

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. IPR2023-00321

U.S. Patent No. 10,689,656

“Expression Constructs and Methods of
Genetically Engineering Methylophilic Yeast”

**DECLARATION OF DR. CARL BATT IN SUPPORT OF
PETITION FOR *INTER PARTES* REVIEW OF U.S. PATENT NO. 10,689,656
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-1002	U.S. Patent No. 10,689,656 (“the ’656 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”)

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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”)
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”)

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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”)
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-1040	Prosecution History of U.S. Patent No. 10,689,656
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”)

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EX-1046	GenPept and Revision History Summaries for GenBank Accession No. AAA33980: leghemoglobin [Glycine max], 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
EX-1051	GenPept and Revision History Summaries for GenBank Accession No. ABD57365: methanol expression regulator I [Komagataella pastoris], NAT'L LIBR. MED.: NAT'L CTR. FOR BIOTECH. INFORMATION, <i>available at</i> https://www.ncbi.nlm.nih.gov/protein/ABD57365.1
EX-1052	GenPept and Revision History Summaries for GenBank Accession No. DQ395124: Pichia pastoris methanol expression regulator I gene, complete cds, NAT'L LIBR. MED.: NAT'L CTR. FOR BIOTECH. INFORMATION, <i>available at</i> https://www.ncbi.nlm.nih.gov/nuccore/DQ395124.1
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/

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EX-1054	Benson et al., <i>GenBank</i> , NUCLEIC ACIDS RESEARCH, 2014, Vol. 42, Database issue, D32-D37
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)
EX-1062	Kurtz et al., Development of Autonomously Replicating Plasmids for <i>Candida albicans</i> , 7 MOLECULAR & CELLULAR BIOLOGY 209-17 (1987)
EX-1063	Roggenkamp et al., <i>Transformation of the methylotrophic yeast Hansenula polymorpha by autonomous replication and integration vectors</i> , 202 MOLECULAR & GEN. GENETICS 302-08 (1986)

LIST OF CHALLENGED CLAIMS

Claim 1	
[1pre]	A methylotrophic yeast cell comprising
[1a]	a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises a first exogenous nucleic acid encoding a methanol expression regulator 1 (Mxr1) transcriptional activator operably linked to at least one methanol-inducible promoter element comprising a sequence to which the Mxr1 transcriptional activator binds,
[1b]	wherein the recombinant nucleic acid molecule comprises a second exogenous nucleic acid encoding a heterologous polypeptide operably linked to at least one methanol-inducible promoter element.
Claim 2	
The yeast cell of claim 1, wherein the methylotrophic yeast cell is of a yeast selected from the group consisting of <i>Candida</i> , <i>Hansenula</i> , <i>Pichia</i> and <i>Torulopsis</i> .	
Claim 3	
The yeast cell of claim 1, wherein the cell is a <i>Pichia</i> cell.	
Claim 4	
The yeast cell of claim 1, wherein the cell is a <i>Pichia pastoris</i> cell.	
Claim 5	
The yeast cell of claim 1, wherein the recombinant nucleic acid molecule is stably integrated into the genome of the methylotrophic yeast cell.	
Claim 6	
The yeast cell of claim 1, wherein the recombinant nucleic acid molecule is extrachromosomally expressed from a replication-competent plasmid.	
Claim 7	
The yeast cell of claim 1, wherein the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from <i>Pichia pastoris</i> .	
Claim 8	
The yeast cell of claim 1, wherein the transcriptional activator comprises the sequence shown in SEQ ID NO: 59.	

Claim 9
The yeast cell of claim 8, wherein the transcriptional activator is encoded by the nucleic acid sequence shown in SEQ ID NO: 60.
Claim 10
The yeast cell of claim 1, wherein the at least one methanol-inducible promoter element is selected from the group consisting of an alcohol oxidase 1 (AOX1) promoter element from <i>Pichia pastoris</i> , an alcohol oxidase (AOD1) promoter element from <i>Candida boidinii</i> , a methanol oxidase (MOX) promoter element from <i>Hansenula polymorpha</i> , a methanolic alcohol oxidase (MOD1) promoter element from <i>Pichia methanolica</i> , a dihydroxyacetone synthase (DHAS) promoter element from <i>Pichia pastoris</i> , a formaldehyde dehydrogenase (FLD1) promoter element from <i>Pichia pastoris</i> , and a peroxin 8 (PEX8) promoter element from <i>Pichia pastoris</i> .
Claim 11
The yeast cell of claim 1, wherein the at least one methanol-inducible promoter element is an alcohol oxidase 1 (AOX1) promoter element from <i>Pichia pastoris</i> .
Claim 12
The yeast cell of claim 1, wherein the second exogenous nucleic acid encodes at least one heterologous polypeptide involved in biosynthesis of an iron co-factor.
Claim 13
The yeast cell of claim 12, wherein the iron co-factor is heme.
Claim 14
The yeast cell of claim 13, wherein the at least one polypeptide involved in the biosynthesis of heme is selected from the group consisting of aminolevulinic acid (ALA) synthase, ALA dehydratase, porphobilinogen deaminase, uroporphyrinogen (UPG) III synthase, UPG III decarboxylase, coproporphyrinogen (CPG) oxidase, protoporphyrin (PPG) oxidase, and ferrochelatase.
Claim 15
The yeast cell of claim 13, wherein the at least one polypeptide involved in the biosynthesis of heme comprises two or more polypeptides selected from the group consisting of ALA synthase, ALA dehydratase, porphobilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and ferrochelatase.

Claim 16
A method for expressing a heterologous polypeptide in a cell, the method comprising: providing the yeast cell of claim 1; and culturing the cell under conditions for expression of the recombinant nucleic acid molecule, thereby expressing the heterologous polypeptide.
Claim 17
The method of claim 16, where in the heterologous polypeptide is a member of globin family PF00042.
Claim 18
The method of claim 16, where in the heterologous polypeptide is a plant hemoglobin.
Claim 19
The method of claim 18, where in the plant hemoglobin is leghemoglobin.
Claim 20
The method of claim 19, where in the leghemoglobin has a sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:6.
Claim 21
The method of claim 16, where in the introducing step comprises a technique selected from the group consisting of transduction, electroporation, biolistic particle delivery, and chemical transformation.
Claim 22
The method of claim 16, where in the conditions under which the cell is cultured comprises addition of iron or a pharmaceutically or metabolically acceptable salt thereof.
Claim 23
The method of claim 16, where in the culturing step comprises culturing the cell in the presence of methanol.
Claim 24
The method of claim 16, where in the culturing step comprises culturing the cell in the absence of methanol.
Claim 25
The method of claim 16, where in the culturing step comprises culturing the cell in the absence of selection.

Claim 26	
[26pre]	A methylotrophic <i>Pichia</i> yeast cell comprising
[26a]	a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises:
[26b]	a nucleic acid encoding a Mxr1 transcriptional activator sequence from <i>P. pastoris</i> ;
[26c]	a nucleic acid encoding a member of globin family PF00042; and a nucleic acid encoding at least one polypeptide involved in heme biosynthesis, wherein each nucleic acid is operably linked to a methanol-inducible promoter element, wherein the methanol-inducible promoter element comprises at least one sequence to which a Mxr1 transcriptional activator binds.
Claim 27	
The yeast cell of claim 26, wherein the member of the globin family is leghemoglobin.	
Claim 28	
The yeast cell of claim 26, where in the methanol-inducible promoter element is an alcohol oxidase 1 (AOX1) promoter element from <i>P. pastoris</i> .	

I, Carl Batt, 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 1-28 (the “Challenged Claims”) of U.S. Patent No. 10,689,656 (the “’656 patent”). Based on the first page of the ’656 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 ’656 patent claims in this declaration (“Declaration”).

3. I am informed that the ’656 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 ’656 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 '656 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 ’656 patent, I have considered the ’656 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 ’656 patent’s earliest possible priority date. I have also reviewed the prosecution (the “file history”) of the ’656 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 ’656 patent.

20. I have relied on my personal knowledge, education, training, and professional experience in order to form my opinions concerning the '656 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 '656 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, in 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.

35. 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 '656 PATENT

A. Level of Ordinary Skill in the Art

36. The '656 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

37. The '656 patent contains 28 claims, 2 independent and 26 dependent claims.

38. Claim 1 is the first independent claim and is reproduced below.

A methylotrophic Pichia yeast cell comprising:

a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises a first exogenous nucleic acid encoding a methanol expression regulator 1 (Mxr1) transcriptional activator operably linked to at least one methanol-inducible promoter element comprising a sequence to which the Mxr1 transcriptional activator binds,

wherein the recombinant nucleic acid molecule comprises a second exogenous nucleic acid encoding a heterologous polypeptide operably linked to at least one methanol-inducible promoter element.

39. The claims dependent on Claim 1 are generally directed to a product and method comprising:

- *A Pichia pastoris (P. pastoris) yeast cell (claims 2, 3, 4);*

- The recombinant nucleic acid molecule is stably integrated into the genome of the *P. pastoris* yeast cell (claim 5);
- The recombinant nucleic acid molecule is extrachromosomally expressed from a replication-competent plasmid (claim 6);
- An Mxr1 transcriptional activator sequence from *P. pastoris* (claim 7);
- The Mxr1 transcriptional activator with the sequence shown in SEQ ID NO: 59 (claim 8);
- The Mxr1 transcriptional activator is encoded by the nucleic acid sequence shown in SEQ ID NO: 60 (claims 9);
- At least one methanol-inducible promoter element selected from the group consisting of an AOX1 promoter element from *P. pastoris*, an AOD1 promoter element from *Candida boidinii*, a MOX promoter element from *Hansenula polymorpha*, a MOD1 promoter element from *Pichia methanolica*, a DHAS promoter element from *P. pastoris*, an FLD1 promoter element from *P. pastoris*, and a PEX8 promoter element from *P. pastoris* (claim 10);
- A *P. pastoris* AOX1 promoter element (claim 11);

- At least one heterologous polypeptide involved in the biosynthesis of an iron co-factor (claim 12);
- The iron co-factor is heme (claim 13);
- The at least one polypeptide involved in the biosynthesis of heme 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 (claim 14);
- And at least two polypeptides involved in the biosynthesis of heme 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 (claim 15).
- A method for expressing a heterologous protein from the *P. pastoris* cell by culturing the cell under certain conditions (claim 16);
- the polypeptide is from globin family PF00042 (claim 17);
- The polypeptide is a plant hemoglobin (claim 18);
- The plant hemoglobin is leghemoglobin (LegH) (claim 19);

- The LegH with the sequence shown in either SEQ ID NO:4 or SEQ ID NO:6 (claim 20);
- The introduction step comprises either transduction, electroporation, biolistic particle delivery, or chemical transformation (claim 21);
- The inclusion of iron or a pharmaceutically or metabolically acceptable salt thereof in the conditions under which the cell is cultured (claim 22);
- The cell is cultured in methanol (claim 23);
- The cell is cultured in the absence of methanol (claim 24);
- The cell is cultured in the absence of selection (claim 25);

40. The second independent claim, Claim 26, is reproduced below.

A methylotrophic Pichia yeast cell comprising

a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises:

a nucleic acid encoding a Mxr1 transcriptional activator sequence from P. pastoris;

a nucleic acid encoding a member of globin family PF00042; and a nucleic acid encoding at least one polypeptide involved in heme biosynthesis, wherein each nucleic acid is operably linked

to a methanol-inducible promoter element, wherein the methanol-inducible promoter element comprises at least one sequence to which a Mxrl1 transcriptional activator binds.

41. The claims dependent on Claim 26 are generally directed to a product comprising:

- a member of the globin family that is LegH (claim 27);
- and the methanol-inducible promoter element is the AOX1 promoter element from *P. pastoris*.

C. Specification

42. The '656 patent describes well-known “[m]ethods and materials for genetically engineering methylotrophic yeast.” EX-1002, Abstract. The '656 patent states that a methylotrophic *P. pastoris* expression system can produce proteins by operably linking the promoter sequence “in such a way as to direct or regulate expression of the [exogenous] nucleic acid.” EX-1002, 4:50-51. The '656 patent states 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-1002, 7:11-14.

43. Many aspects of the '656 patent are admittedly disclosed in the prior art. The '656 patent states that transcriptional activator Mxr1, a key regulator of the

methanol utilization pathway, is “known in the art.” EX-1002, 4:25. The ’656 patent teaches that the Mxr1 sequence should be placed “under control of a promoter that is inducible” for Mxr1 co-expression. EX-1002, 4:44-47. In addition, the ’656 patent identifies “methanol inducible promoters [that] are known in the art,” such as the “commonly used methanol-inducible promoter from *P. pastoris*.” EX-1002, 4:55-59.

44. The ’656 patent refers to several promoter elements that can be constructed into the claimed expression system. For example, the ’656 patent provides that the AOX1 promoter can be used to direct gene expression in methylotrophic *P. pastoris*. EX-1002, 4:56-60. The ’656 patent states that the AOX1 promoter “is strongly transcribed in response to methanol.” The expression system of the ’656 patent claims a construct “wherein the methanol-inducible element from *P. pastoris* is an alcohol oxidase I (AOX1).” EX-1002, 90:16-17.

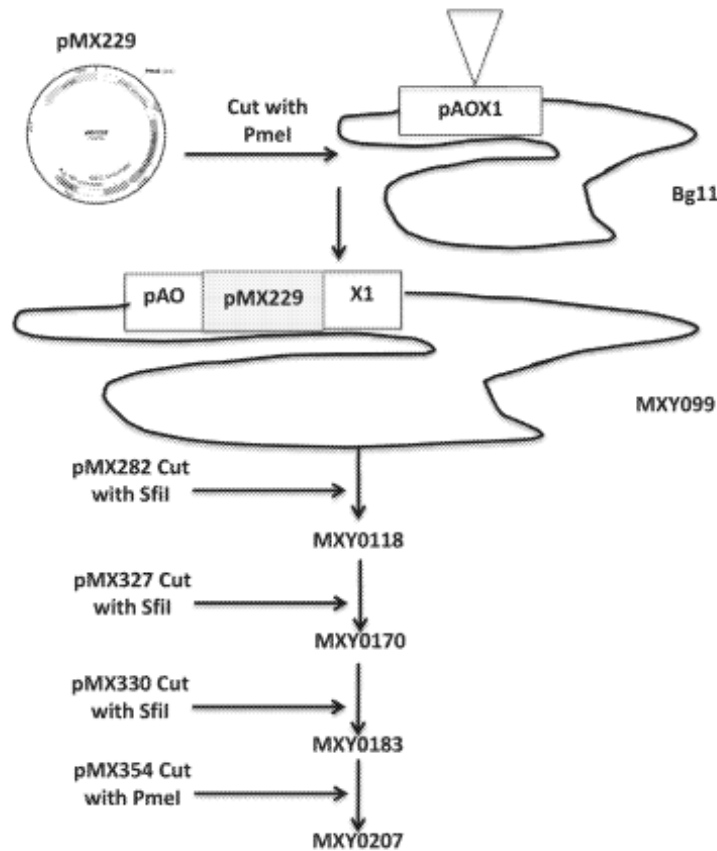


Figure 3

45. 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-1002, 5:58-60, 6:37-44.

46. In order to fully incorporate heme-containing proteins, the ’656 patent recognizes that certain heme biosynthetic enzymes must be coexpressed with heme-containing proteins. For example, a heme-containing protein claimed by the ’656

patent is LegH. EX-1002, 89:1-2, 90:14-15. The '656 patent claims co-expression of "at least one polypeptide involved in the biosynthesis of heme" from the following group: ALA synthase, ALA dehydratase, porphobilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and/or ferrochelatase. EX-1002, 88:45-51.

47. The heme biosynthetic enzyme is encoded within a plasmid that is operably linked to the promoter and MXR1. EX-1002, 87:31-41.

D. Prosecution History

48. Based on my review of the first page of the '656 patent (EX-1002), I see that U.S. Application No. 15/573,300 (the "'300 application") was filed on May 11, 2016, and issued as the '656 patent on June 23, 2020. EX-1002. I understand that the '656 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.

49. I have reviewed the prosecution history of the '656 patent. The '300 application was filed on May 11, 2016. EX-1040, 1. I see that the claims were issued a non-final rejection on July 16, 2019. EX-1040, 1261. The Examiner rejected then-pending claims 1-5, 7, 12-14, and 19 as anticipated by U.S. 2014/008729 ("Zhou") as evidenced by U.S. Pub. No. 2015/0299716. EX-1040, 1266. I understand that the Examiner found that these claims were anticipated by Zhou's disclosure of methylotrophic yeast comprising an exogenous nucleic acid encoding the transcription factor Mit1 linked to a methanol-inducible promoter. EX-1040, 1267. The Examiner found that claims 1-19 and 30-38 were obvious over Zhou in view of PCT Publication No. WO 2015/153,666 (Varadan), Lin-Cereghino (2006), and Liu (2014). EX-1040, 1268. The Examiner found that Varadan taught *P. pastoris* comprising globin and heme biosynthesis molecules, and that the promoter may be *AOXI*. EX-1040, 1269. The Examiner found that Lin-Cereghino disclosed Mxr1 is a transcriptional activator when methanol is utilized, and plasmids comprising Mxr1 under *AOXI/PEX8* control. EX-1040, 1269-70. The Examiner found Liu taught expression of globin polypeptides under control of a constitutive promoter. EX-1040, 1271. The claims were also rejected for nonstatutory double patenting

over the claims of U.S. Patent Nos. 9,938,327 and 10,273,492 and co-pending Application No. 16/123,121. EX-1040, 1271-74.

50. The applicant filed a terminal disclaimer over the '327 patent claims, which was granted. EX-1040, 1293. In response to the non-final rejection, the applicant amended the claims to require Mxrl to avoid anticipation by Zhou and argued that the Varadan should not be prior art prior art. EX-1040, 1402.

51. The Examiner issued a final rejection. EX-1040, 1428-33, maintaining the nonstatutory double patenting rejection over co-pending Application No. 16/123,121. The applicant then asserted that both the '300 and '121 applications were commonly owned. EX-1040, 1445.

52. The claims were thereafter allowed. In the Notice of Allowance, the Examiner stated (EX-1040, 1479):

The prior art fails to disclose or suggest a methylotrophic yeast cell comprising a methanol expression regulator 1 (Mxrl) transcriptional activator, a methanol-inducible promoter element to which the Mxrl transcriptional activator binds, and a nucleic acid encoding a heterologous polypeptide. The yeast can be *Pichia pastoris*. The prior art fails to disclose or suggest a method of producing a polypeptide by culturing the methylotrophic yeast under conditions for expressing the polypeptide, the polypeptide can be a member of the heme family.

V. CLAIM CONSTRUCTION

53. In my opinion, the '656 patent's claims need no construction for purposes of this proceeding. Therefore, they should be given their plain and ordinary meaning.

VI. STATE OF THE ART

A. Recombinant DNA Technology

54. 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.

55. 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.

56. 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.

57. 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.

58. 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 peptide, 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

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.

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

59. 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

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.

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

60. 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.

61. 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.

62. Not every host cell takes up the rDNA. EX-1018(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 the antibiotic. *Id.*

B. Protein Expression Systems

63. 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

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.

64. 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 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.

65. By the early 2000s, POSITAs understood that *P. pastoris*—as well as other “methylophilic” 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.

66. 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

67. 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.

68. 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

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.

