HEM2

over-expression system described above, when used in combination with hemoglobin expression plasmids, can be used to increase the production of hemoglobin and globin expression plasmids can be used for this purpose.” EX-1008 (Mattoon), 7:45-50.

144. A POSITA would be motivated to do so, as taught in Mattoon, in order to improve the yield of the heme-binding protein of interest. EX-1008(Mattoon), 6:45-63 (“By transforming cells with a HEM2-containing plasmid, the present inventors are able to ultimately increase the amount of PBG synthase in the cell, and thus the production of heme, *to increase the production of hemoproteins such as hemoglobin, myoglobin, various cytochromes and hemoprotein enzymes such as catalase.*”). A POSITA would also reasonably expect success doing so in light of the teachings of Mattoon and general knowledge in the art concerning recombinant protein expression.

145. As discussed in X.B.1.b, Mattoon also teaches vectors “controllable by inducible or constitutive promoters” that are “operatively linked” to the nucleic acid encoding proteins of interest and *P. pastoris* cells as preferred host cells. EX-1008(Mattoon), 2:41-44, 5:51-59, 6:9-63; 4:29-40. As previously detailed, a POSITA would have found it obvious in view of Mattoon and Vogl for “a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis” to be “operably linked to a promoter element from *P. pastoris.*” For example, as

explained above, Vogl teaches various *P. pastoris* promoters—e.g., AOX1, TEF1, GAP, PEX8—which were known in the art to be useful for expressing heterologous gene sequences of interest.

146. Mattoon does not disclose “a Mxr1 transcriptional activator sequence from *P. pastoris*.” For reasons discussed in X.B.1.b, however, a POSITA would have been motivated to combine Mattoon with Vogl to engineer *P. pastoris* comprising “a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.”

147. In particular, for reasons discussed in X.B.1.b, a POSITA would have incorporated Mxr1—i.e., the Mxr1 transcriptional activator sequence from *P. pastoris*—in the nucleic acid construct expressing at least one polypeptide involved in heme biosynthesis in *P. pastoris*. As summarized in Vogl, for example, the Takagi study teaches that when Mxr1p was expressed under the strong constitutive promoter of the *GAP* gene (sometimes rendered as P_{GAP}), this approach resulted in the production of a recombinant protein (separately under the control of the methanol-inducible promoter of the *AOX1* gene) that was 7.6-fold higher than that produced by yeast cells without the P_{GAP}-*MXR1* construct. EX-1005(Vogl), 391. In view of this desirable result, a POSITA would have been motivated to incorporate a nucleic acid encoding Mxr1 into the construct to regulate expression of

heterologous proteins that are engineered to be under the control of promoters regulated by Mxr1 binding (e.g., *AOX1*, *DHAS*, and *PEX8* promoters, which are derived from *P. pastoris*).

148. For the same reasons discussed in X.B.1.b, a POSITA would have been motivated to (1) select a promoter from *P. pastoris* such as the *AOX1* promoter to control the expression of at least one polypeptide involved in heme biosynthesis, thus providing “a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis operably linked to a promoter element from *P. pastoris*”; and (2) include in the same nucleic acid molecule “a Mxr1 transcriptional activator sequence from *P. pastoris*” that is expressed under the control of a promoter such as the GAP promoter, where the expressed Mxr1 further regulates a methanol-inducible promoter such as the *AOX1* promoter in *P. pastoris*. EX-1005(Vogl), 391.

149. As further discussed in X.A.1.b, a POSITA would have reasonably expected success in following the well-known techniques disclosed in Vogl and the studies it discusses to construct similar *P. pastoris* expression systems. Moreover, a POSITA would reasonably expect success in incorporating a heme-biosynthesis nucleic acid sequence in light of the teachings of Mattoon as well as the general knowledge in the art, as, e.g., Mattoon teaches methods and sequences for use in expressing heme-biosynthesis enzymes in expression vectors. *E.g.*, EX-

1008(Mattoon), 3:5-6 (“FIG. 4 shows a restriction map of a yeast plasmid for the overexpression of the HEM2 gene.”) (excerpted below).

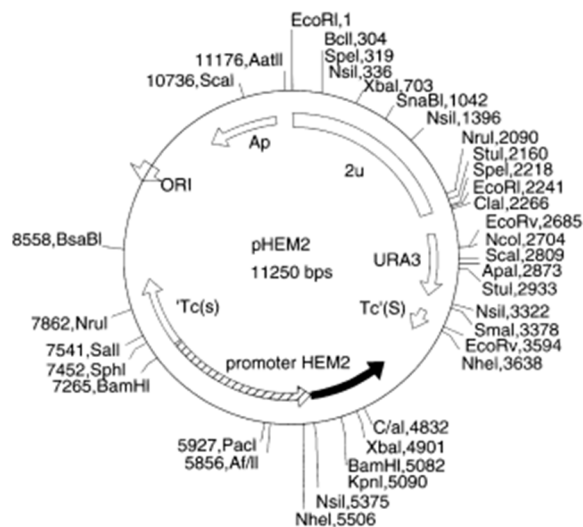


Fig. 4

150. Moreover, as expressly admitted by the '492 patent, incorporating the claimed nucleic acid molecule into *P. pastoris* yeast cells only requires routine techniques, known methods, and available materials for genetic engineering and recombinant protein production. *See supra* § IV.C.

2. Claim 4 (Mattoon and Vogl)

The yeast cell of claim 1, wherein the at least one polypeptide involved in heme biosynthesis is selected from the group consisting of ALA synthase, ALA dehydratase, porphobilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and/or ferrochelatase.

151. In my opinion, Mattoon renders claim 4 obvious.

152. As explained further above with respect to claim 1, Mattoon expressly discloses in Figure 1 all eight heme biosynthetic enzymes recited by Claim 4. EX-1008(Mattoon), Fig. 1. Mattoon also discloses examples in which “the at least one polypeptide involved in heme biosynthesis” is HEM2, ALA dehydratase. EX-1008(Mattoon), 7:63-65. For example, Mattoon would motivate a POSITA to utilize a nucleic acid encoding at least one heterologous polypeptide involved in heme biosynthesis in the claimed recombinant nucleic acid molecule by teaching that “[t]he HEM2 over-expression system described above, when used in combination with hemoglobin expression plasmids, can be used to increase the production of hemoglobin and globin expression plasmids can be used for this purpose.” EX-1008 (Mattoon), 7:45-50. Only ordinary skill would be required, in light of the teachings of Mattoon as explained above.

3. Claim 5 (Mattoon and Vogl)

The yeast cell of claim 1, wherein the promoter element from *P. pastoris* is a constitutive promoter element from *P. pastoris* or a methanol-inducible promoter element from *P. pastoris*.

153. In my opinion, Mattoon alone, or Mattoon in view of Vogl, renders claim 5 obvious.

154. As discussed in §X.B.1.a, Mattoon expressly teaches that “[p]referred host cells” are “yeast of the genus *Pichia*, and *even more preferably, Pichia pastoris*.” EX-1008(Mattoon), 4:32-37, 9:20-10:4. Mattoon also teaches

engineering host cells to comprise nucleic acid sequences encoding proteins of interest that are “operatively linked” or “controllable by inducible or constitutive *promoters*.” EX-1008(Mattoon), 2:41-44, 5:51-59, 6:9-63. A POSITA would understand that Mattoon thus teaches the use of constitutive or methanol-inducible promoter elements from *P. pastoris* within a *P. pastoris* yeast cell expression system.

155. For the reasons discussed in X.B.1.b, a POSITA engineering *P. pastoris* cells to produce recombinant proteins would also have naturally looked to Vogl. Vogl describes and characterizes over twenty promoters used in *P. pastoris*, including both constitutive and inducible promoters. EX-1005(Vogl), 393 (Table 1). Vogl teaches that strong constitutive promoters such as the TEF1 and GAP promoters, or strong methanol-inducible promoters such as the AOX1 and DAS promoters, have been used in *P. pastoris* expression systems to provide “strong” levels of protein expression. EX-1005(Vogl), 392-93.

156. Vogl also teaches the benefits of trying a variety of constitutive and inducible promoters to achieve the most favorable conditions for heterologous protein production. EX-1005(Vogl), 392-93 (explaining that “the strong expression from P_{AOX1} and P_{GAP} is not always the most favorable condition for heterologous protein production,” and that “P_{PEX8} and P_{YPT1} are weak promoters and considered alternatives in case P_{AOX1} or P_{GAP} driven expression is too strong”). For example, Vogl teaches advantages of methanol-inducibility—e.g., “tight regulation” of gene

expression, and that “controllable promoters are a crucial tool for efficient heterologous protein production.” EX-1005(Vogl), 386. Vogl also teaches the advantages of constitutive promoters for the expression of certain proteins. For example, Vogl explains that “[f]or nontoxic proteins, which do not hamper cell growth, a strong constitutive expression system can help to minimize cultivation efforts and lead to a higher space/time yield.” EX-1005(Vogl), 392. Vogl provides examples of strong constitutive promoters such as the GAP and TEF1 promoters. EX-1005(Vogl), 392-93. A POSITA would therefore understand that either inducible or constitutive expression could be advantageous.

