

**UNITED STATES PATENT AND TRADEMARK OFFICE**

---

**BEFORE THE PATENT TRIAL AND APPEAL BOARD**

---

MOTIF FOODWORKS, INC.,

Petitioner,

v.

IMPOSSIBLE FOODS INC.,  
Patent Owner.

---

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

---

**DECLARATION OF DR. GEOFFREY P. LIN-CEREGHINO , PH.D.  
IN SUPPORT OF PETITION FOR *INTER PARTES* REVIEW OF  
U.S. PATENT NOS. 10,273,492 AND 10,689,656**

## **TABLE OF CONTENTS**

|                                                                           | <b><u>Page</u></b> |
|---------------------------------------------------------------------------|--------------------|
| I. INTRODUCTION .....                                                     | 1                  |
| II. EXPERIENCE AND QUALIFICATIONS.....                                    | 2                  |
| III. SUMMARY OF OPINIONS.....                                             | 6                  |
| IV. LEGAL STANDARDS .....                                                 | 8                  |
| A. Person of Ordinary Skill in the Art .....                              | 8                  |
| B. Obviousness.....                                                       | 9                  |
| V. THE '492 and '656 PATENTs .....                                        | 10                 |
| A. Overview of the '492 and '656 Patents.....                             | 10                 |
| B. Person of Skill in the Art for the '492 and '656 Patents .....         | 14                 |
| VI. LITERATURE.....                                                       | 15                 |
| A. Lin-Cereghino (2006).....                                              | 15                 |
| B. U.S. Patent Publication No. 2011/0129874 (Tsutsumi).....               | 19                 |
| C. U.S. Patent No. 8,143,023 (Takagi) .....                               | 21                 |
| D. Vogl (2013) .....                                                      | 22                 |
| VII. RECOMBINANT PROTEIN EXPRESSION IN PICHIA PASTORIS .....              | 25                 |
| A. Overview of Recombinant Protein Expression .....                       | 25                 |
| B. <i>P. pastoris</i> as an Expression System.....                        | 29                 |
| 1. The <i>P. pastoris</i> <i>AOX1</i> Promoter (P <sub>AOX1</sub> ) ..... | 33                 |
| 2. Mxr1p and Its Relationship to P <sub>AOX1</sub> .....                  | 38                 |
| VIII. THE '492 and '656 PATENTS .....                                     | 41                 |
| IX. CONCLUSION.....                                                       | 46                 |

## TABLE OF EXHIBITS

| Exhibit | Description                                                                                                                                                                                           |
|---------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| EX-1001 | U.S. Patent No. 10,273,492 (“the ’492 patent”)                                                                                                                                                        |
| 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. (“Takagi”)                                                                                                                                              |
| EX-1008 | U.S. Patent No. 5,824,511 of Mattoon et al. (“Mattoon”)                                                                                                                                               |
| EX-1010 | U.S. Patent Application No. 2011/0287467 A1 (“Crane”)                                                                                                                                                 |
| EX-1015 | Lin-Cereghino & Cregg, <i>Heterologous protein expression in the methylotrophic yeast Pichia pastoris</i> , FEMS MICROBIOLOGY REVIEWS, 24, 45-66 (2000) (“Lin-Cereghino (2000)”)                      |
| EX-1017 | Hartner et al., <i>Promoter library designed for fine-tuned gene expression in Pichia pastoris</i> , NUCLEIC ACIDS 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-1021 | Sreekrishna, <i>Pichia, optimization of protein expression</i> , ENCYCLOPEDIA OF INDUSTRIAL BIOTECHNOLOGY: BIOPROCESS, BIOSEPARATION, AND CELL TECHNOLOGY, 1-16 (Flickinger ed. 2010) (“Sreekrishna”) |

| Exhibit | Description                                                                                                                                                                                                                                                                                                                          |
|---------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 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 Application No. 2011/0129874 A1 (“Tsutsumi”)                                                                                                                                                                                                                                                                             |
| 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-1032 | Guarna et al., <i>On-line monitoring and control of methanol concentration in shake-flask cultures of Pichia pastoris</i> , BIOTECHNOLOGY & BIOENGINEERING, 56(3), 279-286 (1997) (“Guarna”)                                                                                                                                         |
| EX-1033 | Katakura et al., <i>Effect of methanol concentration on the production of human <math>\beta_2</math>-glycoprotein I Domain V by a recombinant Pichia pastoris: a simple system for the control of methanol concentration using a semiconductor gas sensor</i> , J. FERMENTATION & BIOENGINEERING, 86(5), 482-487 (1998) (“Katakura”) |
| EX-1034 | Hong et al., <i>Fermentation strategies for improved heterologous expression of laccase in Pichia pastoris</i> , BIOTECHNOLOGY & BIOENGINEERING, 79(4), 438-449 (2002) (“Hong”)                                                                                                                                                      |
| EX-1035 | Trinh et al., <i>Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from Pichia pastoris</i> , BIOTECHNOLOGY & BIOENGINEERING, 82(4), 438-444 (2003) (“Trinh”)                                                                                                                            |