69. 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 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), PPER3 (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 (“P_{AOX1}”), the dihydroxyacetone synthase promoter (“P_{DAS}”), and the peroxisomal matrix protein promoter (“P_{PEX8}”), among others. EX-1005(Vogl), 385-87, 392-95; EX-1015(Lin-Cereghino_2000), 46-48; EX-1021(Sreekrishna), 7; EX-1029(Ahmad), 5302-04.

70. 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.

71. 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.

72. 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.

73. 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.

74. 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.

75. 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”):

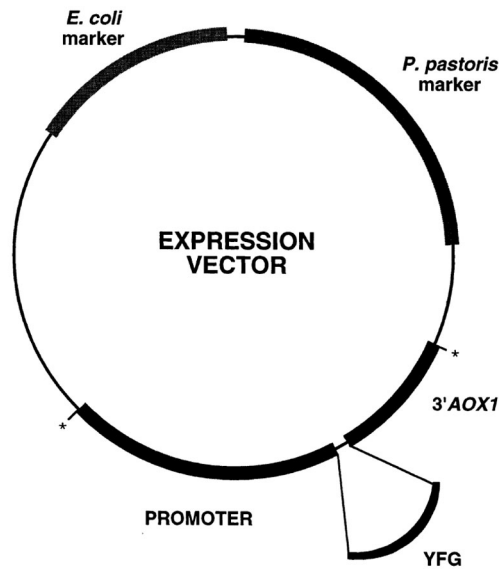


Fig. 3. General diagram of a *P. pastoris* expression vector. YFG, 'Your Favorite Gene'; * sites for cassette amplification.

EX-1015(Lin-Cereghino_2000), 47-48.

76. 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

77. 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_2000), 47, 51-52; EX-1029(Ahmad), 5302.

78. 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_2000), 51-52; EX-1029(Ahmad), 5302.

79. 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.

80. 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_2000), 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.

81. 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

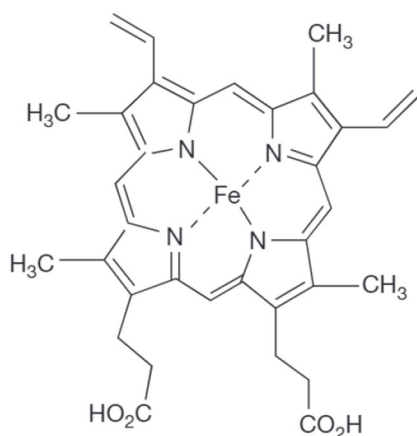
82. The AOX1 promoter is regulated by a transcriptional activator called methanol expression regulator 1 (“Mxr1”), sometimes also referred to as Mxr1 protein (“Mxr1p”). Mxr1 is “an important transcription factor” and “global regulator” of genes involved in methanol utilization in *P. pastoris*. EX-1026(Parua), 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.

83. 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

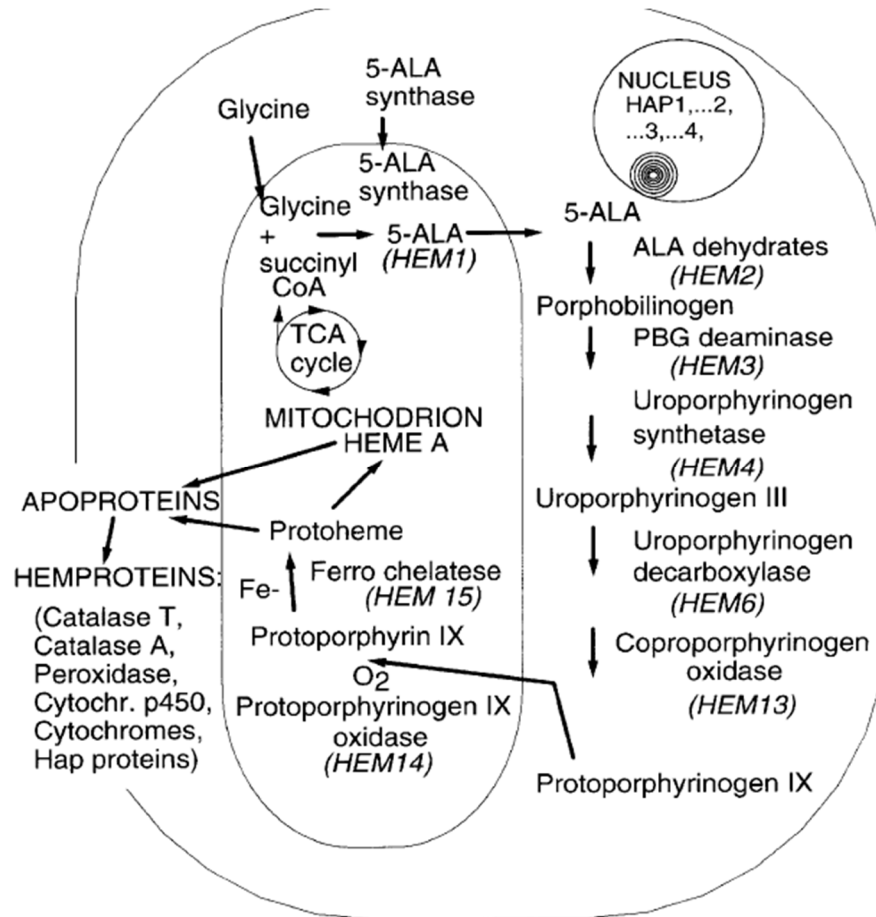
84. Microbial rDNA gene expression systems are employed routinely to produce an important class of proteins known as heme-containing proteins, or hemoproteins. EX-1008(Mattoon); EX-1009(Elrod); EX-1010(Crane); EX-1011(Liu); EX-1031(Krainer). Heme-containing proteins are a group of proteins that contain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



85. 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.

86. 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:

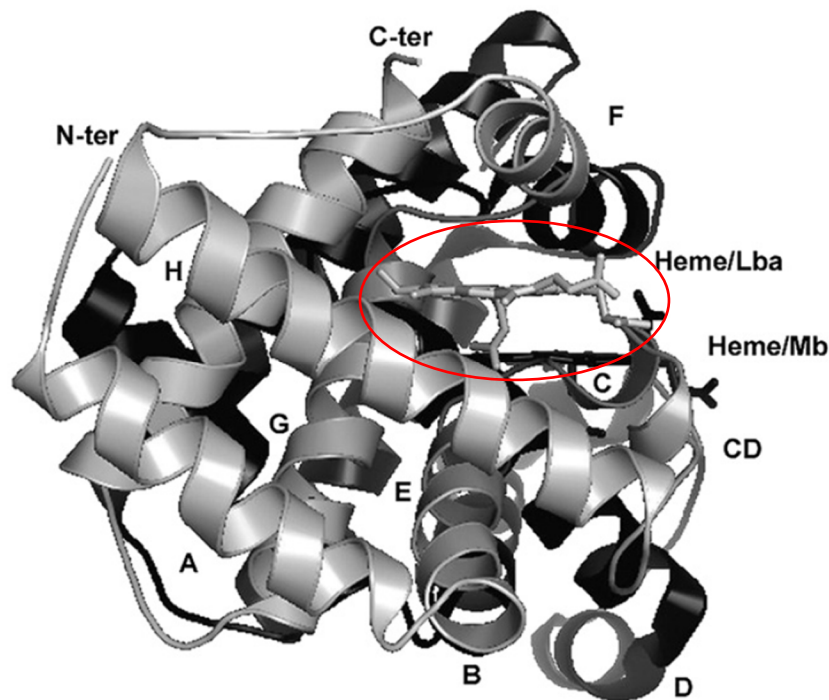


EX-1008(Mattoon), Fig. 1.

87. 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.

88. An exemplary 3-D heme-containing protein structure (with overlays of the soybean (leghemoglobin Lba) and spermwale (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. OVERVIEW OF KEY REFERENCES

A. Tsutsumi

89. I have reviewed U.S. Patent Application Publication No. 2011/0129874 to Tsutsumi and Takagi (“Tsutsumi”), titled “*Pichia Pastoris DAS Promoter Variants*.” I understand that Tsutsumi is considered prior art to the ’656 patent under (post-AIA) 35 U.S.C. § 102(a)(1) as it published on June 2, 2011.

90. Tsutsumi discloses that “it has been known for long that certain promoters are dependent on the presence of methanol in the growth medium for the induction of transcription” and that methanol “[c]ontrolled expression is often desirable.” EX1024(Tsutsumi), [0005], [0041]. Tsutsumi further teaches that Mxr1 is “a key positive regulator required for methanol utilization in *Pichia pastoris*.” .” EX1024(Tsutsumi), [0005].

91. Tsutsumi teaches a methylotrophic yeast expression system for expressing exogenous proteins under the control of a methanol-inducible promoter, particularly the *DAS* promoter. It further teaches co-expressing the transcriptional activator Mxr1 under the control of the *GAP* promoter, where “overexpressed” Mxr1 drives expression of the exogenous protein. EX1024(Tsutsumi), [0002], [0005], [0038], [0040], [0041], [0145]. Tsutsumi teaches increasing expression of Mxr1p in *P. pastoris*, for example, “by controlling the expression of Mxr1 [*e.g.*, by a strong promoter] or by increasing the copy number of the gene encoding Mxr1,” which

resulted in “improved expression” of a protein under the control of a promoter that is activated by Mxr1p. EX-1024(Tsutsumi), [0075], [0137]-[0145].

B. Mattoon

92. I have reviewed U.S. Patent No. 5,824,511, a patent issued to Mattoon et al. on October 20, 1998. (“Mattoon”) (EX-1008). I understand that Mattoon is considered prior art to the ’656 patent under (post-AIA) 35 U.S.C. § 102(a)(1) as it was issued in 1998.

93. 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.

94. 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.

95. 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.

C. Crane

96. 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.). I understand that Crane is considered prior art to the ’656 patent under (post-AIA) 35 U.S.C. § 102(a)(1) as it was published in 2011.

97. 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] *leghemoglobin*.” 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].

98. 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

99. I have reviewed an article authored by Lin-Cereghino et al., entitled *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 2006 (“Lin-Cereghino (2006)”). (EX-1006). 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 2006 is considered prior art to the ’656 patent under (post-AIA) 35 U.S.C. § 102(a)(1), as it was published in 2006.

100. 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.

101. Lin-Cereghino reported that the yeast strains that produced Mxr1p under the weaker methanol-inducible PEX 8 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.

E. Vogl

102. I have reviewed an article authored by Vogl and Glieder, entitled *Regulation of Pichia pastoris promoters and its consequences for protein production*, published in volume 30 of New Biotechnology (pages 385-404) in 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 '656 patent under (post-AIA) 35 U.S.C. § 102(a)(1) as it was published in 2013.

103. 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.

104. 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.

105. 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.

106. 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)).

107. 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)).

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

F. Krainer

109. I have reviewed Krainer et al., *Optimizing cofactor availability for the production of recombinant heme peroxidase in Pichia pastoris*, 14 Microbial Cell Factories 4, which published on January 13, 2015 (“Krainer”). As a person of ordinary skill in the art, I am familiar with the journal “Microbial Cell Factories”, which is a highly-reputable scientific journal reporting research in the field of biotechnology and recombinant protein expression systems, as well as its publisher. 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 Krainer reference was published in and available to a POSITA exercising reasonable diligence on the date of publication, if not before. Accordingly, I understand that Krainer is considered prior art to the ’656 patent under (post-AIA) 35 U.S.C. § 102(a)(1), as it was published in January 2015.

110. Krainer describes certain *P. pastoris* strains that co-expressed (1) the hemoprotein horseradish peroxidase (“HRP”) and (2) one of eight heme biosynthetic enzymes. EX-1031(Krainer), 2-4. Krainer reports that co-expressing HEM1 or HEM3 with HRP increased HRP production. EX-1031(Krainer), 3-4. Krainer further discloses “medium supplementation with iron, heme precursors or hemin (the

ferric chloride species of heme) was assessed as alternative strategy to effectively saturate recombinant apo-species of heme proteins [13-17].” EX-1031(Krainer), 2-3. Krainer concludes “FeSO₄ and hemin ... to be useful medium supplements to increase the yield of active heme protein.” EX-1031(Krainer), 6.

VIII. SUMMARY OF OPINIONS

111. In my opinion, claims 1-28 are unpatentable based on the following grounds:

Ground	Claims	Basis
1	1-17, 21, 23-25	§103, Tsutsumi, Lin-Cereghino, Mattoon
2	18-20	§103, Tsutsumi, Lin-Cereghino, Mattoon, Crane
3	22	§103, Tsutsumi, Lin-Cereghino, Mattoon, Krainer
4	26, 28	§103, Mattoon, Vogl
5	27	§103, Mattoon, Vogl, Crane

IX. GROUND 1: TSUTSUMI, LIN-CEREGHINO, AND MATTOON

A. Overview

112. In my opinion, the Tsutsumi, Lin-Cereghino and Mattoon references render obvious Claims 1-17, 21, and 23-25 of the '656 patent.

113. Tsutsumi is analogous art to the '656 patent because it discloses genetically modifying *P. pastoris* yeast cells to express recombinant proteins, as I

discuss further above in Section VII.A. For example, Tsutsumi, titled “*Pichia Pastoris* Das Promoter Variants,” discloses co-expression of vectors encoding Mxr1 and recombinant proteins of interest in *P. pastoris* in order to enhance the expression of the latter. *E.g.*, EX-1024(Tsutsumi), Example 5.

114. Lin-Cereghino is also analogous art to the '656 patent because it discloses genetically modifying *P. pastoris* yeast cells to express recombinant proteins, as I discuss further above in Section VII.D. The Lin-Cereghino researchers investigated the role of Mxr1 in regulating protein expression in *P. pastoris* by studying *MXR1* mutants. Lin-Cereghino observes, for example, that “[b]ecause *P. pastoris* is a major system for the production of recombinant proteins, an understanding of the factors that regulate methanol induction will further our comprehension of heterologous protein expression in this yeast,” and “[t]he characterization and, ultimately, the optimization of methanol-induced transcription could potentially improve its productivity as a host for important recombinant proteins needed for the treatment of diseases.” EX-1006(Lin-Cereghino), 896.

115. Mattoon is also analogous art to the '656 patent because it discloses genetically modifying *P. pastoris* yeast cells to express recombinant proteins, as I discuss further above in Section VII.B. For example, Mattoon, titled “Method for enhancing the production of hemoproteins,” teaches systems and methods for

enhancing recombinant heme-containing protein expression by co-expressing these proteins with members of their biosynthetic pathway.

116. As I discuss in greater detail below, a POSITA would be motivated to combine the teachings of each of these references in order to achieve the subject matter claimed in the '656 patent claims. A POSITA would reasonably expect success in doing so given the well-known and familiar techniques of vector construction and recombinant protein expression known in the art.

B. Claim 1

1. Preamble: *A methylotrophic yeast cell comprising a recombinant nucleic acid molecule ...*

117. The art long recognized the utility of methylotrophic yeast as a system for the recombinant expression of heterologous proteins. This general understanding is reflected in the Vogl and Crane references, among many others, which teaches the use of *P. pastoris* as an expression system to produce recombinant proteins. Crane further teaches the availability of strains, vectors, and methods employed in the study and use of *P. pastoris*, with emphasis on the production of functional heme-binding proteins using this system.

Methylotrophic yeasts like *Pichia pastoris*, *Candida boidinii*, *Hansenula polymorpha* (*Pichia angusta*) and *Pichia methanolica* are widely used host systems for heterologous protein production [1,2]. They unite the benefits of a eukaryotic expression system (efficient

secretion and post-translational modifications like glycosylations or the introduction of disulfide bonds) with favorable growth properties of robust unicellular organisms (fast growth on minimal media, no production of intrinsic endotoxins or viral DNAs) [3].

EX-1005(Vogl), 386.

Fungal protein expression systems can also be utilized in the methods of the present invention to efficiently produce a functional recombinant heme-binding protein. 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*

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 ... 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), [0043]-[0044].

118. As exemplified below, the Tsutsumi, Lin-Cereghino, and Mattoon references disclose the use of a “methylophilic yeast cell” and the use of “recombinant nucleic acid molecules” (e.g., genes of interest or promoter sequences in expression vectors) to create a recombinant expression system. Tsutsumi teaches:

The present invention relates to the controlled expression of polypeptides from methanol inducible promoters. Examples of these promoters have been described in several *yeast cells belonging to the group of yeast known as methylotrophic yeast*. In the context of the present invention *a methylotrophic yeast is defined as a group of yeast which can utilize methanol as a sole carbon source for their growth*. The promoters for the enzymes involved in methanol metabolism in these organisms are particularly strong, and these promoters (methanol metabolism promoters) are generally used to control the heterologous expression of proteins in yeast.

EX-1024(Tsutsumi), [0038].

119. As I discuss further below, **Tsutsumi** teaches the use of a specific strain of *P. pastoris* that is transformed with “recombinant nucleic acid molecules” that express Mxr1 and a recombinant gene of interest (cutinase). This strain is designated COLs702.

The aim of this experiment was to check whether the expression of a protein of interest . . . could be improved by co-expression of transcription factors, such as Prm1 and/or Mxr1 in *Pichia pastoris*. **The host strain used was COLs702 (Muts)**. COLs702 was constructed from *Pichia pastoris* NRRL Y-15851, which has a mutation in the his4 gene to make the gene inactive.

EX-1024(Tsutsumi), [0137]-[0138].

120. **Lin-Cereghino** teaches the use of *P. pastoris* as an expression system, and, as discussed further below, discloses transforming *P. pastoris* with expression

vectors. For example, in 2006 Lin-Cereghino explained, “*P. pastoris* is best known as a popular system for the production of recombinant proteins. Over 550 such proteins have been synthesized in this yeast.” EX-1006(Lin-Cereghino), 883 (citation omitted).

121. **Mattoon** discloses “[a] method for increasing the production” of heme containing proteins, which Mattoon refers to as “hemoproteins”. EX-1008(Mattoon), Abstract. To achieve the desired levels of expression of heme-containing proteins, Mattoon teaches that “[p]referred host cells are ... yeast of the genus *Pichia*, and even more preferably, *Pichia pastoris*.” EX-1008(Mattoon), 4:29-40.

122. The methylotrophic yeast strains disclosed in the Tsutsumi, Lin-Cereghino, and Mattoon references (*P. pastoris*) each comprise a “recombinant nucleic acid molecule”—namely, an expression vector. This expression vector may or may not be integrated into the host chromosome. **Tsutsumi** teaches several expression vectors expressing cutinase or variants thereof, including improved promoters. Cells comprising these expression vectors were “re-transformed” to add expression vectors containing gene sequences for Prm1 or Mxr1 (e.g., the “pGMxr” expression vector).

A re-transformation of low-expressing *Humicola insolens* cutinase transformants comprising pNori58-HIC (wt *Humicola insolens*

cutinase controlled by the improved DAS promoter), pNori58-RSII0014 (H. insolens cutinase variant controlled by improved DAS promoter), or pNori58-RSII0007 (H. insolens cutinase variant controlled by improved DAS promoter), was carried out with the plasmids harboring Prm1 (pGPrm, SEQ ID NO: 42) or Mxr1 (pGMxr, SEQ ID NO: 43) or both, and their expression was analyzed.

EX-1024(Tsutsumi), [0140].