157. Thus, a POSITA engineering *P. pastoris* cells according to Mattoon’s methods would have been generally motivated to apply Vogl’s teachings regarding constitutive and inducible promoter elements from *P. pastoris* to achieve the most favorable conditions for producing heme-containing proteins. Moreover, a POSITA would reasonably expect success in doing so in light of Vogl’s review of prior art and using only routine techniques and available materials. *See supra* §IV.C.

4. Claim 6 (Mattoon and Vogl)

The yeast cell of claim 5, wherein the constitutive promoter element from *P. pastoris* is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene.

158. In my opinion, Mattoon in view of Vogl renders claim 6 obvious.

159. Vogl teaches that strong constitutive promoters have been used in *P. pastoris* to provide high levels of protein expression. EX-1005(Vogl), 392-93. Vogl explains that “[f]or nontoxic proteins, which do not hamper cell growth, a strong constitutive expression system can help to minimize cultivation efforts and lead to a higher space/time yield.” EX-1005(Vogl), 392. Thus, a POSITA would have been motivated to select a strong constitutive promoter for use with Mattoon to control the expression of a heme-containing protein or one or more heme biosynthesis enzymes for strong levels of protein production.

160. Vogl further teaches that the TEF1 promoter is a strong constitutive promoter from *P. pastoris* that is “similar to P_{GAP},” another well-characterized constitutive promoter. EX-1005(Vogl), 393. Vogl describes the TEF1 promoter as “a crucial component of the eukaryotic translation machinery,” and teaches that under certain conditions, “the activity levels of P_{TEF1} driven expression exceeded P_{GAP} almost twofold.” EX-1005(Vogl), 395. Thus, a POSITA would have been motivated to select the TEF1 promoter to control Mattoon’s expression of heme-containing protein and one or more heme biosynthesis enzymes. Moreover, a POSITA would reasonably expect success doing so in light of Vogl’s teachings and using only routine techniques and available materials. *See supra* §IV.C.

5. Claim 7 (Mattoon and Vogl)

The yeast cell of claim 5, wherein the methanol-inducible promoter element from *P. pastoris* is an alcohol oxidase I (AOX1) promoter element.

161. In my opinion, Mattoon in view of Vogl renders claim 7 obvious.

162. As discussed in §X.B.3, a POSITA would have had a variety of well-known promoters to select among for use in a *P. pastoris* expression system, including methanol-inducible promoters. One of the promoters that could be used in this system is the methanol-inducible promoter of the *AOX1* gene.

163. A POSITA would be familiar with the advantages of the promoter of the *AOX1* gene and would be motivated to utilize it in the claimed recombinant nucleic acid molecule, for example, in order to drive expression of the claimed heterologous polypeptide (gene sequence of interest). These advantages are summarized in the Vogl review. First, as a POSITA would know, and Vogl teaches, this promoter is a promoter for genes of the methanol utilization pathway (MUT). As Vogl explains, MUT gene promoters are “one of the most favorable” aspects of using methylotrophic yeast species such as *P. pastoris* because they afford control over gene expression.

One of the most favorable properties of *P. pastoris* and the other methylotrophic yeasts are the strong methanol inducible promoters present in these organisms. Certain genes of the methanol utilization (MUT) pathway are completely repressed under growth on repressing

carbon sources (e.g. glucose), but are strongly induced on methanol, constituting up to 20–30% of total intracellular protein [15,16]. This tight regulation is exerted on the transcriptional level, making MUT promoters ideal tools for heterologous protein production [17].

EX-1005(Vogl), 386.

164. Due to their well-known advantages, these MUT gene promoters are some of the best-studied promoters for use in a *P. pastoris* expression system. In my experience, a POSITA would be generally motivated to choose a well-studied promoter for use in an expression system as a matter of efficiency. As a general matter, a POSITA would anticipate success based upon the previous results reported in the literature. Thus, as a POSITA would recognize, using a well-studied promoter desirably saves research time and effort. A POSITA would recognize that these benefits apply to use of the promoter of the *AOX1* gene in particular:

Therefore also *most of the published knowledge about the regulation of P. pastoris promoters is focused on the most commonly used classic P. pastoris promoters, the promoters of the MUT pathway gene alcohol oxidase 1 (P_{AOX1}), its rarely used homolog P_{AOX2}, and also the glycolysis gene glyceraldehyde 3-phosphate dehydrogenase (P_{GAP}), which was described as a constitutive promoter in the early days of development.*

EX-1005(Vogl), 386.

165. Vogl teaches other benefits of using the promoter of the *AOX1* gene in order to drive recombinant gene expression. One advantage of using this promoter

is that this promoter drives gene expression at relatively high levels—that is, it is a “strong” promoter. Indeed, Vogl teaches that the promoter of the *AOX1* gene yields “the highest” expression levels of gene sequences of interest. Additionally, as taught in Vogl, this promoter’s activity can be readily controlled or modulated by changing the carbon source consumed by the *P. pastoris* yeast cells. A POSITA would recognize that this level of control over this promoter is advantageous because it affords a POSITA the ability to turn expression of genes regulated by this promoter “on” and “off” with ease. In particular, Vogl teaches using the promoter of the *AOX1* gene enables cells to be grown to high densities before gene expression is induced by methanol. In my experience, it is standard to grow *Pichia* to a high cell density on glycerol prior to feeding with methanol. Otherwise, methanol-induced activation of *AOX1* slows down cell growth.

P_{AOX1} is a ***remarkably strong*** promoter and at the same time ***directly controllable by simply changing the inexpensive carbon source***, it is the ***most commonly used promoter*** for heterologous protein expression in *P. pastoris*. The highest expression levels of heterologous proteins in *P. pastoris* have been achieved with P_{AOX1} (up to 22 g/l intracellular and 15 g/l secreted protein) [25,26]. Concerning controlled bioreactor cultivation, the tight regulation of P_{AOX1} is used to enable cell growth to high densities on a repressing carbon source without heterologous protein expression hampering the cellular metabolism.

EX-1005(Vogl), 386.

The major advantages of P_{AOX1} driven expression are the strength and the tight regulation of this promoter. Especially for the production of toxic or detrimental heterologous proteins, ***the inducibility with methanol is favorable.*** Cultures can at first be grown to high cell densities under repressing conditions and in a second phase, heterologous protein production can be started by methanol induction [3]. However, this requires a two-step cultivation procedure, which increases the process time and handling efforts.

EX-1005(Vogl), 386.

166. Vogl further observed that Takagi “seem[s] to oppose previous findings by Lin-Cereghino et al., where overexpression of *MXR1* under control of the *AOX1* promoter caused a growth defect on methanol and inhibited expression of [methanol utilization] genes.” EX-1005(Vogl), 391 (citing EX-1007(Hartner)). Therefore, a POSITA looking to Vogl would understand that an expression system constructed with *MXR1* and promoters such as the *AOX1* promoter is desirable and would have a reasonable expectation of success when constructing such an expression system.

167. Accordingly, a POSITA would be motivated to select the promoter of the *AOX1* gene for use in driving the expression of gene sequences of interest in a *P. pastoris* expression system. In light of its well-known and well-characterized activity and structure, a POSITA would reasonably expect success in doing so.

168. Moreover, in light of the extensive published literature on this promoter (reviewed in Vogl between pages 386 and 392), only ordinary skill would be

required to insert the sequence of the promoter of the *AOX1* gene into a recombinant expression vector, which could be used to transform a methylotrophic yeast cell such as *P. pastoris*.

6. Claim 8 (Mattoon and Vogl)

The yeast cell of claim 1, wherein the promoter element from *P. pastoris* comprises a constitutive promoter element from *P. pastoris* and a methanol-inducible promoter element from *P. pastoris*.

169. In my opinion, Mattoon in view of Vogl renders claim 8 obvious.

170. As discussed in §X.B.1, Mattoon teaches methods of engineering expression vectors that are “controllable by inducible or constitutive promoters” from *P. pastoris* that are “operatively linked” to the nucleic acid encoding the proteins of interest. EX-1008(Mattoon), 2:41-44, 4:29-40, 5:51-59, 6:9-63.

171. A POSITA would have been motivated to select suitable constitutive and methanol-inducible promoters for the expression of different proteins. Claim 1 of the '492 patent recites two promoters, one controlling the expression of a heme-containing protein and the other the expression of at least one polypeptide involved in heme biosynthesis. EX-1001, 77:49-57. A POSITA would have understood that the two promoters may be the same or different, for example, one being methanol-inducible and the other being constitutively active. The use of both types of promoters in the same *P. pastoris* strains would have been obvious to a POSITA. For example, a POSITA would recognize that it could be advantageous to induce

expression of certain gene sequences while constitutively expressing other gene sequences, as I discuss further below.

172. As discussed in §X.B.1.b, a POSITA desiring to select suitable promoters would also have been motivated to look to Vogl's teachings regarding constitutive and methanol-inducible promoters from *P. pastoris* and would have reasonably expected success in following them. Vogl describes and characterizes over twenty constitutive or methanol-inducible promoters that can be used to provide varying levels of protein expression in *P. pastoris*. EX-1005(Vogl), 393.