| Exhibit | Description                                                                                                                                                                                                                    |
|---------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| EX-1036 | Bawa et al., <i>Functional recombinant protein is present in the pre-induction phases of Pichia pastoris cultures when grown in bioreactors, but not shake-flasks</i> , MICROBIAL CELL FACTORIES 13(127), 1-13 (2014) (“Bawa”) |
| EX-1037 | Ferreira, <i>Heme synthesis</i> , ENCYCLOPEDIA OF BIOLOGICAL CHEMISTRY, 539-42 (2013) (“Ferreira”)                                                                                                                             |
| EX-1042 | Alberts et al., MOLECULAR BIOLOGY OF THE CELL, 305-312, 334-336, 538-540 (4th ed. 2002)                                                                                                                                        |
| EX-1056 | <i>Curriculum Vitae</i> of Dr. Geoffrey Lin-Cereghino                                                                                                                                                                          |

I, Dr. Geoffrey P. Lin-Cereghino, Ph.D., declare as follows:

## **I. INTRODUCTION**

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 which is pending before the Patent Trial and Appeal Board (“the Board”) of the United States Patent and Trademark Office (the “Proceeding”). I understand that, in this Proceeding, Motif is challenging the validity of claims 1-14 of U.S. Patent No. 10,273,492 (the “’492 patent”) and claims 1-28 of U.S. Patent No. 10,689,656 (the “’656 patent”) (the “Challenged Claims”). Based on the first page of each patent, I understand they are assigned to Impossible Foods Inc. (“Patent Owner”).

2. In this declaration (“Declaration”), I have been asked to provide opinions regarding the ’492 and ’656 patents and recombinant expression technology using the *Pichia pastoris* expression system.

3. I am informed that the ’492 and ’656 patents have 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 invalidity (or validity) of the '492 and '656 patents. I understand that neither party has yet proposed any constructions for any claim terms, and that the District Court has not yet construed any claims of the '492 and '656 patents. I reserve the right to amend or supplement my opinions in this Declaration in response to any positions that Patent Owner or its experts may take in the Litigation, or in response to any order of the District Court.

## **II. EXPERIENCE AND QUALIFICATIONS**

5. Exhibit EX-1056 accompanying this Declaration is a true and correct copy of my current *curriculum vitae*. EX-1056 describes in greater detail my education, publications, employment and research history, and professional activities and awards.

6. I am a Professor of Biological Sciences at the University of the Pacific in Stockton, California. My main responsibilities include: teaching four or more courses per year, supervising a research laboratory composed of undergraduate and graduate students, and performing various service functions for the University, particularly advising and committee work. Having performed research on *Pichia pastoris* since 1997 as a postdoctoral fellow and university professor, I am an expert in recombinant protein expression in this yeast species. I also have broad training in the disciplines of microbiology and molecular biology.

7. I received my Bachelor of Science from the University of California, Davis, in May 1989 where I was selected as a Regents Scholar and graduated with Highest Honors. After working as an intern and then laboratory biologist in the immunology department at Syntex Pharmaceuticals, I joined the Ph.D. program at the University of California, San Diego in 1989. As one of two students in my cohort selected for a predoctoral fellowship from the National Science Foundation in 1990, I investigated the use of mRNA turnover as a means of gene regulation in baker's yeast, *Saccharomyces cerevisiae*, for my dissertation.

8. After earning my Ph.D. in 1995, I served as a Visiting Assistant Professor (sabbatical replacement) at Haverford College for one year, where I continued lab studies in mRNA turnover with undergraduate research students and taught courses in basic cell biology, immunology and advanced protein structure.