123. The **Lin-Cereghino** reference teaches the creation of expression vectors using a *P. pastoris* genomic library and conventional cloning techniques. These expression vectors are “transformed” into *P. pastoris* cells for use in experiments.

Plasmid pYL1 was isolated from a *P. pastoris* genomic DNA library using the Sib selection procedure (3, 31). The library was constructed from an *E. coli-P. pastoris* shuttle vector (pYM8) with fragments of *P. pastoris* genomic DNA inserted at a unique BamHI site in the vector (32). pYM8 contains the *Saccharomyces cerevisiae* HIS4 gene for selection in *P. pastoris* and an ampicillin resistance gene for selection in *E. coli*.

Plasmid DNA was purified from each of these cultures using a QIAprep Spin Miniprep kit (QIAGEN, Chatsworth, CA). This DNA (approximately 1 to 2 µg) was then used to individually transform 10 sets of *P. pastoris* JC132 cells using the spheroplast method...

EX-1006(Lin-Cereghino), 884.

124. The **Mattoon** reference teaches various expression vectors encoding either heme-containing proteins (“hemoproteins”) or proteins involved in their synthesis. EX-1008(Mattoon), 3:1-6 (“FIG. 2 shows a restriction and functional map of the catalase T gene and its product.... FIG. 4 shows a restriction map of a yeast plasmid for the overexpression of the HEM2 gene.”).

125. The art recognized methods of creating expression vectors with more than one gene of interest for use in its expression systems, which I discuss below.

(a) **Claim 1[a]-[b]: ... wherein the recombinant nucleic acid molecule comprises a first exogenous nucleic acid ... wherein the recombinant nucleic acid molecule comprises a second exogenous nucleic acid ...**

126. Based on my review of claim 1, it recites that the claimed recombinant nucleic acid molecule (*e.g.*, an expression vector or portions thereof) comprises two exogenous (*e.g.*, heterologous) nucleic acid sequences. As a POSITA would understand, this expression vector may or may not be integrated into the host genome (*e.g.*, claim 5). As explained below, Tsutsumi teaches *Pichia* transformed with multiple expression vectors, each expressing a gene of interest. For example, Tsutsumi claims a “DNA construct” that includes Mxr1 under control of a promoter and a polypeptide under control of a DAS promoter, *i.e.*, a DNA construct comprising first and second nucleic acids encoding a heterologous polypeptide operably linked to a methanol-inducible promoter, *e.g.*, the DAS promoter, and

Mxr1 operably linked to a promoter, e.g., a methanol-inducible promoter, to which it can bind. EX-1024(Tsutsumi), Claim 15 (incorporating Claims 1, 10, 12, and 13). A POSITA would understand that these vectors are integrated into the *Pichia* genome. Accordingly, Tsutsumi teaches *Pichia* comprising a recombinant nucleic acid molecule that comprises the claimed two exogenous nucleic acid sequences.

127. To the extent that the Patent Owner argues that Tsutsumi does not expressly disclose the claimed recombinant nucleic acid molecule comprising two exogenous nucleic acid sequences because Tsutsumi does not disclose a single expression vector comprising the claimed two nucleic acid sequences, it would have been obvious to a POSITA to prepare such a construct. There are ample teachings in the art of this recombinant technique, as I discuss below.

128. In my experience, the creation of vectors expressing more than one heterologous gene sequence was common in the art by 2015. A POSITA in 2015 would recognize certain advantages of preparing vectors expressing more than one heterologous gene sequences. For example, such constructs require only one transformation to introduce multiple heterologous gene sequences into a host cell, which, as a POSITA would recognize, is more efficient at introducing the desired sequences of interest than multiple transformations. It also affords a POSITA more control over the levels of the different genes' respective expression levels, as double transfection events may lead to different levels of vector incorporation in a cell with

the result that there are more copies of one gene sequence than the other. As another exemplary advantage, when multiple vectors are transformed into a host cell, multiple selectable markers (genes encoding proteins that confer antibiotic resistance) are required to select the cells that contain the expression vector of interest, whether or not that construct has been stably integrated into a host genome. Although doing so is feasible, it is less efficient than utilizing one selectable marker.

129. Assembling such constructs uses the same principles and techniques as for preparing a construct that expresses a single heterologous sequence. Preparing an expression vector comprising more than one gene sequence of interest is more complicated than preparing a vector with only one gene sequence, requiring a more advanced strategy insuring that all of the fragments are assembled correctly. However, assembling an expression vector comprising more than one gene sequence of interest for use in *P. pastoris* was technically feasible and well within the ordinary skill of a POSITA.

130. Expression vectors useful for protein expression are known in the art (e.g., as described in the Sambrook Laboratory Manual).

Expression vectors useful for expression of heterologous protein(s) in a multitude of host cells are well known in the art (Sambrook and Russell, Molecular Cloning: a Laboratory Manual 3rd ed. (2001) ...), and some specific examples are provided herein.

EX-1010(Crane), [0034]. In my experience, a POSITA would have been able to create a single expression vector comprising multiple gene sequences of interest using the familiar Sambrook manual.

131. As an example, Crane teaches an approach to combining two genes of interest into a single expression vector. Crane describes an embodiment in which a single expression vector encodes a gene of interest (a heme-binding protein) and another gene of interest expressing the enzyme (ferrochelatase), which is involved in the synthesis of the heme-binding protein:

In another embodiment of the invention, both nucleotide sequences are inserted into one expression vector and the single expression vector encodes both the recombinant heme-binding protein and the recombinant ferrochelatase.

EX-1010(Crane), [0034]-[0035]. Crane provides additional details of an approach to create a “single expression vector” with two nucleotide sequences:

Co-expression of Ferrochelatase with gsNOS. Ferrochelatase (FC) and gsNOS were expressed from the same pACYCduet vector (Novagen). ... The resulting pACYCduet plasmid allows over-expression of gsNOS with a cleavable His-tag and FC with no tag

EX-1010(Crane), [0074]. A POSITA would recognize that similar techniques could be used to prepare an expression vector expressing multiple genes for use in methylotrophic yeast such as *P. pastoris*, as taught in Sambrook and other references, and that doing so would require no more than ordinary skill in the art

given the availability of selectable markers and promoter sequences that could be used in *P. pastoris* (as taught in Tsutsumi, Lin-Cereghino, Mattoon, Vogl, Crane, and many other references). A POSITA would know how to develop a strategy for the sequential assembly of a vector containing more than one gene of interest.

132. **Mattoon.** Mattoon likewise teaches that vectors used to transform host cells are “controllable by inducible or constitutive promoters” that are “operatively linked” to the nucleic acid sequences. EX-1008(Mattoon), 2:41-44, 5:51-59, 6:9-63. In this context, Mattoon teaches that “[b]oth the nucleic acid sequence encoding the [heme] rate-limiting enzyme as well as the heme-binding agent can be present on one expression vector or, alternatively, can be on separate vectors, such as separate multi-copy plasmids.” EX-1008(Mattoon), 5:34-46.

133. Mattoon thus teaches that a gene encoding a protein of interest, and a gene encoding a protein involved in the synthesis of the protein of interest, can be inserted into a single vector. A POSITA would understand that this disclosure in Mattoon could be readily applied to Tsutsumi and Lin-Cereghino, for example, in order to express a gene sequence of interest and a transcription factor that drives the expression of the former in a single vector.

134. For example, **Tsutsumi** teaches that “[t]he modified DAS promoters of the invention *will be useful for expression of any polypeptide of interest in Pichia*,” EX-1024(Tsutsumi), [0068], which a POSITA would understand could

include transcription factors. **Lin-Cereghino** also teaches that “*P. pastoris* is best known as a popular system for the production of recombinant proteins. Over **550** such proteins have been synthesized in this yeast.” EX-1006(Lin-Cereghino), 883. As discussed below, Lin-Cereghino teaches expression of the Mxr1 transcription factor under control of the *PEX8* gene promoter. A POSITA would thus be motivated to combine Mattoon’s teaching (which reflected general knowledge in the art, as exemplified in Crane) with the combination of Tsutsumi and Lin-Cereghino and would have a reasonable expectation of success doing so—for example, by using Mattoon’s teachings, routine techniques, and available materials.

2. ***Claim 1[a] ... wherein the recombinant nucleic acid molecule comprises a first exogenous nucleic acid encoding a methanol expression regulator 1 (Mxr1) transcriptional activator operably linked to at least one methanol-inducible promoter element comprising a sequence to which the Mxr1 transcriptional activator binds ...***

135. As I explain below, Tsutsumi in view of Lin-Cereghino discloses methylotrophic yeast including a recombinant nucleic acid molecule comprising a first exogenous nucleic acid encoding Mxr1 operably linked to a methanol-inducible promoter to which Mxr1 binds.

136. Based on my review of claim 1[a], it generally recites that, in the claimed recombinant nucleic acid molecule, the expression of the nucleic acid encoding the Mxr1 transcriptional activator (transcription factor) is driven by at least

one methanol-inducible promoter element to which Mxr1 binds. The expected consequence of this design is that, as Mxr1 levels increase in the nucleus of the host methylotrophic yeast cell, Mxr1 increases its own expression by binding to the claimed methanol-inducible promoter element in the recombinant nucleic acid molecule.

137. A POSITA would be motivated to engineer a recombinant nucleic acid molecule that expresses Mxr1 under control of a Mxr1-activated and methanol-inducible promoter. A POSITA would understand that this approach would be advantageous if, for example, the gene sequence of interest (the “second nucleic acid” recited in claim 1) is also regulated by Mxr1. Moreover, in this system, the expression of both genes—Mxr1 and the gene sequence of interest—would be controllable due to the presence and level of methanol to which the yeast are exposed.

138. The elements of claim 1[a] were known in the art and would have been obvious to a POSITA. As I describe below, **Tsutsumi** discloses a recombinant nucleic acid molecule comprising an exogenous nucleic acid encoding a methanol expression regulator 1 (Mxr1) transcriptional activator, which is co-expressed in *P. pastoris* in order to drive the expression of a second nucleic acid (the cutinase gene), which is expressed under the control of the methanol-inducible (and Mxr1-activated) promoter *AOX1*. Although Tsutsumi does not expressly disclose Mxr1 under control

of a “methanol-inducible promoter element comprising a sequence to which the Mxr1 transcriptional activator binds,” **Lin-Cereghino** discloses the use of a methanol-inducible promoter, *e.g.*, *PEX8*, to drive expression of Mxr1 in *P. pastoris*. A POSITA would be motivated to combine them for reasons I explain herein, and would reasonably expect success as doing so would require only familiar, well-known molecular biology techniques.

139. **Tsutsumi:** As I explain further below, Tsutsumi teaches a methylotrophic yeast system for expressing exogenous protein and the transcriptional activator Mxr1. Tsutsumi discloses the use of Mxr1 transcription factor as a positive regulator of gene expression, which affords control over the expression of recombinant genes of interest.

Controlled expression is often desirable. In methylotrophic yeast it has been known for long that certain promoters are dependent on the presence of methanol in the growth medium for the induction of transcription. This induction by methanol requires the presence of additional factors, however, the exact mechanism of action for such factors have not been elucidated. Examples of positive factors known from yeast include Mxr1 p, described as a key positive regulator required for methanol utilization in *Pichia pastoris* (Lin-Cereghino et al., 2006, Mol Cell Biol 26(3): 883-897).

EX-1024(Tsutsumi), [0005], [0041] (“In yeast e.g. Mxr1p, has been described as a key positive regulator required for methanol utilization in *Pichia pastoris* ...”).

140. This technology is further described in Tsutsumi’s Example 5. Example 5 is titled “Expression of *Humicola Insolens* Cutinase from Modified DAS Promoter with...Mxr1 Over-Expression.” EX-1024(Tsutsumi), [0136]. Tsutsumi discloses that the “aim of this [Example 5] experiment was to check whether the expression of a protein of interest, exemplified by the *Humicola insolence* [sic] cutinase gene controlled by an improved DAS promoter according to the invention,...could be improved by co-expression of transcription factors, such as ... Mxr1 in *Pichia pastoris*.” EX-1024(Tsutsumi), [0137].

141. Tsutsumi discloses the recombinant methods it used to co-express Mxr1 in Example 5's cutinase-expressing yeast cells under the control of a promoter element. Specifically, “re-transformation of low-expressing *Humicola insolens* cutinase transformants...controlled by the improved DAS promoter...was carried out with the plasmids harboring ... Mxr1... and the regulator gene [Mxr1] is controlled by the GAP promoter.” EX-1024(Tsutsumi), [0140]-[0143]. Tsutsumi further discloses the advantages of its approach: “***The obtained data [from Example 5] indicate an improved expression of the test protein [Humicola insolens cutinase] when Mxr protein was over-expressed.***” EX-1024(Tsutsumi), [0145].

This improved expression driven by Mxr1 co-expression is shown, for example, in a table in Tsutsumi (EX-1024(Tsutsumi), [0144]):

Enzyme expression plasmid	Prm1/Mxr1 plasmid	Expression level
pNori58-HIC	none	+++
	pGPrm	+++
	pGMxr/pGPrm	+++
pNori58-RSII0007	None	—
	pGMxr	+
	pGMxr/pGPrm	—
pNori58-RSII0014	None	++
	pGMxr	+++
	pGPrm	++
	pGMxr/pGPrm	+++

142. As a POSITA would understand, Tsutsumi’s recombinant Mxr1 taught in Example 5 is operably linked to the *GAP* promoter—that is, the expression of recombinant Mxr1 is controlled by the *GAP* promoter. Tsutsumi teaches, for example, that “operably linked” means “a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of the polynucleotide sequence such that the control sequence directs the expression of the coding sequence of a polypeptide.” EX-1024(Tsutsumi), [0037].

143. Although Tsutsumi’s Example 5 utilizes the constitutive GAP promoter to control Mxr1, a POSITA would recognize that Tsutsumi also teaches inducible gene expression and the benefits thereof. For example, Tsutsumi discloses that “[p]romoters suitable for the above activation may in particular *be ... induced by methanol.*” EX-1024(Tsutsumi), [0108]. Tsutsumi, a patent publication, contains claims that recite (for example) “wherein expression of the positive regulator Mxr1

is increased by **controlling** the expression of Mxr 1 or by increasing the copy number of the gene encoding Mxr 1.” EX-1024(Tsutsumi), Claim 15. In context, a POSITA would understand these statements in Tsutsumi to indicate or suggest the utility of inducible expression of Mxr1 rather than constitutive expression, as Mxr1 is constantly produced in a constitutive system and, in consequence, its expression is not “controlled” by a researcher.

144. A POSITA would understand from these teachings that Tsutsumi discloses a methylotrophic yeast cell with Mxr1 under the control of a promoter element, which may be either a constitutive or methanol-inducible promoter. Relatedly, as taught in the art, for example, “studies in *P. pastoris* ... indicat[e] that P_{GAP} driven expression is not entirely constitutive but regulated under certain conditions” and “there is a clear influence of the carbon source on the expression strength” of the *GAP* promoter.” EX-1005(Vogl), 392. Thus, a POSITA would recognize that the *GAP* promoter-driven expression of Mxr1 in Tsutsumi Example 5 could be at least partly methanol-induced.

145. In light of the benefits observed in Example 5 from Mxr1 expression driven by the *GAP* promoter, a POSITA would recognize that an expression system constructed with Mxr1 under the control of a strong promoter is advantageous, and would reasonably be expected to succeed at producing a protein of interest. Indeed, Tsutsumi teaches that “***over-expressing the Mxr 1 protein may have an effect on***

the modified DAS promoter activity” of the invention, (EX1024(Tsutsumi), [0074]), which it further teaches in Example 5 is a desirable enhancement of recombinant protein expression. EX-1024(Tsutsumi), [0137]-[0145]. Tsutsumi also teaches that methanol “[c]ontrolled expression is often desirable” and methanol-inducible promoters are “*particularly strong*” and “*generally used to control the heterologous expression of proteins in yeast.*” EX-1024(Tsutsumi), [0005]-[0006] (citing EX-1006(Lin-Cereghino) as support).

146. A POSITA would have several well-established options to choose from in order to express Mxr1 using a recombinant nucleic acid molecule (e.g., a promoter in an expression vector). For example, Tsutsumi provides that “promoters ... from which the expression of heterologous polypeptides can be controlled by the addition of methanol to the growth medium ... include but are not limited to e.g. the *AOXI promoter* (Alcohol Oxidase promoter), *DHAS promoter (or DAS promoter)* (dihydroxyacetone synthase promoter), *FDH promoter (or FMDH promoter)* (formate dehydrogenase promoter), *MOX promoter (Methanol Oxidase promoter)*, *AOX2 promoter*, *ZZA1*, *PEX5-*, *PEX8-*, *PEX14-promoter.*” EX-1024(Tsutsumi), [0040]. Similarly, “[t]he promoters controlling expression of the enzymes involved in methanol metabolism in these organisms are particularly strong, and these promoters are generally used to control the heterologous expression of proteins in yeast.” EX-1024(Tsutsumi), [0006]; EX-1005(Vogl), 386 (“One of the most

favorable properties of *P. pastoris*...are the methanol inducible promoters....”). A POSITA would recognize that these promoters could also be used for the expression of Mxr1 (itself a heterologous polypeptide in the context of this system).

147. **Lin-Cereghino:** As explained further below, Lin-Cereghino teaches the expression of Mxr1 (which it refers to as Mxr1p) under the control of the methanol-inducible promoter of the *PEX8* gene. EX-1006(Lin-Cereghino), 885, 893-94 (“[W]e expressed *MXR1* under the control of the promoter from the *P. pastoris PEX8* gene (32). *PPEX8* is methanol regulated.”).

148. Lin-Cereghino discloses that *P. pastoris* yeast cells are “best known as a popular system for the production of recombinant proteins.” EX-1006(Lin-Cereghino), 883. It describes generating *P. pastoris* strains transformed with expression vectors expressing Mxr1 under control of either the *AOX1* gene promoter (a strong methanol-inducible promoter) or the *PEX8* gene promoter (a weaker methanol-inducible promoter). EX-1006(Lin-Cereghino), 885 (disclosing “pGC215, in which *MXR1* transcription is regulated by the *AOX1* promoter. pGC216 ... [with] the *MXR1* gene under the regulation of the *PEX8* promoter”). In particular, Lin-Cereghino teaches the advantages of expressing Mxr1 under *PEX8* promoter control. It explains, for example, that the *P. pastoris* strain with Mxr1 under regulation of the methanol inducible promoter P_{PEX8} (pGC217) grew on

methanol and produced “relatively high levels of the product.” EX-1006(Lin-Cereghino), 893.⁵

149. A POSITA would further understand before 2015 that Mxr1 binds to at least one sequence in the *PEX8* and *AOX1* promoters controlling Mxr1 as disclosed by Lin-Cereghino. Indeed, Lin-Cereghino teaches that “Mxr1p specifically bound to one or more sites within the *AOX1* promoter.” EX-1006(Lin-Cereghino), 893. A POSITA would understand from Lin-Cereghino (cited by Tsutsumi) that “since most foreign genes expressed in *P. pastoris* are transcribed under the control of the ... *AOX1* gene, transcription in response to methanol is a key feature of this expression system.” EX-1006(Lin-Cereghino), 883. Additionally, “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. For example, Kranthi and colleagues demonstrated in 2010 the “[i]dentification of Mxr1p-binding sites in the promoters of genes encoding dihydroxyacetone synthase [*DHAS*] and *peroxin 8* [*PEX8*] of the methylotrophic

⁵ Lin-Cereghino discloses that pGC217 was created by mutagenizing the stop codon of P_{PEX8}-MXR1 construct in pGC216 to “provide an in-frame fusion with the c-myc epitope and His₆ tag.” EX1006(Lin-Cereghino), 885.

yeast Pichia pastoris.” EX-1023(Kranthi), Title. Consequently, for example, a POSITA would recognize that the *PEX8* promoter-driven expression of Mxr1 as taught in Lin-Cereghino reflects the methanol-inducible expression of Mxr1 by a promoter element (*PEX8*) to which Mxr1 itself binds.