173. Vogl teaches the advantages of methanol-inducible promoters. Vogl explains that “[o]ne of the most favorable properties of *P. pastoris* and the other methylotrophic yeasts are the strong methanol inducible promoters present in these organisms.” EX-1005(Vogl), 386. Vogl explains that because “P_{AOX1} is a remarkably strong promoter and at the same time directly controllable by simply changing the inexpensive carbon source, it is the most commonly used promoter for heterologous protein expression in *P. pastoris*.” EX-1005(Vogl), 386. Additionally, for example, I discuss advantages of using the methanol-inducible promoter of the *AOX1* gene above with respect to claim 7.

174. Vogl also teaches the advantages of constitutive promoters for the expression of certain proteins. For example, Vogl explains that “[f]or nontoxic proteins, which do not hamper cell growth, a strong constitutive expression system

can help to minimize cultivation efforts and lead to a higher space/time yield.” EX-1005(Vogl), 392. Vogl provides examples of strong constitutive promoters such as the GAP and TEF1 promoters. EX-1005(Vogl), 392-93.

175. Vogl also discusses studies, such as Takagi, in which two different proteins of interest were expressed under two different types of promoters—phytase under the methanol-inducible promoter of the *AOX1* gene and Mxr1 under the constitutive promoter of the GAP gene. EX-1005(Vogl), 391. As taught in Vogl, Takagi’s approach advantageously resulted in the production of a recombinant protein (separately under the control of the methanol-inducible promoter of the *AOX1* gene) that was 7.6-fold higher than that produced by yeast cells lacking Mxr1 expression. EX-1005(Vogl), 391.

176. In light of such teachings, a POSITA would have been motivated to use both constitutive and inducible promoters in a single *P. pastoris* expression system to find a suitable combination for the production of the desired protein of interest. Moreover, because the use of both constitutive and inducible promoters in a single expression system was taught by studies reviewed by Vogl, such as Takagi, and required only ordinary skill to construct, a POSITA would have reasonably expected success in doing so.

7. Claim 9 (Mattoon and Vogl)

The yeast cell of claim 8, wherein the constitutive promoter element from *P. pastoris* is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene.

177. In my opinion, this claim is obvious for reasons discussed above in §X.B.4 and X.B.6. A POSITA would have been motivated to select the TEF1 promoter as one of the promoters recited by claim 8 and would have reasonably expected success in doing so.

8. Claim 10 (Mattoon and Vogl)

The yeast cell of claim 8, wherein the methanol-inducible promoter element from *P. pastoris* is an alcohol oxidase I (AOX1) promoter element.

178. In my opinion, this limitation is obvious for reasons discussed above in X.B.5 and X.B.6. A POSITA would have been motivated to select the AOX1 promoter as one of the promoters recited by claim 8 and would have reasonably expected success in doing so.

9. Claim 11 (Mattoon and Vogl)

The yeast cell of claim 1, wherein the nucleic acid molecule encoding the heme-containing protein is present in the yeast cell in at least two copies.

179. In my opinion, this claim is obvious in light of Mattoon.

180. Mattoon expressly discloses “double transformant yeast cells simultaneously harboring the multicopy plasmids pCTT1” encoding catalase T, a cytoplasmic heme-containing protein, and multicopy plasmids encoding a heme

biosynthetic enzyme. EX-1008(Mattoon), 7:63-8:2. A POSITA would have understood that such double transformant yeast cells comprise multiple copies of the nucleic acid molecule encoding the heme-containing protein of claim 1 would result in higher expression levels of the heme-containing protein.

C. Ground 1B: Mattoon and Vogl further in view of Crane

1. Claim 2 (Mattoon, Vogl, and Crane)

The yeast cell of claim 1, wherein the heme-containing protein is Leghemoglobin (LegH).

181. In my opinion, the combination of Mattoon, Vogl, and Crane renders claim 2 obvious. Mattoon teaches “a method to produce hemoproteins.” EX-1008(Mattoon), 2:9-10. In particular, Mattoon discloses that “[u]sing the present method, desired hemoproteins, *such as* hemoglobin, myoglobin . . . can be produced.” EX-1008(Mattoon), 1:39-41, 2:34-37. Although Mattoon does not expressly disclose leghemoglobin, a POSITA would have known that leghemoglobin, is a member of the heme-containing protein family. EX-1014(Garrocho), 79; Fig. 1. Leghemoglobin was one of the earliest discovered plant hemoglobins and was identified due to its red pigmentation. Garrocho-Villegas explains:

Plant Hbs were first identified by Kubo (1939). This author analyzed the red pigment of soybean root nodules. ... From this evidence Kubo concluded that the red pigment isolated from soybean root nodules was

a hemeprotein. ... Kubo's plant Hb was named as leghemoglobin (Lb) by Virtanen and Laine (1946) and is also known as plant symbiotic Hb. EX-1014(Garrocho), 79.

182. Similar to Mattoon, Crane teaches using *P. pastoris* to co-express hemoglobin and heme biosynthetic enzyme. EX-1010(Crane), Abstract, [0009]. Crane teaches leghemoglobin as an example of heme-containing proteins that can be expressed through its methods. Crane teaches “methods of producing recombinant functional heme-binding proteins with complete heme incorporation and purified preparations” in “*Pichia pastoris* expression systems,” which are “completely functional” and suitable for “commercial applications.” EX-1010(Crane), Abstract, [0021], [0024], [0044], [0060]-[0062].

For instance, Pichia pastoris expression systems, may be used to co-express a recombinant heme-binding protein of interest with ferrochelatase ... *Such systems include suitable Pichia pastoris strains*, vectors, reagents, transformants, sequencing primers, and media. EX-1010(Crane), [0044].

Traditional methods of producing recombinant heme-binding proteins generate recombinant proteins having incomplete heme incorporation. Under incorporation of heme into recombinant proteins can adversely influence their function. In contrast to these traditional methods, the methods of the present invention generate recombinant heme-binding proteins that have complete heme incorporation. *Therefore, these*

proteins are completely functional and are more suitable for research, clinical, and commercial applications.

EX-1010(Crane), [0024]; [0007] (“Full incorporation of heme in recombinant proteins is also important for commercial applications.”).

183. Crane teaches recombinant expression of a plant hemoglobin in *P. pastoris*. Specifically, Crane teaches leghemoglobin—necessarily a plant hemoglobin—as an example of a heme-containing protein that can be produced by its methods.

The various classes of heme-binding proteins that are encompassed by the methods of the present invention include, without limitation, globins (e.g., hemoglobin, myoglobin, neuroglobin, cytoglobin, ***leghemoglobin***), cytochromes (e.g., a-, b-, and c-types, cd1-nitrite reductase, cytochrome oxidase), transferrins (e.g., lactotransferrin, serotransferrin, melanotransferrin), bacterioferrins, hydroxylamine oxidoreductase, nitrophorins, peroxidases (e.g., lignin peroxidase), cyclooxygenases (e.g., COX-1, COX-2, COX-3, prostaglandin H synthase), catalases, cytochrome P-450s, chloroperoxidases, PAS-domain heme sensors, H-NOX heme sensors (e.g., soluble guanylate cyclase, FixL, DOS, HemAT, and CoxA), heme-oxygenases, and nitric oxide synthases.

EX-1010(Crane), [0021].

184. Similar to Mattoon, Crane teaches using *P. pastoris* to co-express hemoglobin and heme biosynthetic enzyme, ferrochelatase. EX-1010(Crane), Abstract, [0009].

Yeast strains and yeast-derived vectors are used commonly for the expression of heterologous proteins. For instance, *Pichia pastoris* expression systems, may be used to co-express a recombinant heme-binding protein of interest with ferrochelatase (see e.g., Ballew N., “Revolutionizing Protein Production in Fungi,” Innovations in Pharmaceutical Technology 70-76 (June 2004), which is hereby incorporated by reference in its entirety). Such systems include suitable *Pichia pastoris* strains, vectors, reagents, transformants, sequencing primers, and media. Available strains include KM71H (a prototrophic strain), SMD1168H (a prototrophic strain), and SMD1168 (a pep4 mutant strain) (Invitrogen).

EX-1010(Crane), [0044].

185. **Motivation to Combine.** In my opinion, it would have been obvious for a POSITA engineering an expression system in accordance with Mattoon’s or Crane’s methods to choose leghemoglobin (e.g., the gene sequence encoding the leghemoglobin protein) as the desired heme-containing protein. A POSITA would recognize that leghemoglobin was one of the earliest-discovered plant hemoglobins and had been studied for decades. EX-1014(Garrocho), 78-79, 81. It also has a structure similar to other hemoglobins and thus would be expected to yield a similar heme-containing protein when produced recombinantly in *P. pastoris*. EX-1044(Nicola), 133 (“Leghaemoglobins are plant haemoproteins which resemble animal myoglobin”); EX-1045(Hargrove), 1032 (“[T]ertiary folding patterns of

... soybean Lb are very similar to those of mammalian myoglobins, and the α and β subunits of human hemoglobin.”); EX-1014(Garrocho), 79 (Fig. 1, Table 1).

186. Mattoon motivates a POSITA to utilize leghemoglobin for reasons I explain further above. For example, Mattoon teaches that “proteins carrying a heme prosthetic group” are “[a]n important class of ... heterologous proteins produced in genetically engineered cells,” EX-1008(Mattoon), 1:13-16, and that “[m]any commercial ventures are working to enhance the biosynthesis of the heme protein family ... in yeast.” EX-1008(Mattoon), 1:17-22; 1:23-30 (discussing the “considerable current interest in the potential of cell-free hemoglobin as a substitute for red blood cells”).