9. In 1996, I joined the laboratory of Michael Gold at the Oregon Graduate Institute of Science and Technology (OGIST) as a postdoctoral research associate, studying the filamentous fungus *Phanerochaete chrysosporium*, a fungus used for bioremediation. I was awarded a National Institute of Environmental Health Sciences Postdoctoral Fellowship and joined the laboratory of James Cregg, also at OGIST. Dr. Cregg, considered the “father of *Pichia*,” was one of the first scientists to develop the molecular tools necessary for using the methylotrophic yeast *P. pastoris* as a recombinant protein expression system. Between 1997 and 2000, under

Dr. Cregg's guidance, I pursued an analysis of the alcohol oxidase I (*AOX1*) promoter in *P. pastoris*, culminating with primary authorship on a publication that identified the transcriptional regulatory protein, methanol expression regulator 1 (Mxr1) protein ("Mxr1p"), which is involved in the control of the *AOX1* promoter (see EX-1005(Vogl)), discussed further in this Declaration). During my work in the Cregg lab, I also created plasmids for the expression of heterologous (non-native) proteins and collaborated with other labs on producing heterologous proteins in *P. pastoris*.

10. In 2000, I became an Assistant Professor at the University of the Pacific ("Pacific") in a joint appointment with my wife, Joan Lin-Cereghino. At Pacific, Joan and I have collaborated for more than two decades on running a research lab dedicated to the expression of recombinant proteins in *P. pastoris*.

11. I have served as a member of the editorial board/associate editor for the journal Protein Expression and Purification since 2001 and have served as a peer reviewer for several other journals. I have published 23 articles and reviews on *P. pastoris* and recombinant protein expression during my time as a faculty member, including Lin-Cereghino et al., *Expression of foreign genes in the yeast Pichia pastoris*, 23 GENETICS ENGINEERING 157-69 (2001); Lin-Cereghino et al., *Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris*, 38 BIOTECHNIQUES 44 (2005); Lin-Cereghino

& Lin-Cereghino, *Vectors and strains for expression*, 389 METHODS IN MOLECULAR BIOLOGY 11-26 (2007); Hartner et al., *Promoter library designed for fine-tuned gene expression in Pichia pastoris*, 36 NUCLEIC ACIDS RESEARCH e76 (2008); Ingram et al., *Overcoming Obstacles in Protein Expression in the Yeast Pichia pastoris: Interviews of Leaders in the Pichia Field*, 4 PACIFIC JOURNAL OF HEALTH 2 (2021).

12. I have served as a PI (principal investigator) or co-PI on five federal research grants from the National Institutes of Health and the National Science Foundation, garnering almost 1.1 million dollars in total, and all involving *P. pastoris*.

13. During the past 22 years at Pacific, my wife and I have mentored 24 Masters and doctoral students as well as 105 undergraduates in our laboratory, all on projects related to *P. pastoris*. I have also been dedicated to teaching, which required me to use and expand my expertise in fungal cell biology and protein expression. In my 22 years at Pacific, for example, I have taught the course Microbiology, which includes a module about recombinant protein production in *P. pastoris*, and I teach about the commercial aspects of *P. pastoris* expression in my course Industrial Microbiology.

14. I am also expected to perform university and professional service as part of my faculty responsibilities. Along with doing committee work related to open educational resources and experiential learning, I have evaluated grants for my

university as well as some local and international funding agencies, usually on topics related to recombinant protein expression.

15. I am being compensated at my customary rate of \$250 per hour for my work in this matter. This compensation is in no way contingent upon the outcome of this matter or upon the opinions I offer in this Declaration. All of the opinions expressed in this Declaration are my own.

### III. SUMMARY OF OPINIONS

16. My opinions are set forth below in greater detail. Briefly, in my opinion:

- A person of ordinary skill in the art for the '492 and '656 patents (a "POSITA," which I define below) would have been motivated to express recombinant proteins in a methylotrophic *Pichia* yeast cell, including *Pichia pastoris*, and would reasonably expect success in doing so. By 2015, *P. pastoris* was a commonly-used and well-studied recombinant expression system.
- A POSITA would have been motivated to express the Mxr1p transcription factor in a nucleic acid expression construct when expressing a recombinant protein in *P. pastoris* and would reasonably expect success in doing so. A POSITA would have known that, upon translocation to the nucleus of a *P. pastoris* cell, native Mxr1p binds

and activates the promoter of the alcohol oxidase 1 gene *AOX1* in *P. pastoris* ( $P_{AOX1}$ ). In consequence, when the  $P_{AOX1}$  sequence is included in an expression construct in order to drive expression (transcription) of a gene sequence of interest, Mxr1p binding to  $P_{AOX1}$  and other inducible promoters can activate that transcription. A POSITA would be motivated to express Mxr1p in *P. pastoris* in order to enhance production of recombinant proteins resulting from Mxr1p binding, and would reasonably expect success in doing so due to Mxr1p's well-characterized ability to activate  $P_{AOX1}$  and other inducible promoters.