150. In light of **Tsutsumi**’s teachings of the advantages of Mxr1 coexpression on recombinant protein production, and the feasibility of controlling Mxr1 expression using a methanol-inducible promoter as taught in **Lin-Cereghino**, a POSITA would have been motivated to combine Lin-Cereghino and Tsutsumi to engineer *P. pastoris* comprising “a recombinant nucleic acid molecule comprising exogenous nucleic acid encoding a methanol expression regulator 1 (Mxr1) transcriptional activator operably linked to at least one methanol-inducible promoter element comprising a sequence to which the Mxr1 transcriptional activator itself binds.”

151. Similar to Tsutsumi, for example, Lin-Cereghino teaches that Mxr1 is “essential for the large accumulations of the AOX1 and DHAS [DAS] transcripts,” such as the DAS promoter controlling the protein of interest in Tsutsumi. EX-1006(Lin-Cereghino), 889. A POSITA would understand this to refer to Mxr1’s regulation of other promoters, which could be used to drive expression of gene sequences of interest.

152. A POSITA would have been generally motivated to increase the expression of Mxr1 by placing it under the control of a methanol-inducible promoter. A POSITA would understand that methanol-inducible promoters have “major advantages” due to their “strength and the tight regulation ... [e]specially 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 non-inducing conditions and in a second phase, heterologous protein production can be started by methanol induction.” EX-1005(Vogl), 392. A methanol-inducible system would allow a POSITA to modulate expression—both of Mxr1 and its regulated targets—using carbon sources and increase expression of a recombinant protein under the control of a methanol. To achieve this goal, a POSITA would have naturally looked to Lin-Cereghino (cited directly by Tsutsumi), which discloses placing the *MXR1* gene under the control of methanol-inducible *P. pastoris* promoters of *PEX8* and *AOX1* in order to increase Mxr1 expression. EX-1006(Lin-Cereghino), 893-94. As taught in Lin-Cereghino, *P. pastoris* cells expressed an increased amount of Mxr1p under the control of the *PEX8* gene promoter. EX-1006(Lin-Cereghino), 893-94.

153. A POSITA would thus be generally motivated to substitute Tsutsumi’s *GAP* promoter controlling Mxr1 with a methanol-inducible promoter in order to tightly control and further overexpress Mxr1 that would bind to the methanol-

inducible promoter, and would reasonably expect success in doing so. As Tsutsumi teaches, for example, “[c]ontrolled expression is often desirable. In methylotrophic yeast it has been known for long that certain promoters are dependent on the presence of methanol in the growth medium for the induction of transcription. This induction by methanol requires...Mxr1 p, described as a key positive regulator required for methanol utilization in *Pichia pastoris* ([citing EX-1006] Lin-Cereghino *et al.*, 2006, Mol Cell Biol 26(3): 883-897)” EX-1024(Tsutsumi), [0005]. Additionally, a POSITA would understand that methanol-inducible promoters are natural alternatives to Mxr1 expression by the *GAP* promoter. EX-1005(Vogl), 392 (“P_{GAP} has emerged as the most commonly used alternative to P_{AOX1}” and “provide[s] similar expression levels under the respective optimal growth conditions.”), 393 (“P_{DAS} is a promoter...showing a similar regulatory pattern and expression level as P_{AOX1}.”).

154. Relatedly, a POSITA would recognize the potential benefits of trying a variety of methanol-inducible promoters to achieve the most favorable conditions for heterologous protein production. EX-1005(Vogl), 392-393 (“the strong expression from P_{AOX1} and P_{GAP} is not always the most favorable condition for heterologous protein production,” and “P_{PEX8} and P_{YPT1} are weak promoters and considered alternatives in case P_{AOX1} or P_{GAP} driven expression is too strong.”).

155. 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 activity. (“[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.”)

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.

156. For at least the reasons I explain above, a POSITA would be motivated to substitute Tsutsumi’s *GAP* promoter controlling Mxr1 expression with a

methanol-inducible promoter such as the promoters of the *DAS*, *PEX8*, or *AOX1* genes. Using this approach, Mxr1 expression is controlled by the presence of methanol and further enhances the expression of gene sequences of interest when activated, as taught in Tsutsumi.

157. A POSITA would reasonably expect success engineering methylotrophic yeast that overexpress Mxr1 under a methanol-inducible promoter to which Mxr1 binds (e.g., *PEX8*) in order to produce higher levels of heterologous protein expression using the teachings of the prior art that I describe above as well as other routinely used molecular biology techniques (e.g., cloning vectors as taught in Sambrook), and materials available for use in such experiments.

158. For example, a POSITA seeking to implement a P_{PEX8} -*MXR1*, P_{DAS} -*MXR1* or P_{AOX1} -*MXR1* expression vector in *P. pastoris* (including strains with multiple copies) would employ well-known strategies to control protein expression in *P. pastoris* with carbon sources. As reviewed in Vogl, for example, a POSITA would first grow the cells to high densities while repressing recombinant protein expression in a medium that contained glucose or glycerol. EX-1005(Vogl), 386 (describing cultivation methods). Once the cells have grown to a high cell density a POSITA would allow the glucose or glycerol to be consumed and then add methanol. *Id.* This would in turn strongly induce recombinant protein expression driven by methanol-inducible promoters (e.g., P_{PEX8}) from a large number of cells. *Id.* A

POSITA would understand that growing the cells to a high density and only then inducing recombinant gene expression with the addition of methanol would result in high levels of protein expression.

3. 1[b] ...wherein the recombinant nucleic acid molecule comprises a second exogenous nucleic acid encoding a heterologous polypeptide operably linked to at least one methanol-inducible promoter element.

159. Tsutsumi discloses, alone or in view of Mattoon, methylotrophic yeast including the recombinant nucleic acid molecule of limitation 1[a] comprising a second exogenous nucleic acid encoding a heterologous polypeptide operably linked to at least one methanol-inducible promoter element. To the extent not disclosed in Tsutsumi, it would be obvious to create such a recombinant nucleic acid molecule, including in a single recombinant nucleic acid molecule as explained above (Section VIII.B.1.a). As I explain further above for claim 1[a], a POSITA would be motivated to express heterologous polypeptides (e.g., a protein of interest) in a methylotrophic yeast species such as *P. pastoris* under the control of a methanol-inducible promoter. *E.g.*, EX-1005(Vogl), 392 (discussing “major advantages” of this approach).

160. Additionally, a POSITA would be motivated to express heterologous polypeptides under the control of a methanol-inducible promoter using a recombinant nucleic acid molecule that also expresses Mxr1 under the control of a Mxr1-driven promoter. For example, using a methanol-inducible system to express

both Mxr1 and the heterologous polypeptide would afford a POSITA control over the expression of both.

161. Mxr1 was known to activate methanol-inducible promoters such as promoters for the *AOXI*, *DAS*, and *PEX8* genes as discussed above. *E.g.*, EX-1006(Lin-Cereghino), Abstract (“We conclude that Mxr1p is a transcription factor that is necessary for the activation of many genes in response to methanol.”). Thus, expression of Mxr1 would be expected to drive expression of a heterologous polypeptide by binding and activating its methanol-inducible promoter if the promoter comprises a Mxr1 binding site. A POSITA would be aware of Mxr1 bindings sites as they had previously been characterized (reviewed in Vogl, EX-1005). A POSITA would be motivated to utilize relatively well-characterized promoters known to be activated by Mxr1 (*e.g.*, promoters for the *AOXI*, *DAS*, and *PEX8* genes) in this system.

162. This approach is taught or suggested in **Tsutsumi**. Tsutsumi describes the creation of improved variants on the endogenous *DAS* promoter sequence. As explained above for claim 1[a], Tsutsumi’s Example 5 discloses a methylotrophic *P. pastoris* yeast expressing cutinase under the control of a *DAS* promoter. The purpose of the Example 5 “experiment was to check whether the expression of a protein of interest “could be improved by co-expression of transcription factors, such as ... Mxr1 in *Pichia pastoris*.” EX-1024(Tsutsumi), [0137]. Tsutsumi discloses the

recombinant methods used to create this construct, including “re-transformation of low-expressing *Humicola insolens* cutinase transformants ... controlled by the improved DAS promoter.” EX-1024(Tsutsumi), [0140].

163. As a POSITA would recognize, and as taught in Tsutsumi, the *DAS* promoter driving expression of cutinase is methanol-inducible.

Several promoters have previously been isolated and described in the literature from which the expression of heterologous polypeptides can be controlled by the addition of methanol to the growth medium. Such promoters include but are not limited to e.g. the *AOX1* promoter (Alcohol Oxidase promoter), ***DHAS promoter (or DAS promoter) (dihydroxyacetone synthase promoter)***, *FDH* promoter (or *FMDH* promoter) (formate dehydrogenase promoter), *MOX* promoter (Methanol Oxidase promoter), *AOX2* promoter, *ZZA1*, *PEX5*-, *PEX8*-, *PEX14*-promoter. ***Particularly the promoter relevant for the present invention is the dihydroxyacetone synthase (DAS or DHAS) promoter.***

Normally ***all of the above promoters require the presence of methanol for their induction.*** This induction by methanol requires the presence of additional factors (such as transcription factors), however, the exact mechanism of action for such factors have not been elucidated.

EX-1024(Tsutsumi), [0040]-[0041]. Table 1 of the Vogl review (excerpted above) also provides that the promoter of the *DAS* gene is “[i]nduced by methanol.” EX-1005(Vogl), 393.

164. Accordingly, Tsutsumi teaches expression of the cutinase gene of interest under control of a methanol-inducible promoter (*DAS*).

The aim of this experiment was to check whether the expression of a protein of interest, exemplified by the ***Humicola insolens [sic] cutinase gene controlled by an improved DAS promoter*** according to the invention, having four repeats of the UAS, could be improved by co-expression of transcription factors, such as Prm1 and/or Mxr1 in *Pichia pastoris*.

EX-1024(Tsutsumi), [0137]. A POSITA would thus understand from these teachings that Tsutsumi discloses a recombinant nucleic acid molecule comprising an exogenous nucleic acid encoding a heterologous polypeptide operably linked to at least one methanol-inducible promoter element (e.g., the *DAS* promoter).

A re-transformation of low-expressing *Humicola insolens* cutinase transformants comprising pNori58-HIC (wt *Humicola insolens* cutinase controlled by the improved *DAS* promoter), pNori58-RSII0014 (*H. insolens* cutinase variant controlled by improved *DAS* promoter), or pNori58-RSII0007 (*H. insolens* cutinase variant ***controlled by improved DAS promoter***), was carried out with the plasmids harboring Prm1 (pGPrm, SEQ ID NO: 42) or Mxr1 (pGMxr, SEQ ID NO: 43) or both, and their expression was analyzed.

EX-1024(Tsutsumi), [0140].

165. Tsutsumi also claims a “DNA construct” that includes Mxr1 under control of a promoter and a polypeptide under control of a DAS promoter. EX-1024(Tsutsumi), Claim 15 (incorporating Claims 1, 10, 12, and 13).

1. An isolated polynucleotide comprising:

- i) a nucleotide sequence consisting of the DAS promoter sequence from *Pichia* or a functional part thereof, wherein the said DAS promoter is comprised in SEQ ID NO: 1; and
- ii) at least one additional UAS, wherein the said UAS is comprised in SEQ ID NO: 2.

10. A DNA construct comprising a polynucleotide sequence of claim 1 operably linked to a structural gene encoding a polypeptide of interest and a terminator.

12. A *Pichia* host cell comprising an expression vector of claim 10.

13. A method of producing a polypeptide of interest comprising:
(a) cultivating the host cell of claim 12, under conditions conducive for the production of the polypeptide of interest; and
(b) recovering the polypeptide.

15. The method according to claim 13, ***wherein expression of the positive regulator Mxr1 is increased by controlling the expression of Mxr1*** or by increasing the copy number of the gene encoding Mxr1.

EX-1024(Tsutsumi), Claims 1, 10, 12, 13, 15.

166. Additionally, Tsutsumi teaches various methanol-inducible promoters from *P. pastoris* that, as a POSITA would understand, could be used in the claimed recombinant nucleic acid molecule in order to express the gene sequence of interest.

EX-1024(Tsutsumi), [0040]; *see also* EX-1005(Vogl), 386, Table 1. For example, Tsutsumi teaches promoters including:

e.g. the *AOX1 promoter* (Alcohol Oxidase promoter), *DHAS promoter (or DAS promoter)* (dihydroxyacetone synthase promoter), FDH promoter (or FMDH promoter) (formate dehydrogenase promoter), MOX promoter (Methanol Oxidase promoter), AOX2 promoter, ZZA1, PEX5-, *PEX8-*, PEX14-promoter.”

EX-1024(Tsutsumi), [0040]. It would be obvious to a POSITA to use a methanol-inducible promoter in the claimed nucleic acid molecule, including options identified by Tsutsumi and Table 1 of the Vogl review, to achieve the most favorable conditions for heterologous protein production. EX-1005(Vogl), 393, Table 1.

167. Accordingly, it is my opinion that, using the teachings of Tsutsumi, Lin-Cereghino and Mattoon, as well as the routinely used techniques and readily available materials that were well-known in the art, a POSITA would have a reasonable expectation of success engineering methylotrophic yeast that overexpress Mxr1 under the control of a methanol-inducible promoter to which Mxr1 binds in order to produce higher levels of heterologous protein expression, when the latter is also expressed under the control of a methanol-inducible promoter.

168. Indeed, as explained in the '656 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.

Constructs or vectors, including expression constructs or vectors, are commercially available or can be produced by recombinant DNA techniques routine in the art.

'656 patent 9:53-55.

A skilled artisan would understand that the recombinant nucleic acid molecule described herein can be stably integrated into the genome of the methylotrophic yeast cell, or can be extrachromosomally expressed from a replication-competent plasmid. Methods of achieving both are well known and routinely used in the art.

'656 patent 5:12-17.

C. Claim 2: *The yeast cell of claim 1, wherein the methylotrophic yeast cell is of a yeast selected from the group consisting of Candida, Hansenula, Pichia and Torulopsis.*

169. In my opinion, claim 2 of the '656 patent is obvious for reasons provided above regarding claim 1. As recognized in the art, for example, “[t]he methylotrophic yeast *Pichia pastoris* is a widely used host for heterologous protein production.” EX-1005(Vogl), Abstract. A POSITA would be motivated to utilize a familiar system to produce recombinant proteins, for example, because of the availability of experimental reagents and materials such as vectors and promoter sequences that would permit efficient experimentation. In light of the availability of

such materials for use with the *P. pastoris* system, a POSITA would reasonably expect success.

170. As I explain further above with respect to claim 1, each of the Tsutsumi, Lin-Cereghino, and Mattoon references each teach the use of methylotrophic yeast cells that are transformed with expression vectors and used to express gene sequences of interest, including *P. pastoris* cells. For example, **Tsutsumi** discloses “[k]nown members of methylotrophic yeast host cells belong[ing] to the genera selected from the group consisting of *Pichia*, *Hansenula*, *Candida*, *Torulopsis*. According to the invention the *Pichia* host cell can in one embodiment be selected from ... *P. pastoris*.” EX-1024(Tsutsumi), [0039]. **Lin-Cereghino** and **Mattoon** also both disclose methylotrophic yeast expression systems, including *P. pastoris*. EX-1006(Lin-Cereghino), 883 (“*P. pastoris* is best known as a popular system for the production of recombinant proteins.”); EX-1008(Mattoon), 2:30-31 (“Preferred yeast strains include *Pichia*, *Hansenula* and strains of *S. cerevisiae*.”), 4:31-37 (“Preferred host cells are eukaryotic and even more preferred host cells include yeast and fungi that are known to be efficient producers of hemoproteins. In particular, yeast of the genus *Pichia*, and even more preferably, *Pichia pastoris*, are desirable for use in the present method.”).

D. Claim 3: *The yeast cell of claim 1, wherein the cell is a Pichia cell.*

171. In my opinion, claim 3 of the '656 patent is obvious for reasons provided above regarding claims 1 and 2.

E. Claim 4: *The yeast cell of claim 1, wherein the cell is a Pichia pastoris cell.*

172. In my opinion, claim 4 of the '656 patent is obvious for reasons provided above regarding claims 1 and 2.

F. Claim 5: *The yeast cell of claim 1, wherein the recombinant nucleic acid molecule is stably integrated into the genome of the methylotrophic yeast cell.*

173. In my opinion, claim 5 of the '656 patent is obvious. A POSITA would be motivated to combine the Tsutsumi, Lin-Cereghino, and Mattoon references with a reasonable expectation of success for reasons explained further above with respect to claim 1. Tsutsumi discloses or renders obvious (whether alone or in view of Mattoon or the knowledge of a POSITA) methylotrophic yeast comprising a recombinant nucleic acid molecule that is stably integrated into the genome. I discuss an alternative to stable genome integration—specifically, extrachromosomal expression—below with respect to claim 6. As a POSITA would recognize, there are a limited, finite number of predictable ways for engineering a methylotrophic yeast cell to comprise recombinant nucleic acid molecules—*i.e.*, the molecules are either stably integrated into the host cell genome or they are extrachromosomally expressed (*e.g.*, via transient transformation with an expression vector, typically in

selection medium in order to identify cells comprising the gene of interest). A POSITA would be generally motivated to try both options, depending on factors such as the purpose and scope of the experimentation, and would have reasonably expected successful incorporation of the nucleic acids into the yeast in either manner. Indeed, the '656 patent acknowledges that methods of “stably integrat[ing]” recombinant nucleic acids “into the genome of the methylotrophic yeast cells,” or “extrachromosomally express[ing]” them “from a replication-competent plasmid,” were “well known and routinely used in the art.” EX-1002, 5:12-17.

174. In particular, a POSITA would have been generally motivated to achieve stable transformation of the methylotrophic yeast used in the claimed system because stable integration of the recombinant nucleic acid sequences into the genome helps to preserve these sequences’ continued expression for subsequent research. Doing so, for example, in order to create host cell lines (“strains”) in yeast and other species was commonplace in the art as of 2015. As taught in the art, for example:

Transient or stable transfection of the host cell with one or more expression vectors is contemplated by the present disclosure.

EX-1010(Crane), [0034].

[O]verexpression can be achieved by coupling a gene to a powerful promoter sequence and placing it on a multicopy plasmid—or integrating it in multiple copies in the genome.

EX-1042(Mol. Bio. Cell.), 539.

Foreign DNA can, however, be rather easily integrated into random positions of many animal genomes. In mammals, for example, linear DNA fragments introduced into cells are rapidly ligated end-to-end by intracellular enzymes to form long tandem arrays, which usually become integrated into a chromosome at an apparently random site.