187. A POSITA would also understand that leghemoglobin’s similarity to animal heme-containing proteins creates attractive commercial applications, such as being a protein alternative for individuals who have concerns about or who otherwise abstain from using proteins that have an animal provenance. Indeed, Crane’s methods are designed to support this type of “commercial application” for heme-containing proteins. EX-1010(Crane), [0024] (“[T]hese proteins are completely functional and are more suitable for research, clinical, and commercial applications.”). A POSITA would be generally motivated to select Crane’s leghemoglobin—e.g., for “commercial applications” involving plant-based

products, as Garrocho-Villegas teaches that leghemoglobin is red-pigmented (EX-1014(Garrocho), 79) and therefore could be used as a colorant.

188. ***Reasonable Expectation of Success.*** A POSITA would have reasonably expected success in combining Mattoon with Vogl and Crane. Crane is directed to methods of constructing expression systems to increase recombinant heme protein production. EX-1010(Crane), Abstract. Crane also teaches that production of heme-containing proteins have been known to be done in *P. pastoris*. EX-1010(Crane), [0047] (“There are several fungal species which have been used as hosts for the expression of recombinant proteins, including, without limitation, yeast (e.g., *Pichia pastoris*[]).”). Mattoon is directed to a method for increasing the production of heme-containing proteins. EX-1008(Mattoon), Abstract. And Vogl is directed to optimizing *P. pastoris* expression systems for desired levels of protein secretion. EX-1005(Vogl), 385. Accordingly, a POSITA combining Mattoon with Vogl and Crane would be able to reasonably expect the successful production of a heme-containing protein, such as leghemoglobin. Doing so would require only ordinary skill in the art in light of the teachings of the prior art and available materials.

2. Claim 3 (Mattoon, Vogl, and Crane)

The yeast cell of claim 2, wherein the LegH has the sequence shown in SEQ ID NO:6.

189. In my opinion, this limitation is obvious over Mattoon, Vogl, and Crane for the same reasons discussed in Section X.C.1. Claim 3 depends from claim 1. For the reasons provided above for claim 1, Mattoon in view of Vogl discloses each limitation of claim 1.

190. As discussed in Section X.C.10, a POSITA would have found it obvious to express leghemoglobin in the *P. pastoris* yeast cells of claim 1. SEQ ID NO:6 in the '492 patent is the amino acid sequence for soybean (*Glycine max*) leghemoglobin. *See* EX-1001, 51-52. This sequence is available in the GenBank/GenPept database under GenBank Accession Number AAA33980, and I accessed it there. *See* EX-1046, 1-2. According to its “Revision History,” Accession Number AAA33980 was first published on the GenBank/GenPept database in April 1993. *Id.* As explained above in Section VI.A, a POSITA could and would have looked to the GenBank/GenPept database to obtain the amino acid sequence for leghemoglobin prior to 2015, as it was the most up-to-date and comprehensive, publicly-available database of DNA sequences at the time. *See* EX-1053(NCBI), EX-1054(Benson), D32.

191. Thus, claim 3 would have been obvious over Mattoon, Vogl, and Crane.

3. Claim 12 (Mattoon, Vogl, and Crane)

The yeast cell of claim 1, wherein the nucleic acid molecule encoding the heme-containing protein operably linked to the promoter element from *P. pastoris* and the Mxr1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence.

192. In my opinion, this limitation is obvious over Mattoon, Vogl, and Crane.

193. As discussed above for claim 2, a POSITA would have readily combined Mattoon and Vogl with Crane. Crane expressly discloses the additional limitation of this claim—“a termination sequence.” Crane claims an expression system “wherein the one or more expression constructs further comprise a promoter sequence, a nucleic acid encoding a ribosome binding sequence, and a nucleic acid encoding a *termination sequence*.” EX-1010(Crane), Claim 22. Moreover, a POSITA would have understood that termination sequences were routinely used in expression vectors for *P. pastoris*. EX-1015(Lin-Cereghino 2000), 48. A POSITA would be motivated to include a termination sequence, e.g., in order to stop the transcription of the gene sequence of interest, and would have a reasonable expectation of success in doing so. The gene transcription is then translated into a protein. EX-1042(Alberts), 306-08, 335-36.

194. Thus, claim 12 would have been obvious over Mattoon, Vogl, and Crane.

4. Claim 13 (Mattoon, Vogl, and Crane)

The yeast cell of claim 1, wherein the nucleic acid encoding the at least one polypeptide involved in heme biosynthesis operably linked to the promoter element from *P. pastoris* and the Mxr1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence.

195. In my opinion, this limitation is obvious in view of Mattoon, Vogl, and Crane. Claim 13 depends from claim 1. For the reasons provided above for Claim 1, Mattoon and Vogl disclose each limitation of claim 1.

196. As discussed above for claim 2, a POSITA would have readily combined Mattoon and Vogl with Crane. Crane discloses a nucleic acid molecule encoding ferrochelatase, which is a heme biosynthetic enzyme. EX-1010(Crane), Claim 16 (“[A]n expression system and one or more expression constructs encoding a recombinant heme-binding protein and a recombinant ferrochelatase.”). In addition, Crane claims this expression construct “further comprise[s] a promoter sequence, a nucleic acid encoding a ribosome binding sequence, and a nucleic acid encoding a termination sequence.” EX-1010(Crane), Claim 22. Moreover, a POSITA would have understood that termination sequences were routinely used in expression vectors for *P. pastoris*. EX-1015(Lin-Cereghino 2000), 48. A POSITA would be motivated to include a termination sequence, e.g., in order to stop the transcription of the gene sequence of interest, and would have a reasonable

expectation of success in doing so. The gene transcription is then translated into a protein. EX-1042(Alberts), 306-08, 335-36.

197. Therefore, Claim 13 would have been obvious over Mattoon, Vogl, and Crane.

D. Ground 1C: Mattoon and Vogl further in view of Lin-Cereghino

1. Claim 14 (Mattoon, Vogl, and Lin-Cereghino)

The yeast cell of claim 1, further comprising a nucleic acid molecule encoding a Mxr1 transcriptional activator operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.

198. In my opinion, Mattoon in view of Vogl and Lin-Cereghino renders this claim obvious. Claim 14 is dependent on Claim 1. For the reasons provided above for Claim 1, Mattoon in view of Vogl discloses each limitation of claim 1.

199. It would be obvious to a POSITA to engineer a yeast cell comprising a Mxr1 sequence from *P. pastoris* and a nucleic acid encoding Mxr1 driven by a *P. pastoris* promoter. A POSITA would recognize, for example, that Mxr1 binds and activates various *P. pastoris* promoters, as I discuss above. When Mxr1 is expressed under the control of a Mxr1-driven promoter, Mxr1 would be expected to bind and further activate its own expression, leading to enhanced Mxr1 expression in the yeast cell. A POSITA would recognize that this would be advantageous if, for example, expression of the gene of interest is also regulated by Mxr. Additionally, as Mxr1

activity is methanol-inducible, expression would be controllable due to the presence and level of methanol to which the yeast are exposed.

200. Vogl discloses that *P. pastoris* could be engineered to produce high yields of heterologous proteins through the use of the “strong, methanol inducible promoter of the alcohol oxidase 1 (*AOX1*) gene,” which is derived from *P. pastoris*. EX-1005(Vogl), 385. Vogl teaches that Mxr1 binds to six places in the *AOX1* promoter. EX-1005(Vogl), 390. Moreover, Vogl describes Mxr1 as “a key activator of methanol induced genes” in methylotrophic *P. pastoris* and “crucial” to the activation of the MUT gene promoters. EX-1005 389. As I explain above with respect to claim 1, Vogl taught various constitutive and inducible promoters (e.g., the constitutive promoters of the GAP and TEF1 genes, and the inducible promoters of the AOX1 and PEX8 genes), which a POSITA would be motivated to use in order to produce recombinant proteins of interest and would reasonably expect success in doing so.

201. Lin-Cereghino would further motivate a POSITA to utilize a nucleic acid molecule encoding Mxr1 operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*. In Lin-Cereghino, for example, *P. pastoris* cells expressed an increased amount of Mxr1 under the control of the *PEX8* promoter—derived from *P. pastoris*—in a methanol-based medium. EX-1006(Lin-Cereghino), 893. Specifically, Lin-Cereghino

screened for pGC217 strains with higher copy numbers of the P_{PEX8}-*MXR1* construct incorporated in the yeast genome and identified a “multicopy strain that grew slowly on methanol” but “contained relatively high levels of the product.” EX-1006(Lin-Cereghino), 893. Lin-Cereghino also teaches *P. pastoris* strains incorporating multiple copies of *MXR1* gene that produced high levels of Mxr1. *Id.* Thus, a POSITA would have been motivated to design rDNA constructs that not only co-express genes encoding heme-containing protein and heme biosynthetic enzymes under methanol-inducible promoters such as the *AOX1* promoter, but also increase Mxr1 expression, for example, by expressing Mxr1 under the *GAP* promoter in glucose-grown cells, or under the *PEX8* promoter in methanol-grown cells, or by increasing the copy number of *MXR1* gene, as taught by Vogl and Lin-Cereghino.

202. Additionally, to achieve recombinant protein expression in *P. pastoris* as I explain above, Vogl provides an overview of other promoters that can be used to drive gene expression, including constitutive promoters of the *GAP* and *TEF* genes. EX-1005(Vogl), 392-95. Vogl explains that promoters have different strengths. EX-1005(Vogl), 392-95. Vogl further explains that the *GAP* and *AOX1* promoters are both strong that “provide similar expression levels under the respective optimal growth conditions.” EX-1005(Vogl), 392. Vogl explains, however, that “the strong expression from the promoters of the *GAP* and *AOX1* genes is not always the most favorable condition for heterologous protein

production”; rather, “a weaker expression level can be more desirable” in some cases. EX-1005(Vogl), 392. Weaker promoters such as the promoters of the *PEX8* gene (inducible) and *YPT1* gene (constitutive) are “alternatives in case [AOX1 or GAP promoter] driven expression is too strong.” EX-1005(Vogl), 393. In Table 1, Vogl lists over 20 promoters that can be used to provide varying levels of protein expression in *P. pastoris* as desired by POSITAs. EX-1005(Vogl), 393.

203. Given this, a POSITA would have been motivated to increase the expression of *MXR1* by placing the gene under the control of a promoter and/or control based on carbon source in growth medium and would reasonably expect success in doing so. To do this, a POSITA would look to the teachings in Lin-Cereghino where *MXR1* was placed under the control of the the *PEX8* promoter. EX-1006(Lin-Cereghino), 885. A POSITA, for example, looking to express Mxr1 (*e.g.*, from a *PEX8* promoter) that is capable of activating a gene operably linked to an *AOX1* promoter in a *P. pastoris* expression system would first grow the cells to high densities while repressing recombinant protein expression in a medium that contained glucose or glycerol. Once the cells have grown to a significant mass, a POSITA would derepress expression by depleting the glucose or glycerol and adding methanol. This would in turn strongly induce *AOX1* promoter-driven recombinant protein expression from a large number of cells. A POSITA would understand that

growing the cells to a high density and then switching on expression would result in high levels of protein secretion.

XI. GROUND 2A-2B: CRANE, VOGL, LIN-CEREGHINO

A. Overview

204. In my opinion, Claims 1-13 are obvious over Crane in view of Vogl. Claim 14 is obvious over Crane in view of Vogl and Lin-Cereghino.

205. As I discuss further below, Crane, Vogl, and Lin-Cereghino are each analogous art to the '492 patent. The '492 patent describes its purported invention as follows:

This disclosure describes the use of *P. pastoris* strains that overexpress the transcriptional activator, Mxr1, from the AOX1 promoter to increase expression of transgenes that also are expressed from the AOX1 promoter, which significantly improves the recombinant production of one or more proteins. In addition, expression of Mxr1 from the AOX1 promoter creates a positive feedback loop that allows for expression of other transgenes from the AOX1 promoter in the absence of methanol, the normally obligate inducer, when repressing carbon sources are depleted. Expression of Mxr1 results in a significant increase in the amount of protein produced.

EX-1001, 1:37-48.

206. Crane discloses nearly all limitations of claim 1 of the '492 except “a Mxr1 transcriptional activator sequence from *P. pastoris*.” As explained above in Section VII.C, Crane teaches that its “invention relates to a purified preparation of

recombinant functional heme-binding proteins.” EX-1010(Crane), [0060]. Crane teaches that heme-containing proteins, which Crane calls heme-binding proteins, include “hemoglobin, myoglobin, [and] *legghemoglobin*.” EX-1010(Crane), [0021]. Crane recognizes that “[y]east strains and yeast-derived vectors are used commonly” for heterologous protein expression and “*Pichia pastoris* expression systems[] may be used to co-express a recombinant heme-binding protein of interest with ferrochelatase.” EX-1010(Crane), [0043]-[0044].

207. As explained above in Section VII.B, Vogl discloses commonly used methanol-inducible promoters from *P. pastoris*, such as the AOX1 promoter, and Mxr1 as a key transcriptional activator that regulates such methanol-inducible promoters from *P. pastoris*. Vogl explains that methylotrophic *P. pastoris* is a “wildly used host cell for heterologous protein production” due to having “favorable properties such as growth to high cell density and high capacities for protein.” EX-1005(Vogl), 385. Vogl teaches transcription constructs for “tailor-made production strains” of methylotrophic *P. pastoris* for protein secretion. EX-1005(Vogl), 385. Vogl explains that *P. pastoris* and its methanol inducible promoter AOX1 have been “extensively studied” and compiles over 170 studies related to *P. pastoris*, its promoters, and its transcription factors. EX-1005(Vogl), 385.

208. Further, as detailed more fully above in Section VII.D, Lin-Cereghino describes *P. pastoris* strains with increased expression of Mxr1 under the control of

either AOX1 promoter (a strong methanol-regulated promoter) or PEX8 promoter (a weaker methanol-regulated promoter). EX-1006(Lin-Cereghino), 885.

B. Ground 2A: Crane and Vogl

1. Claim 1 (Crane and Vogl)

Claim 1[pre]: A methylotrophic *Pichia* yeast cell comprising...

209. In my opinion, this claim element is obvious over Crane alone, or Crane in view of Vogl.

210. Crane teaches that “[y]east strains and yeast-derived vectors are used commonly” for heterologous protein expression and “*Pichia pastoris* expression systems[] may be used to co-express a recombinant heme-binding protein of interest with ferrochelatase.” EX-1010(Crane), [0043]-[0044]. As discussed above in Section X.B.1, Vogl also discloses a methylotrophic *Pichia* yeast cell as an attractive host for the expression of a protein of interest.

211. Thus, either Crane alone, or Crane in view of Vogl, discloses “[a] methylotrophic *Pichia* yeast cell.”

(a) Claim 1[a]: ...a nucleic acid molecule encoding a heme-containing protein operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*...

212. In my opinion, Crane in view of Vogl renders claim element 1[a] obvious.

213. Crane teaches “methods of producing recombinant functional heme-binding proteins” EX-1010(Crane), Abstract. Crane teaches an expression system with “one or more expression constructs encoding a recombinant heme-binding protein” that “further comprise a promoter sequence” EX-1010(Crane), Claim 16; Claim 22. Crane discloses *P. pastoris* expression system. EX-1010(Crane), Claim 21 (“[T]he host cell is selected from the group consisting of a bacterial cell, fungal cell, insect cell, plant cell, archaeal cell, and mammalian cell.”); *id.*, [0043] (“There are several fungal species which have been used as hosts for the expression of recombinant proteins, including, without limitation, yeast [e.g., *Pichia pastoris*”). Thus, a POSITA would have understood that Crane teaches an expression system in a *P. pastoris* host cell comprising “a nucleic acid molecule encoding a heme-containing” that is “operably linked to a promoter element from *P. pastoris*.”

214. Crane does not disclose “a Mxr1 transcriptional activator sequence from *P. pastoris*.” As discussed below, however, a POSITA would have been motivated to combine Crane with Vogl to engineer *P. pastoris* with “a nucleic acid molecule encoding a heme-containing protein operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.”

215. As discussed more fully in Section X.B.1, Vogl teaches that methylotrophic *P. pastoris* expression systems must have a Mxr1 transcriptional activator sequence because, without Mxr1, the methanol utilization pathway will not

be induced. EX-1005(Vogl), 390 (“Deletion of MXR1 in the wild type strain resulted in abolished growth on the peroxisomal substrates methanol and oleate. In conclusion, the mxr1 deletion strain showed under methanol induction highly reduced protein and mRNA levels”).

216. **Motivation to Combine.** A POSITA would understand that Crane was ripe for modifications that would yield “a high level of transcription” of heme-containing proteins. EX-1010(Crane), [0040], [0087]. A POSITA would have naturally looked to combine Crane’s expression system with Vogl, which discloses commonly used strong *P. pastoris* promoters such as the AOX1 promoter, regulated by the known transcriptional activator Mxr1, and “their application to improve heterologous protein production.” EX-1005(Vogl), 385-87. Crane teaches that “it is desirable to use strong promoters to obtain a high level of transcription and, hence, expression.” EX-1010(Crane), [0040]. As explained above in Section X.A.1.b, Vogl discloses the construct of a promoter element and MXR1 transcriptional activator sequence to produce a high yields of a protein of interest in methylotrophic *P. pastoris*. A POSITA would have been motivated to maximize the recombinant expression of heme-containing proteins through Crane’s methods by combining them with Vogl’s expression optimization methods.

217. **Reasonable Expectation of Success.** Crane teaches that “[t]here are several fungal species which have been used as hosts for the expression of

recombinant proteins, including, without limitation, yeast (e.g., *Pichia pastoris*[]).” Crane further teaches that “[a]ny plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.” EX-1010(Crane), [0047]. Therefore, a POSITA would reasonably expect successful expression of recombinant heme-containing proteins in *P. pastoris* when adding the transcriptional activator Mxr1 to Crane’s methods of recombinant heme-containing protein production. Moreover, only ordinary skill would have been required to genetically modify the expression system in Crane to include Mxr1.

218. Thus, claim element 1[a] is obvious over Crane in view of Vogl.

(b) Claim 1[b]: ...a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.