EX-1042(Mol. Bio. Cell.), 540. Indeed, the ‘656 patent acknowledges that methods of “stably integrat[ing]” recombinant nucleic acids “into the genome of the methylotrophic yeast cells,” or “extrachromosomally express[ing]” them “from a replication-competent plasmid,” were “well known and routinely used in the art.”

EX-1002, 5:12-17.

175. In my experience, vectors used for expression in *Pichia* are designed to facilitate stable integration. For example, the gene sequences of interest in an expression vector are typically flanked by sequences that can recombine with the host genome. As discussed below, at least the Tsutsumi and Mattoon references teach the stable integration of recombinant nucleic acid molecules into the genome of methylotrophic (*Pichia*) yeast cells.

176. **Tsutsumi:** Tsutsumi teaches that its “host cells” include “any cell type that is susceptible to transformation, transfection, transduction, and the like with a

nucleic acid construct or expression vector comprising a polynucleotide of the present invention.” EX-1024(Tsutsumi), [0037]. Additionally, Tsutsumi discloses vectors that can be transformed into *P. pastoris*.

The modified DAS promoters of the invention may also advantageously be used in any suitable *Pichia* expression plasmid. The skilled person will know how to clone the promoter into such a construct. In a further embodiment the invention therefore relates to an expression vector comprising a DNA construct of the invention, further comprising a signal peptide coding region.

In a still further aspect the invention relates to a *Pichia* host cell comprising an expression vector of the invention. Particularly the *Pichia* host cell is a *Pichia pastoris* host cell.

EX-1024(Tsutsumi), [0069]-[0070].

177. Tsutsumi teaches the concept of stable integration of recombinant nucleic acids into the genome: “[c]onventionally, maximal expression of a gene in a eukaryotic organism is achieved by amplifying *in the chromosome* an expression cassette containing a single promoter operably linked to a gene encoding the polypeptide of interest.” EX-1024(Tsutsumi), [0004]. As a POSITA would understand from Tsutsumi, a “nucleic acid construct” is synonymous with “expression cassette” when the nucleic acid construct contains the control sequences required for expression of a coding sequence. EX-1024(Tsutsumi), [0004].

178. Tsutsumi further discloses screening transformants for the presence of stable integration of the gene sequence of interest at a particular locus using conventional PCR amplification techniques.

Transformation of *Pichia Pastoris*:

Pichia pastoris strains were transformed by electroporation. ...

Colony PCR for Screening of His4 Locus Integration

The cell was subjected to PCR using Expand High Fidelity plus (Roche). ... The PCR primers were primer139; 5'-ctgctctagccagtttgctg-3' (SEQ ID NO: 10) (upstream of His4 marker in genome) and S98; 5'-gccgcccagtcctgctcgct-3' (SEQ ID NO: 11) (between His4 marker and AOX terminator in expression plasmids).

The ***strains in which the expression cassette was integrated*** at His4 locus showed about 2.9 kb band.

EX-1024(Tsutsumi), [0115]-[0117].

Resulting expression plasmids (pDd-2 through pDd-10) were linearized by digestion with Sall and transformed into *Pichia pastoris* his4 strain.

The strains in which the expression cassette was integrated at the HIS4 locus were screened by colony PCR. The selected strains were cultivated in liquid medium with methanol induction and phytase activity in the culture broth was measured. The results are shown in Table 3.

EX-1024(Tsutsumi), [0124].

Each expression plasmid was integrated at HIS4 locus of Pichia pastoris, and the expression level with methanol induction was evaluated in YPD medium. The results are shown in Table 6.

EX-1024(Tsutsumi), [0136].

179. Based on these exemplary disclosures, a POSITA would understand that Tsutsumi discloses the stable integration of recombinant nucleic acid molecules in a methylotrophic yeast cell.

180. **Mattoon:** Similarly, Mattoon teaches “transfection includes any means for introducing a nucleic acid sequence or expression vector into a host cell, including, but not limited to, ... expression of a vector, such as a multi-copy plasmid or *the use of a vector that integrates into the genum [sic] of the host cell.*” EX-1008(Mattoon), 4:41-48. Mattoon provides that “a vector may be one in which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosomes into which it has been integrated.” EX-1008(Mattoon), 4:60-5:4. A POSITA would be motivated to combine Mattoon with with Tsutsumi and Lin-Cereghino, with a reasonable expectation of success, for reasons I explain above (claim 1).

G. Claim 6: *The yeast cell of claim 1, wherein the recombinant nucleic acid molecule is extrachromosomally expressed from a replication-competent plasmid.*

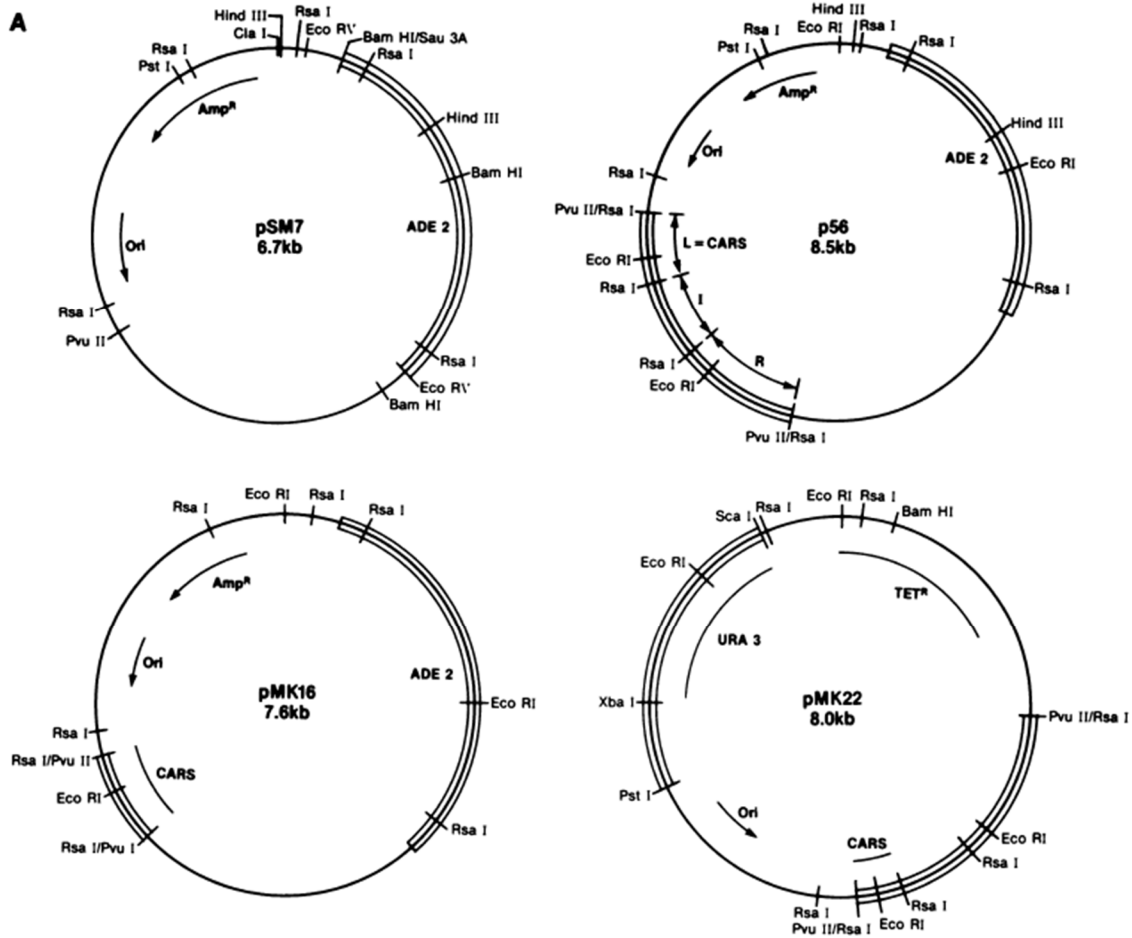
181. In my opinion, claim 6 of the '656 patent is obvious. A POSITA would be motivated to combine the Tsutsumi, Lin-Cereghino, and Mattoon references with a reasonable expectation of success for reasons explained further above with respect to claim 1. As I explain above with respect to claim 5, a POSITA would recognize

that there are a limited, finite number of predictable ways for engineering a methylotrophic yeast cell to express recombinant nucleic acid molecules (such as the nucleic acid sequences encoding Mxr1 and a heterologous polypeptide).

182. One approach available to a POSITA is “extrachromosomal” expression. EX-1010(Crane), [0034], EX-1042(Mol. Bio. Cell.), 539-540. A POSITA would understand that, following transformation of a recombinant nucleic acid molecule (e.g., in an expression vector), the expression of the recombinant nucleic acid molecules in a methylotrophic yeast host cell occurs “extrachromasomally” when the expression vector is not integrated into the host genome.

183. To this end, a POSITA would be motivated to use a replication-competent expression vector, which contains an origin of replication sequence (frequently annotated as “ori”). The presence of the ori sequence in the expression vector allows the initiation of replication within a vector in order for the vector to be propagated in the host cells. Consequently, the expression vector can be replicated independently of the host genome. It would be obvious to a POSITA to prepare replication-competent constructs—*e.g.*, vectors containing a familiar origin of replication (ori) sequence—in order to replicate and propagate the vectors during proliferation of host cells.

184. In my experience, ori sequences would have been available to a POSITA working with methylotrophic yeast in 2015, which could have been included in an expression vector for use in methylotrophic yeast. In addition, there were vectors available for use in at least the methylotrophic yeast species *Candida* and *Hansenula*, which permit extrachromosomal expression in those yeast. EX-1062(Kurtz), 209, 216; EX-1063(Roggenkamp), 303-07 (both disclosing “autonomous” replication, which a POSITA would understand to refer to expression using a non-integrated, “extrachromosomal” expression vector). Below I reproduce certain plasmids used in the Kurtz study, which comprise an ori sequence (and are therefore replication competent).



EX-1062(Kurtz), Fig. 4A. Similarly, **Mattoon** teaches “the vector may be an autonomously replicating vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, such as a plasmid.” EX-1008(Mattoon), 4:60-5:4. A POSITA would be motivated to combine Mattoon with Tsutsumi and Lin-Cereghino, with a reasonable expectation of success, for reasons I explain above (claim 1).

H. Claim 7: *The yeast cell of claim 1, wherein the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from Pichia pastoris.*

185. In my opinion, claim 7 of the '656 patent is obvious for reasons I explain further above with respect to claim 1. For example, Lin-Cereghino discloses a methylotrophic *P. pastoris* expression system constructed to include the gene sequence that encodes the Mxr1 transcription factor—*i.e.*, the *P. pastoris MXR1* gene. As I explain further above with respect to claim 1, a POSITA would be motivated to utilize Mxr1 taught in the Lin-Cereghino reference in the claimed recombinant nucleic acid molecule in order to drive expression of the gene sequence of interest (linked to a methanol-inducible promoter element), and would reasonably expect success in light of the teachings of Tsutsumi (for example). It would therefore be obvious to a POSITA to incorporate the *MXR1* gene sequence in the claimed recombinant nucleic acid molecule.

186. As a POSITA would recognize, the Lin-Cereghino reference teaches that *MXR1* has its origins in the *P. pastoris* genome. Indeed, Lin-Cereghino teaches the discovery of the *MXR1* gene in the search for genes that natively regulate the expression of methanol-inducible genes in *P. pastoris*:

Growth of the yeast *Pichia pastoris* on methanol induces the expression of genes whose products are required for its metabolism. ***To elucidate the molecular mechanisms responsible for methanol regulation, we identify genes required for the expression of AOX1.***

Mutations in one gene, named MXR1 (methanol expression regulator 1), result in strains that are unable to (i) grow on the peroxisomal substrates methanol and oleic acid, (ii) induce the transcription of AOX1 and other methanol pathway and PEX genes, and (iii) form normal appearing peroxisomes in response to methanol. MXR1 encodes a large protein with a zinc finger DNA-binding domain near its N terminus that has similarity to *Saccharomyces cerevisiae* Adr1p. We conclude that Mxr1p is a transcription factor that is necessary for the activation of many genes in response to methanol. *We propose that MXR1 is the P. pastoris homologue of S. cerevisiae ADR1*

EX-1006(Lin-Cereghino), 883, 887-88; *see also* EX-1005(Vogl), 389. Accordingly, a POSITA would understand that the *MXR1* gene sequence is derived from *P. pastoris*. Additionally, as I explain below with respect to claim 9, the gene sequence for *MXR1* was available to a POSITA via the NCBI's public GenBank/GenPept database.

I. Claim 8: *The yeast cell of claim 1, wherein the transcriptional activator comprises the sequence shown in SEQ ID NO: 59.*

187. In my opinion, claim 8 of the '656 patent is obvious. For reasons I explain further above with respect to claim 1, a POSITA would have been motivated to express the Mxr1 transcription factor in methylotrophic yeast using the claimed recombinant nucleic acid molecule in order to drive expression of the gene sequence of interest, where expression of the Mxr1 transcription factor's gene sequence is

driven by a methanol-inducible promoter element as taught in Lin-Cereghino. A POSITA would reasonably expect success in light of the teachings of Tsutsumi, among others. It would therefore be obvious to a POSITA to incorporate the *MXR1* gene sequence in the claimed recombinant nucleic acid molecule (see below with respect to claim 9).

188. As a POSITA would recognize, the sequence covered in SEQ ID NO: 59 is the amino acid sequence of the Mxr1 transcriptional activator native to *P. pastoris*. EX-1002, 77-84; EX-1051. This sequence was known by 2015 and available to a POSITA. Specifically, the SEQ ID NO: 59 amino acid sequence is translated from the gene sequence that encodes it—namely, the *MXR1* gene sequence native to *P. pastoris*. The *MXR1* gene sequence that encodes the SEQ ID NO: 59 amino acid sequence was available to a POSITA via the GenBank/GenPept database. This *MXR1* gene sequence is identified as SEQ ID NO: 60 in the '656 patent, as I discuss further below regarding claim 9. Accordingly, a POSITA would be readily able to translate the amino acid sequence from the known nucleic acid sequence encoding Mxr1 (SEQ ID NO: 60) using routine methods in the art.

189. For context, as I discuss above (Section VI.A), the GenBank database is publicly accessible and contains “the most up-to-date and comprehensive DNA sequence information” from multiple databases around the world. EX-1053(NCBI). GenBank also comprises a repository of amino acid sequences from several sources

called “GenPept,” including translations from annotated coding regions of nucleic acid sequences in GenBank. In my experience, as shown below, the sequences in GenBank/GenPept database were available to a POSITA and a POSITA would be motivated to utilize these databases to obtain these sequences.

190. The sequence covered by SEQ ID NO: 59 is available in the GenBank/GenPept database. Specifically, as excerpted below, SEQ ID NO: 59 is available under GenBank Accession Number ABD57365 for the protein “methanol expression regulator I” (Mxr1), which was available in the GenBank/GenPept database at least as of March 2006. EX-1051, 1-3.

[methanol expression regulator I \[Komagataella pastoris\]](#)
1155 aa protein
Accession: ABD57365.1 GI: 88866603
Current status: live

I	II	Version	Gi	Accession	Update Date
☒	☐	1	88866603	ABD57365.1	Jan 9, 2012 11:46 AM
☐	☒	1	88866603	ABD57365.1	Mar 4, 2006 12:07 AM

Accession [ABD57365](#) was first seen at NCBI on Mar 4, 2006 12:07 AM

EX-1051, 3. As disclosed in the GenBank/GenPept database (excerpted and highlighted below), *Komagataella pastoris* is another name to indicate *P. pastoris*, and the amino acid sequence associated with “methanol expression regulator I” of *Komagataella pastoris* is associated with the research disclosed in the Lin-Cereghino (2006) reference, a study of Mxr1 (Mxr1p) in *P. pastoris*.

LOCUS ABD57365 1155 aa linear PLN 04-MAR-2006
DEFINITION methanol expression regulator I [Komagataella pastoris].
ACCESSION ABD57365
VERSION ABD57365.1
DBSOURCE accession [DQ395124.1](#)
KEYWORDS .
SOURCE Komagataella pastoris (Pichia pastoris)
ORGANISM [Komagataella pastoris](#)
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina;
Saccharomycetes; Saccharomycetales; Phaffomycetaceae; Komagataella.
REFERENCE 1 (residues 1 to 1155)
AUTHORS Lin-Cereghino,G.P., Godfrey,L., de la Cruz,B.J., Johnson,S.,
Khuongsathiene,S., Tolstorukov,I., Yan,M., Lin-Cereghino,J.,
Veenhuis,M., Subramani,S. and Cregg,J.M.
TITLE Mxr1p, a key regulator of the methanol utilization pathway and
peroxisomal genes in Pichia pastoris
JOURNAL Mol. Cell. Biol. 26 (3), 883-897 (2006)
PUBMED [16428444](#)
REFERENCE 2 (residues 1 to 1155)
AUTHORS Lin-Cereghino,G.P., Godfrey,L. and Cregg,J.M.

191. Accordingly, prior to 2015, a POSITA would reasonably expect success in obtaining the amino acid sequence of *P. pastoris* Mxr1, including as shown in SEQ ID NO: 59 under Accession Number ABD57365.

J. Claim 9: *The yeast cell of claim 8, wherein the transcriptional activator is encoded by the nucleic acid sequence shown in SEQ ID NO: 60.*

192. In my opinion, claim 9 of the '656 patent is obvious for reasons I explain further above regarding claims 1 and 8. Additionally, as a POSITA would recognize, the sequence covered by SEQ ID NO: 60 is the nucleic acid sequence encoding the *MXR1* gene in *P. pastoris*. EX-1002, 83-88. As I explain further above with respect to claim 8, in my experience, a POSITA desiring to know the nucleic acid sequence encoding the *P. pastoris* Mxr1 transcription factor prior to 2015 could have identified the sequence claimed in SEQ ID NO: 60 under Accession Number DQ395124 in the publicly-available GenBank database.

193. Specifically, from my investigation of the GenBank database, the gene sequence recited in SEQ ID NO: 60, described as the “*Pichia pastoris* methanol expression regulator I gene,” is available under Accession Number DQ395124. EX-1052, 1-2. As shown below (EX-1052, excerpts from pages 1 and 3), this sequence first appeared in the GenBank database in March 2006. This sequence is also associated with the research disclosed in the Lin-Cereghino (2006) reference, a study of Mxr1 (Mxr1p) in *P. pastoris*.

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LOCUS      DQ395124                3468 bp    DNA        linear    PLN 04-MAR-2006
DEFINITION Pichia pastoris methanol expression regulator I gene, complete cds.
ACCESSION  DQ395124
VERSION    DQ395124.1
KEYWORDS   .
SOURCE      Komagataella pastoris (Pichia pastoris)
  ORGANISM  Komagataella pastoris
            Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina;
            Saccharomycetes; Saccharomycetales; Phaffomycetaceae; Komagataella.
REFERENCE   1 (bases 1 to 3468)
  AUTHORS   Lin-Cereghino,G.P., Godfrey,L., de la Cruz,B.J., Johnson,S.,
            Khuongsathiene,S., Tolstorukov,I., Yan,M., Lin-Cereghino,J.,
            Veenhuis,M., Subramani,S. and Cregg,J.M.
  TITLE      Mxr1p, a key regulator of the methanol utilization pathway and
            peroxisomal genes in Pichia pastoris
  JOURNAL    Mol. Cell. Biol. 26 (3), 883-897 (2006)
  PUBMED     16428444
REFERENCE   2 (bases 1 to 3468)
  AUTHORS   Lin-Cereghino,G.P., Godfrey,L. and Cregg,J.M.

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I	II	Version	Gi	Accession	Update Date
		1	88866602	DQ395124.1	Jan 9, 2012 11:46 AM
		1	88866602	DQ395124.1	Mar 4, 2006 12:07 AM

Accession [DQ395124](#) was first seen at NCBI on Mar 4, 2006 12:07 AM

194. Accordingly, because the sequence claimed in SEQ ID NO: 60 was publicly available beginning in 2006, a POSITA would reasonably have expected success in obtaining this sequence.

K. Claim 10: *The yeast cell of claim 1, wherein the at least one methanol-inducible promoter element is selected from the group consisting of an alcohol oxidase 1 (AOX1) promoter element from Pichia pastoris, an alcohol oxidase (AOD1) promoter element from Candida boidinii, a methanol oxidase (MOX) promoter element from Hansenula polymorpha, a methanolic alcohol oxidase (MOD1) promoter element from Pichia methanolica, a dihydroxyacetone synthase (DHAS) promoter element from Pichia pastoris, a formaldehyde dehydrogenase (FLD1) promoter element from Pichia pastoris, and a peroxin 8 (PEX8) promoter element from Pichia pastoris.*

195. In my opinion, claim 10 of the '656 patent is obvious. As I explain above with respect to claim 1, each of the Tsutsumi and Lin-Cereghino references teach the selection and use of a variety of methanol-inducible promoters derived from *P. pastoris* in order to achieve the production of heterologous gene sequences of interest. EX-1024(Tsutsumi), [0040]; EX-1006(Lin-Cereghino), 893-94. Methanol-inducible promoters from *P. pastoris* were also generally known in the art. E.g., EX-1005(Vogl), 392-393, Table 1.

196. Among these options are the methanol-inducible promoters for the *DAS*, *AOX1*, and *PEX8* genes, among others. EX-1024(Tsutsumi), [0040]; EX-1006(Lin-Cereghino), 893-94; EX-1005(Vogl), 392-393. Prior to 2015, 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. As noted in the Vogl review, the Tsutsumi reference teaches “optimizing” the promoter for the *DAS* gene. EX-1005(Vogl), 394.

197. 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).

198. Moreover, although '656 patent claim 1 requires two promoters—one methanol-inducible promoter element operably linked to Mxr1 and another methanol-inducible promoter element operably linked to the heterologous polypeptide—claim 1 does not require that they be the same promoter. A POSITA would recognize that it could be advantageous to utilize different methanol-inducible promoters to drive expression of the two claimed nucleic acid sequences.

199. As an example, Tsutsumi uses different promoters that can be used in a methylotrophic yeast cell in order to drive the expression of different nucleic acid sequences. Specifically, Example 5 of Tsutsumi teaches expression of a gene sequence of interest (cutinase) under the methanol-inducible promoter of the *DAS* gene and the Mxr1 transcription factor (Mxr1p) under the control of the constitutive promoter P_{GAP}. EX-1024(Tsutsumi), [0042]; EX-1005(Vogl), 391 (reviewing prior Tsutsumi disclosure).

200. Thus, as taught or suggested by Tsutsumi, a POSITA would also be motivated to use two different promoters in the same *P. pastoris* cells—e.g., a heterologous polypeptide under the control of a strong methanol-inducible promoter such as the promoter of the *AOX1* or *DAS* genes (as taught in Tsutsumi) in order to maximize its production—and Mxr1p under a different, weaker methanol-inducible

promoter such the *PEX8* gene (as taught in Lin-Cereghino). A POSITA would reasonably expect success doing so in light of the prior art teachings of these and other references, routine techniques, and available materials.

L. Claim 11: *The yeast cell of claim 1, wherein the at least one methanol-inducible promoter element is an alcohol oxidase 1 (AOX1) promoter element from Pichia pastoris.*

201. In my opinion, claim 11 of the '656 patent is obvious. As I explain further above with respect to claim 10, 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.

202. 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.

203. 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.

204. Vogl teaches other benefits of using the promoter of the *AOXI* gene in order to drive recombinant gene expression. One advantage of using this promoter in at least some circumstances is that this promoter produces gene sequences at relatively high levels—that is, it is a “strong” promoter. Indeed, Vogl teaches that this promoter 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 *AOXI* 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 *AOXI* slows down cell growth.

P_{AOXI} 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.

205. 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.

206. 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*.

M. Claim 12: *The yeast cell of claim 1, wherein the second exogenous nucleic acid encodes at least one heterologous polypeptide involved in biosynthesis of an iron co-factor.*

207. In my opinion, claim 12 of the '656 patent is obvious. As I explain further above with respect to claim 1, the combination of Tsutsumi, Lin-Cereghino and Mattoon discloses every limitation of the yeast cell of claim 1.

208. Mattoon further discloses a nucleic acid molecule encoding at least one polypeptide—a protein, such as an enzyme—involved in heme biosynthesis of “heme,” which a POSITA would understand is an iron co-factor as claimed. The '656 patent provides, for example, that “[i]n some embodiments, the at least one heterologous nucleic acid encodes one or more polypeptides involved in the biosynthesis of *an iron co-factor such as heme.*” EX-1002, 2:15-20.

209. For example, Mattoon teaches the “simultaneous production of *heme* and heme binding agents (*e.g.*, apoprotein)” in yeast cells. EX-1008(Mattoon), 2:22-26. Mattoon teaches coordinating the amount of apoprotein produced by yeast cells with the amount of heme synthesized by 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.

210. 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:

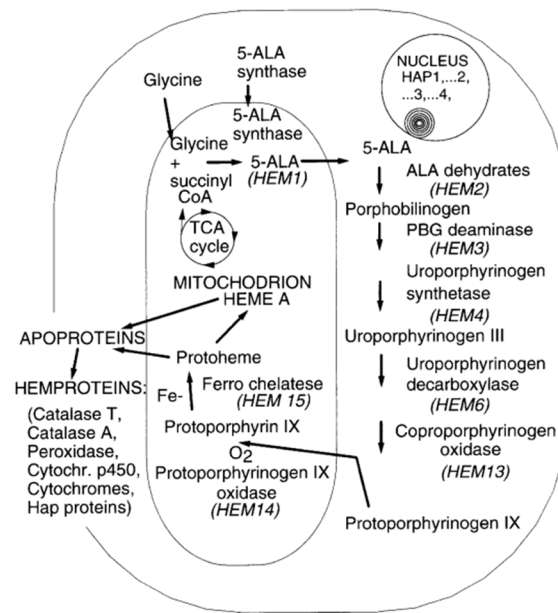


Fig. 1

211. Mattoon 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), 7:62-65, Figs. 6-7. Accordingly, a POSITA would understand that yeast cells transformed with HEM2, HEM3, HEM13, or HEM15, which encode enzymes involved in the heme biosynthetic pathway, 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.

212. Mattoon further motivates 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 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”).

213. Moreover, a POSITA would reasonably expect success in incorporating a heme-biosynthesis nucleic acid sequence in the claimed recombinant nucleic acid molecule 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).

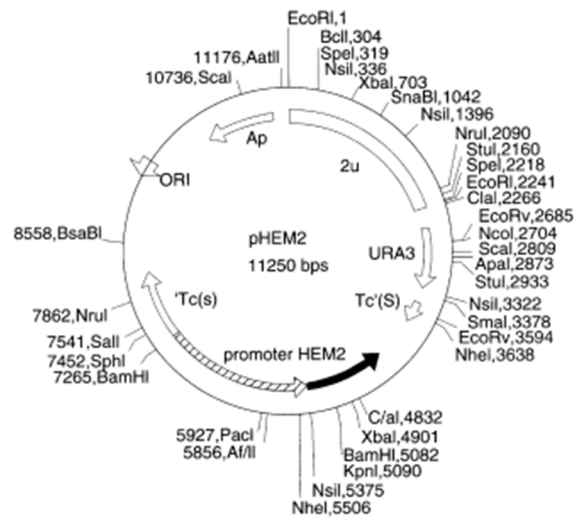


Fig. 4

N. Claim 13: *The yeast cell of claim 12, wherein the iron co-factor is heme.*

214. In my opinion, claim 13 of the '656 patent is obvious for reasons provided above with respect to claims 1 and 12. As provided more fully above with respect to claim 12, for example, a POSITA would be motivated to incorporate in the claimed recombinant nucleic acid molecule a nucleic acid sequence encoding at least one heterologous polypeptide involved in biosynthesis of the iron co-factor heme (for example, the gene sequences *HEM1*, *HEM2*, or *HEM3* encoding biosynthesis enzymes), and would reasonably expect success doing so in light of the teachings of Mattoon, the availability of experimental reagents and materials for use with the *P. pastoris* system, and the general knowledge in the art.

- O. Claim 14: *The yeast cell of claim 13, wherein the at least one polypeptide involved in the biosynthesis of heme is selected from the group consisting of aminolevulinic acid (ALA) synthase, ALA dehydratase, porphobilinogen deaminase, uroporphyrinogen (UPG) III synthase, UPG III decarboxylase, coproporphyrinogen (CPG) oxidase, protoporphyrin (PPG) oxidase, and ferrochelatase.***

215. In my opinion, claim 14 of the '656 patent is obvious for reasons provided above with respect to claims 1 and 12-13. As I explain in greater detail above for claim 12, for example, Mattoon teaches the expression of at least one of the claimed polypeptides involved in heme biosynthesis.

216. As I explain above with respect to claim 12, for example, Mattoon teaches “identified enzymes in the heme biosynthetic pathway,” which include enzymes claimed in claim 14: “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 [porphobilinogen] deaminase (encoded by HEM3)*.” EX-1008(Mattoon), 1:54-58, Fig. 1.

217. Mattoon further discloses expressing the heme biosynthesis enzyme along with the gene sequence of interest in *P. pastoris*. For example, Mattoon teaches “double transformant yeast cultures simultaneously harboring the multicopy plasmid pCCT1,” which expresses the gene sequence encoding the heme-containing

protein catalase T, “and one of the following multicopy plasmids: pHEM2, pHEM3, pHEM13, and pHEM15.” EX-1008(Mattoon), 7:62-65, Figs. 6-7.

218. 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.

P. Claim 15: *The yeast cell of claim 13, wherein the at least one polypeptide involved in the biosynthesis of heme comprises two or more polypeptides selected from the group consisting of ALA synthase, ALA dehydratase, porphobilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and ferrochelatase.*

219. In my opinion, claim 15 of the '656 patent is obvious for reasons provided above with respect to claims 1 and 12-13. As I explain in greater detail above for claim 12, for example, Mattoon teaches the expression of at least one of the claimed polypeptides involved in heme biosynthesis. A POSITA would be motivated to express two or more such polypeptides, and would reasonably expect

success doing so at least in light of the teachings of Mattoon and general knowledge in the art concerning recombinant protein expression.

220. For example, Mattoon teaches that transforming *P. pastoris* cells with expression vectors encoding multiple enzymes (*e.g.*, *HEM2*) can advantageously increase production of heme-containing proteins by modulating expression levels of these enzymes. Specifically, Mattoon teaches:

The present inventors have discovered that the second enzyme in the yeast heme biosynthetic pathway is the primary rate limiting enzyme. ***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.*** By doing so, the normal rate limiting step in the pathway at the second enzyme is shifted back to the first enzyme. For example, in the yeast heme pathway between 5-aminolevulinate and uroporphyrinogen, the rate limiting enzyme is PBG synthase. When excess PBG synthase is introduced into a cell by transformation with, for example, a multicopy plasmid bearing the *HEM2* gene, ALA synthase becomes the rate limiting enzyme. ***Similar transformation of cells to achieve the overexpression of HEM1 can then be performed to further adjust the production of desired hemoproteins.***

EX-1008(Mattoon), 6:45-63.

Q. Claim 16: *A method for expressing a heterologous polypeptide in a cell, the method comprising: providing the yeast cell of claim 1; and culturing the cell under conditions for expression of the recombinant nucleic acid molecule, thereby expressing the heterologous polypeptide.*

221. In my opinion, claim 16 of the '656 patent is obvious for reasons provided above with respect to claim 1. As I explain more fully above, Tsutsumi, Lin-Cereghino and Mattoon render obvious the yeast cell of claim 1. It would be obvious to a POSITA to utilize the yeast cell of claim 1 in order to express a heterologous polypeptide (the protein product of the gene sequence of interest) by culturing the cell under conditions for expression of the recombinant nucleic acid molecule.

222. This method is exemplified in, and obvious in view of, at least the Tsutsumi reference. In its Example 5, for example, Tsutsumi discloses culturing methylotrophic *P. pastoris* yeast cells under conditions for expression of the recombinant nucleic acid molecule to produce a heterologous polypeptide, including by co-expressing the Mxr1 transcription factor. Although Tsutsumi does not disclose that Mxr1 is produced under control of a methanol-inducible promoter, it would be obvious to a POSITA to engineer this expression using conventional techniques as discussed further above with respect to claim 1.

223. Specifically, Tsutsumi discloses that the Example 5 “experiment was to check whether the expression of a protein of interest, exemplified by the *Humicola*

insolence [sic] cutinase gene controlled by an improved DAS promoter according to the invention,...could be improved by co-expression of transcription factors, such as...Mxr1 in *Pichia pastoris*.” EX-1024(Tsutsumi), [0137]. Tsutsumi discloses that the yeast cells of Example 5 were cultured under conditions for expression of the *Humicola insolens* cutinase and Mxr1 of the DNA construct. Specifically, Tsutsumi discloses the following small-scale cell culture conditions:

After heat shock at 42° C, the cells were harvested and re-suspended in 1 ml YPDS w/o Zeocin and incubated with shaking at 30° C, 200 rpm for 3 h. The cells were then harvested, resuspended in 100 µl of the supernatant and plated on YPDS plates containing 100 µg/ml Zeocin.” EX-1024(Tsutsumi), [0142].

224. For large-scale expression, Tsutsumi discloses the following cell culture conditions:

One transformant from each of the above transformations that showed expression in small scale was chosen for expression in large scale: inoculation in shake flasks with 150 ml Buffered Sorbitol Complex media (BSCM) in 500 ml baffled flasks. After 24 h of growth, the cultures were induced with 100% methanol to a final concentration of 1 %. Once induction was started, the cotton plugs were replaced with four layers of gauze cloth+1 layer of Cheese cloth. Induction was carried out every 24 h at 22° C, 150 rpm for 3 days with induction and without induction for 2.5 days (over the weekend). An aliquot of culture (500 µl) was pelleted by centrifugation at 13000 rpm for 5 min

and the supernatants (12 µl) analyzed for protein expression on a 12% SDS-PAGE with coomassie stain.”

EX-1024(Tsutsumi), [0143].

225. Tsutsumi discloses that, under these conditions, the “test protein”—i.e., heterologous polypeptide (*Humicola insolens* cutinase)—was expressed and that this expression was improved when the Mxr transcription factor was “over-expressed.” EX-1024(Tsutsumi), [0144]-[0145].

226. Additionally, as discussed further above with respect to claim 1, Lin-Cereghino discloses culturing methylotrophic *P. pastoris* yeast cells under conditions for over-expression of Mxr1 under the control of methanol-inducible promoters. EX-1006(Lin-Cereghino), 884 (see “Media, strains, and microbial techniques” and “Induction on carbon sources”), 893 (describing results of over-expression).

R. Claim 17: *The method of claim 16, wherein the heterologous polypeptide is a member of globin family PF00042.*

227. In my opinion, claim 17 of the ’656 patent is obvious for reasons I explain more fully above with respect to claims 1 and 16.

228. As exemplified in GenBank, for example, a POSITA would understand that PF00042 is a reference number to the family of globin containing proteins. EX-1048(GenBank), 1. The “molecular function” of these proteins is “heme binding.”

Accordingly, a POSITA would understand that the PF00042 designation refers to a class of heme-containing (heme-binding) proteins.

globin domain	
Details	
NCBI HMM accession	NF012272.2
Source identifier	EMBL-EBI PF00042
Product name ?	globin domain-containing protein
Label ?	Globin
Family type ?	domain
GO term(s) ?	Molecular function: heme binding (GO:0020037)

EX-1048(GenBank), 1.

229. It would be obvious to a POSITA to utilize a heme-binding protein gene sequence to encode the claimed heterologous polypeptide in light of the teachings of Mattoon. For example, Mattoon discloses “recombinant cells” comprising “a gene encoding a heme binding agent” (*e.g.*, apoprotein) “that when expressed, is capable of” binding to heme. EX-1008(Mattoon), 2:22-24, 2:57-60. Mattoon further discloses yeast cells that produce the hemoprotein catalase “as a model for producing other hemoproteins, such as hemoglobin [and] myoglobin.” EX-1008(Mattoon), 7:26-33.

230. A POSITA would understand that hemoproteins such as catalase, hemoglobin, and myoglobin are also members of the globin family PF00042. For

example, the Crane reference teaches that these proteins are “globins” and that they bind heme: “[t]he 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, **legghemoglobin**).” EX-1010(Crane), [0021].

S. **Claim 21: *The method of claim 16, wherein the introducing step comprises a technique selected from the group consisting of transduction, electroporation, biolistic particle delivery, and chemical transformation.***

231. In my opinion, claim 21 of the '656 patent is obvious, including for reasons provided more fully above with respect to claims 1 and 16. Moreover, a POSITA would be generally motivated to utilize conventional techniques for transforming methylotrophic yeast host cells with recombinant nucleic acid molecules, such as the techniques recited in claim 21. A POSITA would understand that these techniques would likely be successful methods to achieve the desired ultimate goal of heterologous protein expression in the host cell. The art taught that such approaches and reagents were available to a POSITA:

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].

232. As further example, Tsutsumi and Mattoon disclose the use of a conventional “electroporation” introducing step for integrating recombinant nucleic acid molecules into *P. pastoris*. For example, Tsutsumi describes “transform[ing]” recombinant DNA into *P. pastoris* strains by “electroporation.”

Transformation of Pichia Pastoris:

Pichia pastoris strains were transformed by electroporation. Fresh competent cells were prepared by the following procedure. Linearized plasmid DNA (1~2 µg) was mixed with 80 µl of fresh competent cells and stored on ice for 5 min. Cells were transferred to an ice-cold 0.2 cm electroporation cuvette. Transformation was performed using a BioRad™ GenePulser II. Parameters used were 1500 V, 25 µF and 200Ω. Immediately after pulsing, cells are suspended in 1 ml of ice cold 1 M sorbitol. The mixtures were plated on the relevant selection plates.

EX-1024(Tsutsumi), [0115].

233. Mattoon similarly teaches that electroporation is a familiar technique to introduce nucleic acids (e.g., the claimed recombinant nucleic acid molecule) into host cells such as methylotrophic *P. pastoris* cells:

As used herein, transfection includes any means for introducing a nucleic acid sequence or expression vector into a host cell, including, but not limited to, transformation (e.g., with DNA coated microbeads), electroporation, microinjection, absorption, and protoplast fusion.

EX-1008(Mattoon), 4:41-48.