219. In my opinion, the combination of Crane and Vogl renders this limitation obvious for largely the same reasons discussed above in limitation 1[a].

220. Crane discloses co-expressing a recombinant heme-binding protein in the presence of one or more heme precursors. EX-1010(Crane), [0029], Claims 2, 17, 18. A POSITA would understand that the described heme precursors are “polypeptides involved in heme-biosynthesis.”

221. As discussed in limitation 1[a], Crane teaches that “[e]xpression vectors include (among other elements) regulatory sequences (e.g., *promoters*) that can be

operably linked to the desired protein-encoding nucleic acid molecule” (*i.e.*, a heme-containing protein) to “cause the expression of such nucleic acid molecule in the host cell.” EX-1010(Crane), [0033]. As previously detailed, a POSITA would have found “operably linked to a promoter element from *P. pastoris*” obvious over Crane.

222. As I explained above for claim element 1[a], a POSITA would have been motivated to construct the recombinant heme-containing protein expression system of Crane, including the at least one polypeptide involved in heme biosynthesis, with the Mxr1 transcription factor as disclosed by Vogl and would have a reasonable expectation of success in doing so. As explained above for claim element 1[a], Vogl also discloses the construct of a promoter element and MXR1 transcriptional activator sequence for protein expression in methylotrophic *P. pastoris*.

223. Accordingly, claim element 1[b] is obvious over Crane in view of Vogl.

2. Claim 2 (Crane and Vogl)

The yeast cell of claim 1, wherein the heme-containing protein is Leghemoglobin (LegH).

224. In my opinion, this claim is obvious over Crane in view of Vogl. Claim 2 depends from claim 1. For the reasons provided above for claim 1, Crane in view of Vogl discloses each limitation of claim 1.

225. Crane discloses constructing the *P. pastoris* expression system with genes encoding Leghemoglobin. Ex-1010(Crane), [0021] (“The various classes of

heme-binding proteins that are encompassed by the methods of the present invention include, without limitation, globins (e.g., hemoglobin, myoglobin, neuro globin, cytoglobin, **leghemoglobin**)”).

226. Accordingly, claim 2 is obvious over Crane in view of Vogl.

3. Claim 3 (Crane and Vogl)

The yeast cell of claim 2, wherein the LegH has the sequence shown in SEQ ID NO:6.

227. In my opinion, this claim is obvious over Crane in view of Vogl. Claim 3 depends from claim 2. For the reasons provided above for claim 2, Crane in view of Vogl discloses claim 2.

228. Crane discloses constructing the *P. pastoris* expression system with the genes for Leghemoglobin and hemoglobins generally “without limitation.” Ex-1010(Crane), [0021] (“The various classes of heme-binding proteins that are encompassed by the methods of the present invention include, without limitation, globins (e.g., hemoglobin, myoglobin, neuro globin, cytoglobin, leghemoglobin)”). Thus, a POSITA would have found it obvious to express leghemoglobin in the *P. pastoris* yeast cells of claim 1.

229. SEQ ID NO:6 in the ’492 patent is the amino acid sequence for soybean (Glycine max) leghemoglobin. See EX-1001, 51-52. This sequence is available in the GenBank/GenPept database under GenBank Accession Number AAA33980. See EX-1046, 1-2. According to its “Revision History,” Accession Number

AAA33980 was first published on the GenBank/GenPept database in April 1993.

Id. As explained above in Section VI.A, a POSITA could and would have looked to the GenBank/GenPept database to obtain the amino acid sequence for leghemoglobin prior to 2015, as it was the most up-to-date and comprehensive, publicly-available database of DNA sequences at the time. *See* EX-1053(NCBI); EX-1054(Benson), D32.

230. Accordingly, claim 3 is obvious over Crane in view of Vogl.

4. Claim 4 (Crane and Vogl)

The yeast cell of claim 1, wherein the at least one polypeptide involved in heme biosynthesis is selected from the group consisting of ALA synthase, ALA dehydratase, porphobilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and/or ferrochelatase.

231. In my opinion, Crane in view of Vogl renders this limitation obvious. Claim 4 depends from claim 1. For the reasons provided above for claim 1, Crane in view of Vogl discloses each limitation of claim 1.

232. Crane discloses constructing the *P. pastoris* expression system for complete heme-binding protein production by “co-expressing a recombinant ***heme-binding protein*** and [a] recombinant ***ferrochelatase protein***,” a heme biosynthetic enzyme, i.e., at least one polypeptide involved in heme biosynthesis. EX-1010(Crane), Abstract, [0009].

233. Accordingly, claim 4 is obvious over Crane in view of Vogl.

5. Claim 5 (Crane and Vogl)

The yeast cell of claim 1, wherein the promoter element from *P. pastoris* is a constitutive promoter element from *P. pastoris* or a methanol-inducible promoter element from *P. pastoris*.

234. It is my opinion that Crane in view of Vogl renders this claim obvious.

Claim 5 depends from claim 1. For the reasons provided above for claim 1, Crane in view of Vogl discloses each limitation of claim 1.

235. Vogl expressly discloses this limitation. Vogl teaches that “[o]ne of the *most favorable properties* of *P. pastoris* and the other methylotrophic yeasts are the strong methanol inducible promoters present in these organisms.” EX-1005(Vogl), 386. In addition, Vogl discloses that constitutive promoter elements can be used for protein secretion in *P. pastoris*—noting that the prior art has “characterized the *P. pastoris* glyceraldehyde 3-phosphate dehydrogenase gene (GAP) and showed that its promoter is strong and constitutive.” EX-1005(Vogl), 392.

236. A POSITA would have been motivated to select a methanol-inducible promoter from *P. pastoris*, such as the AOX1 promoter, to control the expression of Crane’s heme-binding protein and one or more heme biosynthetic enzymes in *P. pastoris*. Moreover, Vogl teaches, as shown in Table 1, that *P. pastoris* expression systems can be constructed with numerous other constitutive and methanol inducible promoters. A POSITA would have reasonably expected success in following the well-known techniques disclosed in Vogl’s prior studies to construct similar *P.*

pastoris expression systems using only routine techniques, known methods, and readily available materials for genetic engineering and recombinant protein production. *See supra* § IV.C.

237. Thus, a POSITA would have found claim 5 obvious over Crane in view of Vogl.

6. Claim 6 (Crane and Vogl)

The yeast cell of claim 5, wherein the constitutive promoter element from *P. pastoris* is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene.

238. In my opinion, Crane in view of Vogl renders claim 6 obvious. Claim 6 depends from claim 5. For the reasons provided above for claim 5, Crane in view of Vogl discloses claim 5.

239. Vogl teaches that constitutive promoters have been used in *P. pastoris* to provide high levels of protein expression. EX-1005(Vogl), 392-93. Vogl explains that “[f]or nontoxic proteins, which do not hamper cell growth, a strong constitutive expression system can help to minimize cultivation efforts and lead to a higher space/time yield.” EX-1005(Vogl), 392. Thus, a POSITA would have been motivated to select a strong constitutive promoter to control the expression of a heme-binding protein or one or more heme biosynthesis enzymes for strong levels of protein production.

240. Vogl further teaches that TEF1 is a strong constitutive promoter from *P. pastoris*. Vogl describes TEF1 as “a crucial component of the eukaryotic translation machinery.” EX-1005(Vogl), 395. Vogl discloses that “the activity levels of PTEF1 driven expression exceeded” the activity of most other *P. pastoris* promoters. EX-1005(Vogl), 395. Thus, a POSITA would have been motivated to select the TEF1 promoter to control Crane’s expression of heme-containing protein and one or more heme biosynthesis enzymes. A POSITA would have reasonably expected success in following the well-known techniques disclosed in Vogl’s prior studies to construct similar *P. pastoris* expression systems using only routine techniques, known methods, and readily available materials for genetic engineering and recombinant protein production. *See supra* § IV.C.

241. Thus, a POSITA would have found claim 6 obvious over Crane and Vogl.

7. Claim 7 (Crane and Vogl)

The yeast cell of claim 5, wherein the methanol-inducible promoter element from *P. pastoris* is an alcohol oxidase I (AOX1) promoter element.

242. In my opinion, Crane in view of Vogl renders claim 7 obvious. Claim 7 depends from claim 5. For the reasons provided above for claim 5, Crane in view of Vogl discloses claim 5.

243. As discussed in §X.B.3, a POSITA would have had a variety of well-known promoters to select among for use in a *P. pastoris* expression system, including methanol-inducible promoters. One of the promoters that could be used in this system is the methanol-inducible promoter of the *AOX1* gene.

244. A POSITA would be familiar with the advantages of the promoter of the *AOX1* gene and would be motivated to utilize it in the claimed recombinant nucleic acid molecule, for example, in order to drive expression of the claimed heterologous polypeptide (gene sequence of interest). These advantages are summarized in the Vogl review. First, as a POSITA would know, and Vogl teaches, this promoter is a promoter for genes of the methanol utilization pathway (MUT). As Vogl explains, MUT gene promoters are “one of the most favorable” aspects of using methylotrophic yeast species such as *P. pastoris* because they afford control over gene expression.

One of the most favorable properties of P. pastoris and the other methylotrophic yeasts are the strong methanol inducible promoters present in these organisms. Certain genes of the methanol utilization (MUT) pathway are completely repressed under growth on repressing carbon sources (e.g. glucose), but are strongly induced on methanol, constituting up to 20–30% of total intracellular protein [15,16]. This tight regulation is exerted on the transcriptional level, making MUT promoters ideal tools for heterologous protein production [17].