T. Claim 23: *The method of claim 16, wherein the culturing step comprises culturing the cell in the presence of methanol.*

234. In my opinion, claim 23 of the '656 patent is obvious for reasons I explain more fully above with respect to claims 1 and 16. Additionally, a POSITA would be motivated to culture methylotrophic yeast cells such as *P. pastoris* in the presence of methanol for several reasons. When methanol is present in the environment at certain (non-toxic) levels, for example, it serves as an energy source for methylotrophic yeast. When a recombinant nucleic acid molecule (e.g., an expression vector) is designed so that nucleic acids are expressed in methylotrophic yeast host cells under the control of a methanol-inducible promoter, a POSITA would be motivated to culture the cells in methanol in order to induce gene expression.

235. A POSITA would recognize, however, that culturing in methanol could be desirable at certain times during the recombinant protein production process but not at other times. These considerations are exemplified in the Vogl review, which teaches certain advantages of culturing methylotrophic yeast both in the presence and absence of methanol.

236. Vogl describes how expression can be “repressed under growth on repressing carbon sources (e.g. glucose)” and “strongly induced on methanol.” EX-1005(Vogl), 386. First, a POSITA would grow the cells to high densities while repressing recombinant protein expression in a medium that contained glucose or glycerol, i.e., in the absence of methanol, to prevent interference by the expressed recombinant protein on the growth of the cells. EX-1005(Vogl), 386. Once the cells have grown to a significant mass (tight cell density), a POSITA would derepress expression by depleting the glucose or glycerol and adding methanol. EX-1005(Vogl), 386. This would in turn strongly induce recombinant protein expression driven by methanol-inducible promoters from a large number of cells. EX-1005(Vogl), 386. According to Vogl, methanol induction of the AOX1 promoter has been used to produce the “highest expression levels” of recombinant proteins:

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.

237. Similarly, **Tsutsumi** discloses that its culturing conditions involve steps in the absence and presence of methanol. For example, Tsutsumi describes certain steps of its large-scale culturing processes as occurring in the presence or absence of methanol (for example, by adding methanol as it is depleted):

One transformant from each of the above transformations that showed ***expression in small scale was chosen for expression in large scale: inoculation in shake flasks with 150 ml Buffered Sorbitol Complex media (BSCM) in 500 ml baffled flasks. After ~24 h of growth, the cultures were induced with 100% methanol*** to a final concentration of 1%. Once induction was started, the cotton plugs were replaced with four layers of gauze cloth+1 layer of Cheese cloth. Induction was carried out every 24 h at 22° C., 150 rpm for 3 days ***with induction and without induction for 2.5 days (over the weekend)***. An aliquot of culture (500 µl) was pelleted by centrifugation at 13000 rpm for 5 min and the supernatants (12 µl) analyzed for protein expression on a 12% SDS-PAGE with coomassie stain.

EX-1024(Tsutsumi), [0143]. A POSITA would understand that Buffered Sorbitol Complex media does not contain methanol.

238. A POSITA would understand from these disclosures that Tsutsumi describes conventional methods for culturing *P. pastoris* yeast cells with and without methanol where the cells contain methanol-inducible promoters and that such methods could be desirably used to maximize protein expression, with a reasonable likelihood of success in light of the teachings of Tsutsumi and other prior art (e.g., Vogl).

U. Claim 24: *The method of claim 16, wherein the culturing step comprises culturing the cell in the absence of methanol.*

239. In my opinion, claim 24 of the '656 patent is obvious for reasons I explain more fully above with respect to claims 1 and 16. Additionally, as I explain more fully above with respect to claim 23, a POSITA would recognize the advantages of culturing methylotrophic yeast in the absence of methanol—e.g., during the expansion phase, in order to maximize cellular growth and avoid the metabolic costs to the yeast cells of devoting resources to recombinant protein production—and would reasonably expect success in doing so in light of the teachings of the prior art, such as Tsutsumi and Vogl.

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.

V. Claim 25: *The method of claim 16, wherein the culturing step comprises culturing the cell in the absence of selection.*

240. In my opinion, claim 25 of the '656 patent is obvious for reasons I explain more fully above with respect to claims 1 and 16. In addition, claim 25 is obvious because a POSITA would recognize that cells transformed with the claimed recombinant nucleic acid molecule need to express the protein that confers selection (antibiotic) resistance in order to have resistance to the antibiotic. A POSITA would recognize that the cells would need a few hours to become resistant (a selection marker) or else the cells will die when exposed to the antibiotic.

241. These general motivations are taught in Tsutsumi, which discloses a culturing step comprising culturing methylotrophic yeast cells in the absence of selection. Tsutsumi's large-scale culturing phase, which I discuss above with respect to claims 23-24, does not involve any selection. However, Tsutsumi discloses that the transformants used in its large-scale culturing were initially grown and induced in medium without the Zeocin antibiotic that was later used to select

transformants expressing the heterologous polypeptide (*Humicola insolens* cutinase) during its small-scale culturing phase.

After heat shock at 42° C., the cells were harvested and re-suspended in 1 ml YPDS *w/o Zeocin* and incubated with shaking at 30° C., 200 rpm for 3 h. The cells were then harvested, resuspended in 100 µl of the supernatant and plated on YPDS plates containing 100 µg/ml Zeocin.

EX-1024(Tsutsumi), [0142].

242. A POSITA would understand from these disclosures that Tsutsumi discloses culturing the cell in the absence of selection. Thus, a POSITA would be motivated to culture methylotrophic yeast cells comprising the claimed recombinant nucleic acid molecule during at least certain stages of the recombinant protein production process as explained above, and, in light of the teachings of Tsutsumi, would reasonably expect success in doing so.

X. GROUND 2: TSUTSUMI, LIN-CEREGHINO, MATTOON, AND CRANE

243. In my opinion, the combination of the Tsutsumi, Lin-Cereghino, Mattoon and Crane references renders obvious Claims 18-20. Similarly, Crane is analogous art because Crane describes methods of producing recombinant heme-containing proteins in microbial expression systems including methylotrophic yeast (*P. pastoris*). Similar to Mattoon, Crane discloses “methods of producing recombinant functional heme-binding proteins with complete heme incorporation”

by “co-express[ing] a recombinant heme-binding protein of interest with ferrochelatase” (*i.e.*, co-expressing a nucleic acid molecule encoding a heme-containing protein with a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis). EX-1010(Crane), Abstract, [0044]. As taught in Crane, the recombinant heme-binding proteins can be produced in “*Pichia pastoris* expression systems.” *Id.* Crane similarly teaches that it is “desirable to use strong promoters” to direct “high level[s]” of expression of the recombinant heme-binding protein. *Id.*, [0040].

A. Claim 18: *The method of claim 16, wherein the heterologous polypeptide is a plant hemoglobin.*

244. In my opinion, claim 18 of the ’656 patent is obvious for reasons I explain more fully above with respect to claims 1 and 16. Additionally, claim 18 is obvious because electing a “plant hemoglobin”—specifically, “leghemoglobin”—as the heterologous polypeptide of claim 16 would have been obvious to a POSITA based on Tsutsumi, Lin-Cereghino and Mattoon alone, or further in view of Crane.

245. Plant hemoglobins, and their gene sequences, were known in the art. Below I reproduce Table 1 of a 2007 review article by Garrocho-Villegas, titled “Hemoglobin sequences identified in land plants.” EX-1014(Garrocho), 81. This table also provides the “Accession numbers in the GenBank database” for each

sequence. Accordingly, a POSITA would have access to these sequences for cloning into the claimed recombinant nucleic acid molecule.

Table 1
Hemoglobin sequences identified in land plants

Organism	Hb ^a (accession number) ^b	
I. Seed plants		
a) Angiosperms		
Monocotyledonous		
<i>Hordeum vulgare</i> (barley)	nsHb-1 (U94968), tHb (AF376063)	<i>Medicago sativa</i> (alfalfa)
<i>Oryza sativa</i> (rice)	nsHb1–1 (U76029), nsHb2–1 (U76031), nsHb3–1 (AF335504), nsHb4–1 (AF335504), tHb (BADJ32857)	<i>M. truncatula</i> (barrel medic)
<i>Saccharum spp.</i> (sugarcane)	tHb-I (C0373680)	<i>Sesbania rostrata</i>
<i>Triticum aestivum</i> (wheat)	nsHb-1 (AY151390), tHb (AY151391)	<i>Vicia faba</i> (broad bean)
<i>Zea mays ssp. mays</i> (maize)	nsHb-1 (AY005818), nsHb-2 (DQ171946)	<i>V. sativa</i> (common vetch)
<i>Z. mays ssp. parviglumis</i> (teosinte)	nsHb-1 (AF291052)	<i>Vigna unguiculata</i> (cowpea)
Dicotyledonous (legumes)		
<i>Astragalus sinicus</i>	symbHb (IQ199647)	Dicotyledonous (no-legumes)
<i>Canavalia lineata</i> (bay bean)	symbHb (UD9671)	<i>Alnus firma</i>
<i>Glycine max</i> (soybean)	symbHb (V00453), nsHb-1 (U47143), tHb (AA548191)	<i>Arabidopsis thaliana</i>
<i>Lotus japonicus</i>	symbHb (AB238218), nsHb-1 (AB238220)	<i>Beta vulgaris</i> (sugar beet)
<i>Lupinus luteus</i> (yellow lupine)	symbHb (Y00401)	<i>Brassica napus</i> (canola)
<i>Phaseolus vulgaris</i> (kidney bean)	symbHb (P02234)	<i>Casuarina glauca</i> (swamp oak)
<i>Pisum sativum</i> (pea)	symbHb (P02233)	<i>Cichorium intybus × C. endivia</i> (chicory)
<i>Psophocarpus tetragonolobus</i> (winged bean)	symbHb (X65874)	<i>Citrus unshiu</i> (satsuma orange)
		<i>Datisca glomerata</i> (durango root)
		<i>Euryale ferox</i> (fox nut)
		<i>Gossypium hirsutum</i> (cotton)
		<i>Lycopersicon esculentum</i> (tomato)
		<i>Malus domestica</i> (apple)
		<i>Myrica gale</i>
		<i>Nicotiana tabacum</i> (tobacco)
		<i>symHb</i> (M32883), nsHb-1 (AF172172)
		<i>symHb</i> (X57733), tHb (ABE93362)
		<i>symHb</i> (M23313)
		<i>symHb</i> (Z54159)
		<i>symHb</i> (YO0229)
		<i>symHb I</i> (U33206), <i>symHb II</i> (U33207)
		I. Seed plants
		a) Angiosperms
		Dicotyledonous (no-legumes)
		<i>Parasponia anderssonii</i> nsHb-1 (U27194)
		<i>P. rigida</i> nsHb-1 (P68169)
		<i>Pyrus communis</i> (pear) nsHb-1 (AAY224133)
		<i>Raphanus sativus</i> (horseradish) nsHb-1 (AY286331)
		<i>Solanum tuberosum</i> (potato) nsHb2–1 (AY151389)
		<i>Trema orientalis</i> nsHb-1 (AF027215)
		<i>T. tomentosa</i> nsHb-1 (P07803)
		<i>T. virgate</i> nsHb-1 (AJ131349)
		b) Gymnosperms
		<i>Pinus taeda</i> (pine) tHb (BQ291271)
		II. Bryophytes
		a) Mosses
		<i>Physcomitrella patens</i> nsHb (AF218049)
		<i>Ceratodon purpureus</i> nsHb (AF309562)
		b) Liverworts
		<i>Marchantia polymorpha</i> (liverwort) nsHb (AY026341)

246. One of these options was leghemoglobin—one of the earliest discovered plant hemoglobins. 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.

247. 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. A POSITA would have understood that Mattoon’s disclosures would be applicable to heme-containing proteins such as plant hemoglobins.

248. Like Tsutsumi, Lin-Cereghino, and Mattoon, the Crane reference teaches expressing proteins in *P. pastoris*, and specifically, co-expressing the sequences of heme-containing proteins and heme biosynthetic enzymes in *P. pastoris*. EX-1010(Crane), Abstract, [0009], [0044]. Indeed, similar to the Mattoon reference, 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.”).

249. 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].

250. For essentially the same reasons that I explain above for the Mattoon reference, a POSITA would be generally motivated to select Crane’s leghemoglobin for inclusion in the recombinant protein expression system taught by Tsutsumi, Lin-Cereghino, and Mattoon—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. The use of plant heme-containing proteins could appeal to individuals who have concerns about or who otherwise abstain from using proteins that are sourced from animals, for example. A POSITA would reasonably expect success in doing so in light of the teachings of the prior art and routine techniques, and available materials.

B. Claim 19: *The method of claim 18, wherein the plant hemoglobin is leghemoglobin.*

251. In my opinion, claim 19 of the ’656 patent is obvious for reasons I explain more fully above with respect to claims 1 and 16. In particular, as I explain above for claim 18, a POSITA would have been generally motivated to select the leghemoglobin nucleic acid sequence to encode the claimed heterologous polypeptide, e.g., for “commercial applications” as taught in Crane. A POSITA would reasonably expect success in doing so because, as explained further above for claim 18, leghemoglobin was a well-known plant hemoglobin with a known sequence. 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). As shown in Table 1 of Garrocho-Villegas, leghemoglobin was one of a finite number of hemoglobins (plant or otherwise) to a POSITA to choose from, and a POSITA would be motivated to use it in plant-based commercial applications as I discuss above for claim 18. A POSITA would have reasonably expected success in doing so given the knowledge of the prior art, the use of routine techniques, and available materials.

C. Claim 20: *The method of claim 19, wherein the leghemoglobin has a sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:6.*

252. In my opinion, claim 20 of the '656 patent is obvious for reasons I explain more fully above with respect to claims 1, 16 and 18-19.

253. It would also be obvious to use the sequence identified as SEQ ID NO: 4 of the '656 patent, which is the ***known amino-acid*** sequence of the soybean (*Glycine max*) leghemoglobin. EX-1002, 51-52; EX-1046. As discussed above, Crane teaches the use of methylotrophic *P. pastoris* expression systems expressing leghemoglobin.

254. A POSITA desiring to produce leghemoglobin could have readily obtained the amino-acid sequence found in SEQ ID No: 4 prior to 2015. In particular, the sequence shown in SEQ ID NO: 4 is available in the NCBI's GenBank/GenPept database under Accession Number AAA33980, which first appeared in the database in April 1993. EX-1046, 1-6. The GenBank/GenPept database is publicly available and contains "the most up-to-date and comprehensive DNA sequence information" from multiple databases around the world. EX-1053(NCBI). Thus, a POSITA desiring to know the amino acid sequence of leghemoglobin prior to 2015 would have been able to find the sequence shown in SEQ ID NO: 4 under Accession Number AAA33980 in the GenBank/GenPept databases as explained above (claims 8-9).

255. Additionally, for example, as exemplified in Table 1 of Garrocho-Villegas (2007), excerpted below, a POSITA would know how to locate the nucleic acid sequence encoding *G. max* leghemoglobin, also noted as symbiotic hemoglobin (symHb). EX-1014(Garrocho), 79, 81. A POSITA would be readily able to derive the amino acid sequence from the known nucleic acid sequence encoding this protein using routine methods in the art.

Table 1
Hemoglobin sequences identified in land plants

Organism	Hb ^a (accession number ^b)
<i>I. Seed plants</i>	
a) Angiosperms	
Monocotyledonous	
<i>Hordeum vulgare</i> (barley)	nsHb-1 (U94968), tHb (AF376063)
<i>Oryza sativa</i> (rice)	nsHb1-1 (U76029), nsHb2-1 (U76031), nsHb3-1 (AF335504), nsHb4-1 (AF335504), tHb (BAD32857)
<i>Saccharum</i> spp. (sugarcane)	nsHb-1 (C0373680)
<i>Triticum aestivum</i> (wheat)	nsHb-1 (AY151390), tHb (AY151391)
<i>Zea mays</i> ssp. <i>mays</i> (maize)	nsHb-1 (AY005818), nsHb-2 (DQ171946)
<i>Z. mays</i> ssp. <i>parviglumis</i> (teosinte)	nsHb-1 (AF291052)
Dicotyledonous (legumes)	
<i>Astragalus sinicus</i>	symHb (DQ199647)
<i>Canavalia lineata</i> (bay bean)	symHb (UD9671)
<i>Glycine max</i> (soybean)	symHb (V00453), nsHb-1 (U47143), tHb (AA548191)
<i>Lotus japonicus</i>	symHb (AB238218), nsHb-1 (AB238220)
<i>Lupinus luteus</i> (yellow lupine)	symHb (Y00401)
<i>Phaseolus vulgaris</i> (kidney bean)	symHb (P02234)
<i>Pisum sativum</i> (pea)	symHb (P02233)
<i>Psophocarpus</i> <i>tetragonolobus</i> (winged bean)	symHb (X65874)

XI. GROUND 3: TSUTSUMI, LIN-CEREGHINO, MATTOON, AND KRAINER

256. Like Tsutsumi, Lin-Cereghino, and Mattoon (discussed above for Ground 1, the Krainer reference is analogous art to the '656 patent because it also discloses genetically modifying *P. pastoris* yeast cells to express recombinant proteins. For example, the Krainer study “investigated the effect of both, pathway engineering and medium supplementation, [sic] to optimize the recombinant production of the heme protein horseradish peroxidase in the yeast *Pichia pastoris*.” EX-1031(Krainer), Abstract.

257. As I discuss above for Ground 1, the combination of Tsutsumi, Lin-Cereghino, and Mattoon teach the limitations of claims 1 and 16. It would also have been obvious to a POSITA to add iron or iron salts to the culture medium of claim 16 in light of the teachings of the Krainer reference I discuss further below.

A. Claim 22: *The method of claim 16, wherein the conditions under which the cell is cultured comprises addition of iron or a pharmaceutically or metabolically acceptable salt thereof.*

258. In my opinion, claim 22 of the '656 patent is obvious for reasons I explain more fully above with respect to claims 1 and 16. In addition, adding iron or iron salts to the culture medium of claim 16 would have been obvious to a POSITA based on the teachings of the Krainer reference.

259. Similar to Mattoon, Krainer discloses using methylotrophic *P. pastoris* yeast to co-express (1) the heme-containing protein horseradish peroxidase (“HRP”) and (2) one of the eight heme biosynthetic enzymes (*i.e.*, co-expressing a nucleic acid molecule encoding a heme-containing protein with a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis). EX-1031(Krainer), 3-4.

260. Similar to the teachings of the Mattoon reference, Krainer relates to “[o]ptimizing cofactor availability for the production of recombinant heme peroxidase in *Pichia pastoris*.” EX-1031(Krainer), Title. Krainer teaches

In this study, we aimed at optimizing heme availability and thus boost the amount of recombinant active heme protein, namely horseradish peroxidase (HRP), in the yeast *P. pastoris* by evaluating pathway engineering and medium supplementation. In a systematic approach, we co-overexpressed HRP with genes of the endogenous heme biosynthesis pathway of *P. pastoris*, and assessed the effect of medium supplementation with iron, ALA and hemin on the yield of target enzyme activity.

EX-1031(Krainer), 2. As one means of achieving this optimization, Krainer expressly discloses “medium supplementation with *iron*, heme precursors or hemin (the *ferric chloride* species of heme) ... to effectively saturate recombinant apo-species of heme proteins.” EX-1031(Krainer), 1-2. As a POSITA would recognize, ferric chloride is an iron salt.