EX-1005(Vogl), 386.

245. Due to their well-known advantages, these MUT gene promoters are some of the best-studied promoters for use in a *P. pastoris* expression system. In my experience, a POSITA would be generally motivated to choose a well-studied promoter for use in an expression system as a matter of efficiency. As a general matter, a POSITA would anticipate success based upon the previous results reported in the literature. Thus, as a POSITA would recognize, using a well-studied promoter desirably saves research time and effort. A POSITA would recognize that these benefits apply to use of the promoter of the *AOX1* gene in particular:

Therefore also *most of the published knowledge about the regulation of P. pastoris promoters is focused on the most commonly used classic P. pastoris promoters, the promoters of the MUT pathway gene alcohol oxidase 1 (P_{AOX1}), its rarely used homolog P_{AOX2}, and also the glycolysis gene glyceraldehyde 3-phosphate dehydrogenase (P_{GAP}), which was described as a constitutive promoter in the early days of development.*

EX-1005(Vogl), 386.

246. Vogl teaches other benefits of using the promoter of the *AOX1* gene in order to drive recombinant gene expression. One advantage of using this promoter in at least some circumstances is that this promoter drives gene expression at relatively high levels—that is, it is a “strong” promoter. Indeed, Vogl teaches that the promoter of the *AOX1* gene yields “the highest” expression levels of gene sequences of interest. Additionally, as taught in Vogl, this promoter’s activity can

be readily controlled or modulated by changing the carbon source consumed by the *P. pastoris* yeast cells. A POSITA would recognize that this level of control over this promoter is advantageous because it affords a POSITA the ability to turn expression of genes regulated by this promoter “on” and “off” with ease. In particular, Vogl teaches using the promoter of the *AOX1* gene enables cells to be grown to high densities before gene expression is induced by methanol. In my experience, it is standard to grow *Pichia* to a high cell density on glycerol prior to feeding with methanol. Otherwise, methanol-induced activation of *AOX1* slows down cell growth.

P_{AOX1} is a ***remarkably strong*** promoter and at the same time ***directly controllable by simply changing the inexpensive carbon source***, it is the ***most commonly used promoter*** for heterologous protein expression in *P. pastoris*. The highest expression levels of heterologous proteins in *P. pastoris* have been achieved with P_{AOX1} (up to 22 g/l intracellular and 15 g/l secreted protein) [25,26]. Concerning controlled bioreactor cultivation, the tight regulation of P_{AOX1} is used to enable cell growth to high densities on a repressing carbon source without heterologous protein expression hampering the cellular metabolism.

EX-1005(Vogl), 386.

The major advantages of P_{AOX1} driven expression are the strength and the tight regulation of this promoter. Especially for the production of toxic or detrimental heterologous proteins, ***the inducibility with methanol is favorable.*** Cultures can at first be grown to high cell

densities under repressing conditions and in a second phase, heterologous protein production can be started by methanol induction [3]. However, this requires a two-step cultivation procedure, which increases the process time and handling efforts.

EX-1005(Vogl), 386.

247. Vogl further observed that Takagi “seem[s] to oppose previous findings by Lin-Cereghino et al., where overexpression of *MXR1* under control of the AOX1 promoter caused a growth defect on methanol and inhibited expression of [methanol utilization] genes.” EX-1005(Vogl), 391 (citing EX-1007(Hartner)). Therefore, a POSITA looking to Vogl would understand that an expression system constructed with *MXR1* and promoters such as the AOX1 promoter is desirable and would have a reasonable expectation of success when constructing such an expression system.

248. Accordingly, a POSITA would be motivated to select the promoter of the *AOX1* gene for use in driving the expression of gene sequences of interest in a *P. pastoris* expression system. In light of its well-known and well-characterized activity and structure, a POSITA would reasonably expect success in doing so.

249. Moreover, in light of the extensive published literature on this promoter (reviewed in Vogl between pages 386 and 392), only ordinary skill would be required to insert the sequence of the promoter of the *AOX1* gene into a recombinant expression vector, which could be used to transform a methylotrophic yeast cell such as *P. pastoris*.

250. Accordingly, claim 7 is obvious over Crane in view of Vogl.

8. Claim 8 (Crane and Vogl)

The yeast cell of claim 1, wherein the promoter element from *P. pastoris* comprises a constitutive promoter element from *P. pastoris* and a methanol-inducible promoter element from *P. pastoris*.

251. In my opinion, Crane in view of Vogl renders claim 8 obvious. Claim 8 depends from claim 1. For the reasons provided above for claim 1, Crane in view of Vogl discloses each limitation of claim 1.

252. As explained more fully in Section X.A.6, Vogl discloses using constitutive and methanol-inducible promoters from *P. pastoris* to control recombinant protein expression in *P. pastoris*, a POSITA would have been motivated to select suitable constitutive and methanol-inducible promoters, including two different promoters, for the expression of different proteins within Crane's system. A POSITA would recognize that it could be advantageous to induce expression of certain gene sequences while constitutively expressing other gene sequences, as I discuss more fully in in Section X.A.6 above. For example, in Table 1, Vogl lists over twenty constitutive or methanol-inducible promoters that can be used to provide varying levels of protein expression in *P. pastoris*, and it discusses studies, such as Takagi, in which two different proteins of interest were expressed under two different types of promoters—phytase under the methanol-inducible promoter of the *AOX1* gene and Mxr1 under the constitutive promoter of the GAP

gene. EX-1005(Vogl), 391, 393. As taught in Vogl, Takagi's approach advantageously resulted in the production of a recombinant protein (separately under the control of the methanol-inducible promoter of the *AOX1* gene) that was 7.6-fold higher than that produced by yeast cells lacking Mxr1 expression. EX-1005(Vogl), 391. EX-1005(Vogl), 393. For all the reasons explained in Section X.A.6, the use of both types of promoters in the same *P. pastoris* strains would have been obvious to a POSITA.

253. Thus, claim 8 would have been obvious over Crane and Vogl.

9. Claim 9 (Crane and Vogl)

The yeast cell of claim 8, wherein the constitutive promoter element from *P. pastoris* is a promoter element from a transcriptional elongation factor EF-1 (TEF1) gene.

254. In my opinion, this claim is obvious for reasons discussed above in Sections XII.A.6 and XII.A.8. A POSITA would have been motivated to select the TEF1 promoter as one of the promoters recited by claim 1 and would have a reasonable expectation of success in doing so.

10. Claim 10 (Crane and Vogl)

The yeast cell of claim 8, wherein the methanol-inducible promoter element from *P. pastoris* is an alcohol oxidase I (AOX1) promoter element.

255. In my opinion, this claim is obvious for reasons discussed above in Sections XII.A.7 and XII.A.8. A POSITA would have been motivated to select the

AOX1 promoter as one of the promoters recited by claim 1 and would have a reasonable expectation of success in doing so.

11. Claim 11 (Crane and Vogl)

The yeast cell of claim 1, wherein the nucleic acid molecule encoding the heme-containing protein is present in the yeast cell in at least two copies.

256. In my opinion, Crane in view of Vogl renders this claim obvious. Claim 11 depends from claim 1. For the reasons provided above for claim 1, Crane in view of Vogl discloses each limitation of claim 1.

257. Crane expressly discloses “an expression system and one or more expression constructs encoding a recombinant heme-binding protein.” EX-1010(Crane), Claim 16. A POSITA would have understood that an expression system comprising **more than one** expression construct would include at least two copies of the nucleic acid molecule encoding a recombinant heme-binding protein and would result in higher expression levels of the heme-binding protein.

258. Thus, a POSITA would have found claim 11 obvious over Crane in view of Vogl.

12. Claim 12 (Crane and Vogl)

The yeast cell of claim 1, wherein the nucleic acid molecule encoding the heme-containing protein operably linked to the promoter element from *P. pastoris* and the Mxr1

transcriptional activator sequence from *P. pastoris* further comprises a termination sequence.

259. In my opinion, Crane in view of Vogl renders claim 12 obvious. Claim 12 depends from claim 1. For the reasons provided above for claim 1, Crane in view of Vogl discloses each limitation of claim 1. As explained more fully for claim 1, Vogl teaches, for example, a POSITA would be motivated to express the nucleic acid molecule encoding Crane's heme-containing protein operably linked to a promoter element from *P. pastoris* in a yeast cell comprising the Mxr1 transcriptional activator sequence from *P. pastoris*, and would reasonably expect success doing so, because Mxr1 was well-known to drive transcription via binding various *P. pastoris* promoters (e.g., PEX8, AOX1, DAS). Thus, Mxr1 would be expected to drive expression of the heme-containing protein operably linked to a promoter that Mxr1 binds and activates.

260. As explained more fully in X.C.3, Crane further expressly discloses the limitation of claim 12. Crane claims an expression system "wherein the one or more expression constructs further comprise a promoter sequence, a nucleic acid encoding a ribosome binding sequence, and a nucleic acid encoding a **termination sequence**." EX-1010(Crane, Claim 22. Moreover, a POSITA would have understood that termination sequences were routinely used in expression vectors for *P. pastoris*. EX-1015(Lin-Cereghino 2000), 48.

261. Thus, claim 12 would have been obvious over Crane and Vogl.

13. Claim 13: (Crane and Vogl)

The yeast cell of claim 1, wherein the nucleic acid encoding the at least one polypeptide involved in heme biosynthesis operably linked to the promoter element from *P. pastoris* and the Mxr1 transcriptional activator sequence from *P. pastoris* further comprises a termination sequence.

262. In my opinion, Crane discloses the limitations of Claim 13. Claim 13 depends from claim 1. For the reasons provided above for claim 1, Crane in view of Vogl discloses each limitation of claim 1. As explained more fully for claim 1, Vogl taught, for example, a POSITA would be motivated to express Crane's nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis operably linked to the promoter element from *P. pastoris* in a yeast cell comprising the Mxr1 transcriptional activator sequence from *P. pastoris*, and would reasonably expect success doing so, because Mxr1 was well-known to drive transcription via binding various *P. pastoris* promoters (e.g., PEX8, AOX1, DAS). Thus, Mxr1 would be expected to drive expression of the heme biosynthesis polypeptide operably linked to a promoter that Mxr1 binds and activates.

263. As explained more fully in X.C.3 and X.C.4, Crane further expressly discloses the limitation of claim 13. Crane discloses a nucleic acid molecule encoding ferrochelatase, which is a heme biosynthetic enzyme. EX-1010(Crane), Claim 16 (“[A]n expression system and one or more expression constructs encoding a recombinant heme-binding protein and a recombinant ferrochelatase.”). In

addition, Crane claims an expression construct that “further comprise[s] a promoter sequence, a nucleic acid encoding a ribosome binding sequence, and a nucleic acid encoding a **termination sequence.**” EX-1010(Crane), Claim 22. Moreover, a POSITA would have understood that termination sequences were routinely used in expression vectors for *P. pastoris*. EX-1015(Lin-Cereghino), 48.

264. Therefore, Claim 13 would have been obvious over Crane in view of Vogl.

C. Ground 2B: Crane and Vogl in further view of Lin-Cereghino

1. Claim 14 (Crane, Vogl, and Lin-Cereghino)

The yeast cell of claim 1, further comprising a nucleic acid molecule encoding a Mxr1 transcriptional activator operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.

265. In my opinion, Crane in view of Vogl and Lin-Cereghino renders claim 14 obvious. Claim 14 depends from claim 1. For the reasons provided above for claim 1, Crane in view of Vogl discloses each limitation of claim 1.

266. As I explain above for Ground 1C, it would be obvious to a POSITA to engineer a yeast cell comprising a Mxr1 sequence from *P. pastoris* and a nucleic acid encoding Mxr1 driven by a *P. pastoris* promoter. A POSITA would recognize, for example, that Mxr1 binds and activates various *P. pastoris* promoters as I discuss above. When Mxr1 is expressed under the control of a Mxr1-driven promoter, Mxr1 would be expected to bind and further activate its own expression, leading to enhanced Mxr1 expression in the yeast cell. A POSITA would recognize that this

would be advantageous if, for example, expression of the gene of interest is also regulated by Mxr. Additionally, as Mxr1 activity is methanol-inducible, expression would be controllable due to the presence and level of methanol to which the yeast are exposed.

267. Vogl discloses that *P. pastoris* could be engineered to produce high yields of heterologous proteins through the use of the “strong, methanol inducible promoter of the alcohol oxidase 1 (*AOX1*) gene,” which is derived from *P. pastoris*. EX-1005(Vogl), 385. Vogl teaches that Mxr1 binds to six places in the *AOX1* promoter. EX-1005(Vogl), 390. Moreover, Vogl describes Mxr1 as “a key activator of methanol induced genes” in methylotrophic *P. pastoris* and “crucial” to the activation of the MUT gene promoters. EX-1005 389. As I explain above with respect to claim 1, Vogl taught various constitutive and inducible promoters (e.g., the constitutive promoters of the GAP and TEF1 genes, and the inducible promoters of the AOX1 and PEX8 genes), which a POSITA would be motivated to use in order to produce recombinant proteins of interest.

268. Lin-Cereghino would further motivate a POSITA to utilize a nucleic acid molecule encoding a Mxr1 operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*. In Lin-Cereghino, for example, *P. pastoris* cells expressed an increased amount of Mxr1 under the control of the *PEX8* promoter—derived from *P. pastoris*—in a methanol-

based medium. EX-1006(Lin-Cereghino), 893. Specifically, Lin-Cereghino screened for pGC217 strains with higher copy numbers of the P_{PEX8} -*MXR1* construct incorporated in the yeast genome and identified a “multicopy strain that grew slowly on methanol” but “contained relatively high levels of the product.” EX-1006(Lin-Cereghino), 893. Lin-Cereghino also teaches *P. pastoris* strains incorporating multiple copies of *MXR1* gene that produced high levels of Mxr1. *Id.* Thus, a POSITA would have been motivated to design rDNA constructs that not only co-express genes encoding heme-containing protein and heme biosynthetic enzymes under methanol-inducible promoters such as the *AOX1* promoter, but also increase Mxr1 expression, for example, by expressing Mxr1 under the *GAP* promoter in glucose-grown cells, or under the *PEX8* promoter in methanol-grown cells, or by increasing the copy number of *MXR1* gene, as taught by Vogl and Lin-Cereghino.

269. Additionally, to achieve recombinant protein expression in *P. pastoris* as I explain above, Vogl provides an overview of other promoters that can be used to drive gene expression, including constitutive promoters of the *GAP* and *TEF* genes. EX-1005(Vogl), 392-95. Vogl explains that promoters have different strengths. EX-1005(Vogl), 392-95. Vogl further explains that the *GAP* and *AOX1* promoters are both strong that “provide similar expression levels under the respective optimal growth conditions.” EX-1005(Vogl), 392. Vogl explains, however, that “the strong expression from the promoters of the *GAP* and *AOX1*

genes is not always the most favorable condition for heterologous protein production”; rather, “a weaker expression level can be more desirable” in some cases. EX-1005(Vogl), 392. Weaker promoters such as the promoters of the *PEX8* gene (inducible) and *YPT1* gene (constitutive) are “alternatives in case [AOX1 or GAP promoter] driven expression is too strong.” EX-1005(Vogl), 393. In Table 1, Vogl lists over 20 promoters that can be used to provide varying levels of protein expression in *P. pastoris* as desired by POSITAs. EX-1005(Vogl), 393.

270. As described more fully in Section X.D.1, Vogl further supports this. In particular, Vogl teaches that high levels of protein expression can be achieved by co-expressing a protein of interest under the control of the AOX1 promoter and high levels of *MXR1* under the control of the GAP promoter, a constitutive promoter. EX-1005(Vogl), 391. Vogl teaches that the methanol-inducible the AOX1 promoter provides “strong” levels of protein expression and that Mxr1 is a transcription factor that is crucial for methanol induced expression. EX-1005(Vogl), 393. Therefore, a POSITA looking to Vogl would understand that an expression system constructed with *MXR1* and promoters such as the AOX1 promoter is desirable and would have a reasonable expectation of success when constructing such an expression system.

271. Given this, a POSITA would have been motivated to increase the expression of *MXR1* by placing the gene under the control of a promoter and/or to regulate the promoter based on carbon source in growth medium. To do this, a

POSITA would look to the teachings in Lin-Cereghino which disclose the expression of *MXR1* using the methanol inducible PEX8 and AOX1 promoters. EX-1006(Lin-Cereghino), 885. As detailed above in Section X.D.1, a POSITA looking to implement expression of Mxr1 (*e.g.*, from a PEX8 promoter) that is capable of activating a gene operably linked to a AOX1 promoter in a *P. pastoris* expression system would first grow the cells to high densities while repressing recombinant protein expression. Then, once the cells have grown to a significant mass, a POSITA would derepress expression by depleting the glucose or glycerol in the medium and adding methanol. EX-1005(Vogl), 387; EX-1015(Lin-Cereghino_2000), 51-52; EX-1021(Sreekrishna), 7-8; EX-1022(Zhang), 149.

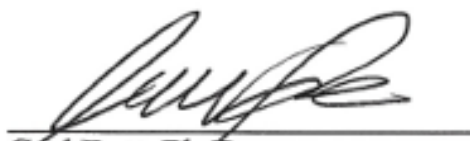
272. Accordingly, Claim 14 is obvious over Crane in view of Vogl and Lin-Cereghino.

XII. SECONDARY CONSIDERATIONS

273. I understand that “secondary considerations” may be legally relevant to an obviousness analysis. If Impossible presents such evidence, I will consider it; however, I do not believe that any commercial success of the claimed invention or other objective evidence would be attributable to the merits of the claimed invention given my analysis of the lack of novelty and the obviousness of the ’492 patent.

XIII. CONCLUSION

274. In signing this Declaration, I recognize that the Declaration will be filed as evidence in a contested case before the Patent Trial and Appeal Board of the United States Patent and Trademark Office. I also recognize that I may be subject to cross-examination in the case and that cross-examination will take place within the United States. If cross-examination is required of me, I will appear for cross-examination within the United States during the time allotted for cross-examination. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on the information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.



Carl Batt, Ph.D.

January 27, 2023

Date