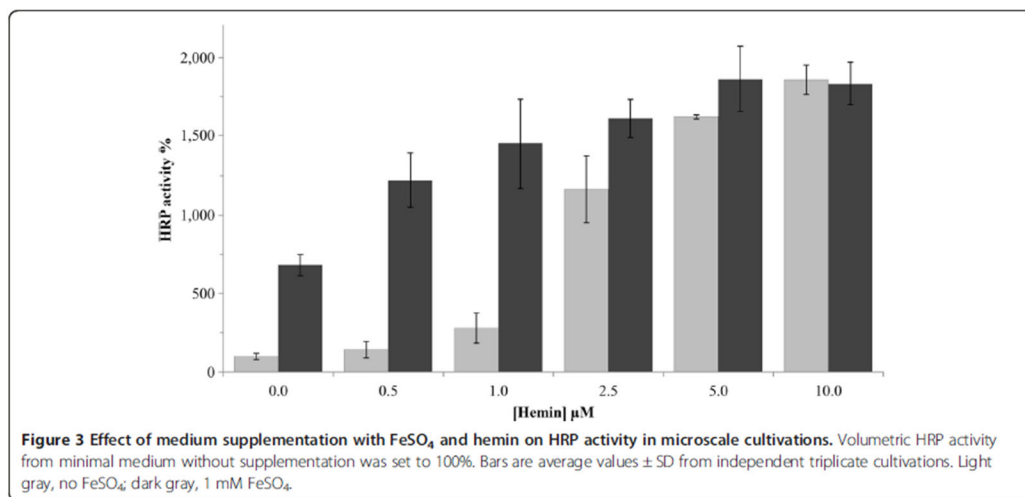
261. Krainer teaches that medium supplementation is beneficial in order to enhance the production of heme-containing proteins. Krainer teaches that “*addition of 1 mM FeSO₄ boosted the amount of active HRP up to 7-fold* (Figure 3), hinting at a potential shortage of iron in heme biosynthesis.” EX-1031(Krainer), 4. As a POSITA would recognize, FeSO₄ (iron sulfate) is a kind of iron salt. As summarized in Krainer:

In this study *we present a systematic approach to optimize heme availability and thus boost the amount of active heme protein produced in the yeast P. pastoris* by evaluating metabolic pathway

engineering and medium supplementation. The results can be summarized as: ...

Medium supplementation with the traditionally used and pricy heme precursor ALA did not increase the yield of active product and can be omitted in future cultivations. *FeSO₄* and hemin on the other hand *turned out to be useful medium supplements to increase the yield of active heme protein.*

EX-1031(Krainer), 6. These results, as measured using HRP (heme-containing protein) activity are further shown in Figure 3 of Krainer:



Krainer thus discloses that “medium supplementation” with iron proved to be an efficient strategy to increase the yield of active heme-containing enzyme and further Krainer’s goal of producing proteins saturated with heme. EX-1031(Krainer), 1-2.

262. Accordingly, in light of the teachings of Krainer, a POSITA would have been motivated to supplement the culture medium of *P. pastoris* cells expressing a heme-containing protein (e.g., as taught in Mattoon) with iron and hemin and would

reasonably expect success in doing so in light of the teachings of Krainer as well as other prior art disclosures and the availability of routine techniques and available materials.

XII. GROUND 4: MATTOON AND VOGL

A. Overview

263. In my opinion, the combination of the Mattoon and Vogl references render claims 26 and 28 obvious. Both Mattoon and Vogl are analogous art to the '656 patent because each discloses genetically modifying *P. pastoris* yeast cells to express recombinant proteins. For example, as explained above (Section VII , Vogl discloses commonly used constitutive and methanol-inducible promoters and transcriptional activators from *P. pastoris* and Mxr1, a key transcriptional activator that regulates methanol-inducible promoters. For reasons I explain further below, a POSITA would be motivated to combine the teachings of Mattoon and Vogl and would reasonably expect success in doing so.

B. Claim 26

264. In my opinion, claim 26 of the '656 patent is obvious over the combination of the teachings of the Mattoon and Vogl references as explained below.

2. **26[pre]: *A methylotrophic Pichia yeast cell comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises: ...***

265. In addition to my analysis above regarding the use of methylotrophic yeast to express recombinant proteins, I note that the **Mattoon** and **Vogl** references both disclose the use of “[a] methylotrophic *Pichia* yeast cell” for the purposes of recombinant expression. For example, 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. Vogl similarly 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.

3. **26[a]: *a nucleic acid encoding a Mxr1 transcriptional activator sequence from *P. pastoris****

266. The **Vogl** reference teaches “a Mxr1 transcriptional activator sequence from *P. pastoris*,” EX-1005(Vogl), 391 as well as the advantages of utilizing Mxr1-driven expression of gene sequences of interest in a recombinant nucleic acid molecule. The nucleic acid sequence encoding Mxr1 was available to a POSITA as I explain above regarding claim 9 (Section VIII.J).

267. Vogl describes the importance of Mxr1 as a regulator of native gene expression in *P. pastoris*. For example, Vogl teaches that Mxr1 is “a key regulator

of methanol inducible genes,” and is a “well characterized” transcriptional activator of methanol-inducible promoters from *P. pastoris*. EX-1005(Vogl), 390-91, 400. Vogl explains that “*MXR1* was shown to be crucial for the activation of numerous genes involved in MUT [methanol utilization pathway].” EX-1005(Vogl), 390. It reviews research concerning the biological activity of Mxr1, including that the deletion of the *MXR1* gene causes “highly reduced protein” expression from genes controlled by methanol-inducible promoters. EX-1005(Vogl), 390 (citing Ex-1006(Lin-Cereghino)). A POSITA would therefore understand from Vogl and the references cited therein that Mxr1 can drive expression of gene sequences from methanol-inducible promoters (e.g., promoters containing sites to which Mxr1 binds). Such sites were known in the art and I discuss them further above with respect to claim 1 (e.g., *AOX1*, *DHAS*, and *PEX8* genes). E.g., EX-1023(Kranthi).

268. Vogl also reviews studies that demonstrate the benefit of co-expressing Mxr1 with gene sequences of interest. 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 lacking the P_{GAP} -*MXR1* construct. EX-1005(Vogl), 391⁶. 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 methanol-inducible promoters regulated by Mxr1 binding (e.g., *AOX1*, *DHAS*, and *PEX8* promoters).

269. A POSITA also would have reasonably expected success in following the well-known techniques disclosed in Vogl and the prior studies it reviews in order to construct similar *P. pastoris* expression systems. 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, which would have been available to a POSITA. As acknowledged in the '656 patent, incorporating the claimed nucleic acid molecule into *P. pastoris* yeast cells only requires routine techniques, known methods, and

⁶ Although Takagi used glucose (a repressing carbon source) growth medium in its experiments, a POSITA would have understood that glucose does not completely shut off P_{AOX1} -driven transcription. EX-1036(Bawa), 1 (describing “leaky expression” from P_{AOX1} in both glucose- and glycerol-grown cells); EX-1006(Lin-Cereghino), 893 (“Mxr1p was *mostly* cytoplasmic in glucose-grown cells”).

available materials for genetic engineering and recombinant protein production.
'656 patent 5:12-17 (see claim 1 above).

270. 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-1005(Crane), 386; EX-1018(De Schutter), 561; EX-1015(Lin-Cereghino_2000), 45.

271. 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 operably linked to a promoter element from *P. pastoris*.

272. 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 *Pichia* (e.g., *P. pastoris*) cells to contain a nucleic acid molecule encoding a heme-containing protein operably linked to a methanol-inducible promoter element, wherein the methanol-inducible promoter element comprises at least one sequence to which a Mxr1 transcriptional activator binds.

273. Moreover, to the extent that claim 26 requires each of the nucleic acids—Mxr1 sequence, PF00042 family member, and heme biosynthesis enzyme—to be on the same recombinant nucleic acid molecule, this approach would have been obvious to a POSITA in light of the state of the art and as taught in Mattoon, as I explain in Section VIII.B.1.a above.

4. 26[b] a nucleic acid encoding a member of globin family PF00042;

274. As a POSITA would recognize, PF00042 is a reference number used by the European Molecular Biology Laboratory/European Bioinformatics Institute (EMBL-EBI) to indicate globin containing proteins, which bind heme. EX-1048

(excerpt below). **Mattoon** discloses yeast cells that produce the hemoprotein catalase “as a model for producing other hemoproteins, such as hemoglobin [and] myoglobin.” EX-1008(Mattoon), 7:26-33.

275. As I explain further above regarding claim 17, for example, a POSITA would understand that heme-containing proteins are members of the globin family PF00042. As exemplified in GenBank and excerpted below, a POSITA would understand that PF00042 is a reference number to the family of globin containing proteins. EX-1048(GenBank), 1. The “molecular function” of these proteins is “heme binding.” Accordingly, a POSITA would understand that the PF00042 number refers to heme-containing (heme-binding) proteins, which would include hemoglobin and myoglobin taught by Mattoon.

globin domain	
Details	
NCBI HMM accession	NF012272.2
Source identifier	EMBL-EBI PF00042
Product name ?	globin domain-containing protein
Label ?	Globin
Family type ?	domain
GO term(s) ?	Molecular function: heme binding (GO:0020037)

EX-1048(GenBank), 1.

276. Additionally, Mattoon exemplifies “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. Thus, 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.

277. Mattoon also teaches that more than one nucleic acid “can be present on one expression vector.” EX-1008(Mattoon), 5:34-46. As I discuss in greater detail above (Section VIII.B.1.a), a POSITA would therefore understand that the nucleic acids of limitations 26[a] and 26[b] can be present on one recombinant nucleic acid molecule.

5. 26[c] and a nucleic acid encoding at least one polypeptide involved in heme biosynthesis,

278. **Mattoon** discloses the inclusion of a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis in its systems for expressing heme-containing proteins (e.g., catalase). For example, Mattoon 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.”). I discuss these teachings of Mattoon further above regarding claims 12-14. Additionally, Mattoon would motivate a POSITA to utilize a nucleic acid encoding at least one 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.

279. Additionally, 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. Mattoon further discloses the “simultaneous production of heme and heme binding agents

(e.g., apoprotein)” in yeast cells. EX-1008(Mattoon), 2:22-26. 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* nucleic acid sequences would comprise “a nucleic acid encoding at least one polypeptide involved in heme biosynthesis.”

280. Additionally, as previously noted, Mattoon teaches that more than one nucleic acid “can be present on one expression vector.” EX-1008(Mattoon), 5:34-46. As I discuss in greater detail above (Section VIII.B.1.a), a POSITA would therefore understand that the nucleic acids of limitations 26[a] and 26[b] can be present on one recombinant nucleic acid molecule.

6. **26[d]: *wherein each nucleic acid is operably linked to a methanol-inducible promoter element, wherein the methanol-inducible promoter element comprises at least one sequence to which a Mxrl1 transcriptional activator binds.***

281. **Mattoon** discloses or suggests that “each nucleic acid is operably linked to a methanol-inducible promoter” with at least one sequence to which Mxr1 binds. For example, Mattoon discloses linking nucleic acid sequences (e.g., of

heme-binding proteins and their biosynthesis enzymes to inducible promoters. As I explain above, Mattoon teaches that expression vectors can be “controllable by inducible or constitutive promoters” that are “operatively linked” to the nucleic acid encoding the protein(s) of interest. EX-1008(Mattoon), 2:41-44, 5:51-59, 6:9-63; 4:29-40. Mattoon teaches, for example, using inducible (conditional) promoters:

Expression vectors of the present invention should be operatively connected to a suitable promoter (e.g., conditional promoters), being any DNA sequence which shows transcriptional activity in the host cell of choice, and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Mattoon, 5:5-10.

282. In order to achieve inducible expression of genes of interest when using a *P. pastoris* system to produce recombinant proteins—as was widely done in the art—a POSITA would have been motivated to and naturally looked to a reference such as **Vogl** (which reviews the state of the art regarding gene expression in *P. pastoris*) to select suitable inducible promoters, such as the promoters for *AOX1*, *DAS*, and *PEX8* genes, to control the expression of the nucleic acid encoding a member of the globin family of proteins, a polypeptide involved in heme biosynthesis, and Mxr1. EX-1005(Vogl), 385-87, 391; EX-1008(Mattoon), 2:41-44, 5:51-59, 6:9-63.

283. A POSITA would have also been motivated to express Mxr1 in a methanol-inducible *P. pastoris* expression system. For example, Vogl teaches that Mxr1 plays an important role in the expression of recombinant proteins as Mxr1 is a “positive regulator of methanol inducible genes” in methylotrophic *P. pastoris*. EX-1005(Vogl), 387, 390. For reasons that I explain further above with respect to claim 1, a POSITA would understand that the promoters of the *AOX1*, *DAS*, and *PEX8* genes are methanol-inducible promoters containing sequences that the Mxr1 transcriptional activator binds and activates. EX-1005(Vogl), 393 (citing EX-1023(Kranthi (2010))); EX-1006(Lin-Cereghino), 893. Additionally, in reviewing the Takagi research (summarized above), Vogl teaches that high levels of Mxr1 expression (driven by the strong *GAP* promoter) advantageously results in higher expression of a protein of interest controlled by a promoter activated by Mxr1. EX-1005(Vogl), 391. Vogl also teaches advantages of methanol-inducibility, as I discuss above—e.g., “tight regulation” of gene expression, and that “controllable promoters are a crucial tool for efficient heterologous protein production.” EX-1005(Vogl), 386.

284. Accordingly, based on Vogl’s teachings, a POSITA would have been motivated to utilize a methanol-inducible, Mxr1-regulated promoter (for example, the promoters of the *AOX1*, *DAS*, and *PEX8* genes) as well as to express Mxr1 itself in Mattoon’s *P. pastoris* expression system in order to produce recombinant heme-

containing proteins. As I explain further above with respect to claim 1, a POSITA would have reasonably expected success in operably linking each nucleic acid to a methanol-inducible promoter using well-known, routinely-used techniques and available materials.

C. Claim 28: *The yeast cell of claim 26, wherein the methanol-inducible promoter element is an alcohol oxidase 1 (AOX1) promoter element from P. pastoris.*

285. In my opinion, claim 28 of the '656 patent is obvious. As I discuss further above (for example, with respect to claims 1 and 26), a POSITA would have been able to choose from among multiple well-known promoters for use in driving gene expression in a *P. pastoris* expression system—either constitutive or methanol-inducible. One of the promoters that could be used in this system is the methanol-inducible promoter of the *AOX1* gene.

286. The **Vogl** reference teaches a variety of reasons why a POSITA would select the promoter of the *AOX1* gene in a *P. pastoris* expression. First, as a POSITA would know, and as Vogl teaches, this promoter is a promoter for genes of the methanol utilization pathway (MUT). Vogl explains, for example, that MUT gene promoters are “one of the most favorable” aspects of using methylotrophic yeast species such as *P. pastoris*.

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.

287. 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 be better able to predict the results of using a well-studied promoter because, for example, using a promoter with well-known structure and function would make it easier to interpret or troubleshoot any unexpected results. Thus, for example, as a POSITA would recognize, using a well-studied promoter desirably saves research time and effort. Vogl teaches 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.

288. Vogl teaches other benefits of using the promoter of the *AOXI* gene. One advantage of using this promoter in at least some circumstances is that this promoter produces gene sequences at relatively high levels—that is, it is a “strong” promoter. Indeed, Vogl teaches that this promoter 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 that using the promoter of the *AOXI* gene enables cells to be grown to high densities before gene expression is induced by methanol, which, as a POSITA would recognize, may be desirable for the industrial production of biologics (in bioreactors) or if the protein generated from the gene sequence of interest may be toxic to the yeast cells.

P_{AOXI} 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.

289. I note that Lin-Cereghino contains an experiment in which *P. pastoris* transformed with construct expressing Mxr1 under the control of the *AOX1* promoter failed to grow. EX-1006(Lin-Cereghino), 893 (“[T]his strain, which was otherwise the wild type, did not grow on methanol and did not induce Mxr1p or other methanol pathway components in response to methanol. Apparently, high levels of Mxr1p are toxic to *P. pastoris*.”). In my opinion, this statement would not discourage a POSITA in 2015 from using the *AOX1* promoter in the claimed recombinant nucleic acid molecule. Based on my review of Lin-Cereghino, for example, experiments were

not reported to investigate Mxr1 toxicity in *P. pastoris* and alternative causes for why *P. pastoris* harboring pGC215 failed to grow. Moreover, a POSITA would understand from the subsequent teaching in the art, such as Tsutsumi and Vogl (discussed above with respect to claim 1), that Mxr1 overexpression is not toxic to *P. pastoris*. Accordingly, a POSITA would understand that it would be feasible to enhance expression of the claimed Mxr1, globin, and heme biosynthesis polypeptide nucleic acid sequences using the *AOX1* promoter.

290. Accordingly, a POSITA would be motivated to select the promoter of the *AOX1* gene for use in driving the expression of genes 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. 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 clone 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*.

XIII. GROUND 5: MATTOON, VOGL, AND CRANE

291. In my opinion, the combination of Mattoon and Vogl in further view of Crane renders claim 27 obvious. Mattoon is analogous art to the '656 patent as I discuss above. Vogl is analogous art to the '656 patent because, as I explain in this Declaration, Vogl (a review of the technical literature) describes promoters and

techniques for producing recombinant proteins in *P. pastoris*, and the results of such experiments. As discussed in Ground 4, Mattoon and Vogl render obvious the claimed methylotrophic *Pichia* yeast cell comprising a recombinant nucleic acid molecule of claim 26. For essentially the same reasons as I provide above for Ground 2 (in particular, for claims 18-19), it would be obvious to a POSITA to produce leghemoglobin as taught in Crane in the claimed *Pichia* recombinant expression system.

A. Claim 27: *The yeast cell of claim 26, wherein the member of the globin family is leghemoglobin.*

292. In my opinion, it would be obvious to utilize leghemoglobin (e.g., the gene sequence encoding the leghemoglobin protein) in the claimed yeast cell. As I explain further above with respect to Ground 2 and claim 18, for example, 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. The gene sequence of leghemoglobin was therefore available to a POSITA and could be combined in the claimed recombinant nucleic acid molecule using conventional and familiar cloning techniques as previously explained.

293. In particular, as discussed further above (e.g., claims 18-19), Crane discloses “methods of producing recombinant functional heme-binding proteins,” including by constructing a *P. pastoris* expression system that can be used to express

a wide variety of heme-containing proteins. EX-1010(Crane), Abstract, [0009], [0044]. One of these genes is 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., ... *leghemoglobin*), ...”).

294. Accordingly, and for all the reasons that I discuss above regarding claim 26 with respect to Mattoon and Vogl, whether alone or further in view of Crane, a POSITA would have been motivated to select the leghemoglobin nucleic acid sequence as the member of the globin family to be used in the claimed recombinant nucleic acid molecule.

XIV. NO OBJECTIVE EVIDENCE OF NONOBVIOUSNESS

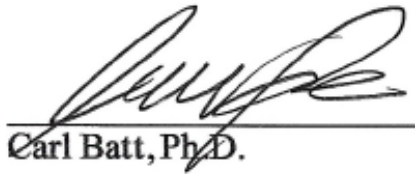
295. I am unaware of any objective indicia of nonobviousness for the '656 patent. Should Patent Owner come forward with any objective indicia evidence, I reserve the right to respond and rebut such evidence.

XV. CONCLUSION

296. 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.

297. 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