- A POSITA would have been motivated to express Mxr1p in *P. pastoris* under control of  $P_{AOX1}$  or other promoters and would reasonably expect success in doing so. By 2015,  $P_{AOX1}$  was recognized in the field as a strong promoter and desirable to use in expressing recombinant proteins. A POSITA would therefore recognize that  $P_{AOX1}$  or other promoters could be used to drive expression of recombinant Mxr1p (Mxr1p is also natively produced in *P. pastoris*). A POSITA would not be discouraged from doing so. For example, the literature had shown that increasing Mxr1p levels in *P. pastoris* cells under the control of promoters was advantageous.

- The '492 and '656 patents disclose certain experiments relating to the production of recombinant hemoproteins in *P. pastoris*, including experiments using Mxr1p expressed under the control of P<sub>AOX1</sub>. In my opinion, the results of these experiments would not be surprising to a POSITA. Rather, these results are in line with what a POSITA would have expected based on the teachings of the prior art, including foundational work in *P. pastoris* performed by me and my colleagues.

#### **IV. LEGAL STANDARDS**

17. In this section I describe my understanding of certain legal principles that I have relied upon in forming my opinions set forth in this Declaration.

18. I have been informed of these legal standards by counsel for Motif. I am not an attorney and I have not researched the law on patent invalidity. I am relying only on Motif's attorneys relating to these legal standards.

##### **A. Person of Ordinary Skill in the Art**

19. 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, conventional wisdom in the art, and is a person of ordinary creativity—not an automaton.

20. 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;
- the sophistication of the technology.

**B. Obviousness**

21. I am informed and understand that, under 35 U.S.C. § 103, a patent claim is invalid if the subject matter of the claim as a whole would have been obvious to a person of ordinary skill in the art at the time of the invention. I further understand 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 between each claim of the patent and the prior art; (3) the level of ordinary skill in the art at the time the patent was filed; and (4) any objective indicia of non-obviousness, also known as “secondary considerations.” Such objective indicia include: long-felt but unresolved needs; commercial success; failure of others to solve the problem that the inventor solved; and surprising/unexpected results. I am further informed and understand that an obviousness determination generally requires a finding that all claimed limitations are disclosed in the prior art, and that a person of ordinary skill in the art would have been motivated to combine or modify the teachings in the prior art and would have

had a reasonable expectation of success in doing so. I am further informed and understand that absolute predictability of success is not required, only a reasonable expectation.

## **V. THE '492 AND '656 PATENTS**

### **A. Overview of the '492 and '656 Patents**

22. I am informed and understand that the '492 and '656 patents both claim priority to eight provisional applications, the earliest of which was filed on May 11, 2015. EX-1001, Cover Page; EX1002, Cover Page. In rendering my opinions in this Declaration, I have assumed that the '492 and '656 patents have a priority date of May 11, 2015.

23. From my review of the '492 and '656 patents, I see that the '492 patent contains 14 claims. EX-1001, 77:49-79:17. I further see that the '656 patent contains 28 claims. EX-1002, 87:31-90:18. These claims generally relate to the production of “heme-containing protein[s]” in methylotrophic *Pichia* cells. Claim 1 of the '492 patent recites:

1. A methylotrophic *Pichia* yeast cell comprising:

a nucleic acid molecule encoding a heme-containing protein operably linked<sup>1</sup> to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*; and  
a nucleic acid molecule encoding at least one polypeptide involved in heme biosynthesis operably linked to a promoter element from *P. pastoris* and a Mxr1 transcriptional activator sequence from *P. pastoris*.

24. Certain dependent claims of the '492 patent recite the use of an inducible "AOX1" promoter element.

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

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

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

---

<sup>1</sup> The '492 and '656 patents both state that "'operably linked' means that a promoter or other expression element(s) are positioned relative to a nucleic acid coding sequence in such a way as to direct or regulate expression of the nucleic acid (e.g., in-frame)." EX-1001, 4:47-51; EX-1002, 4:47-51.

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

25. Independent claims 1, 16 and 26 of the '656 patent recite:

1. 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.

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.

26. 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 Mxr11 transcriptional activator binds.

26. Certain dependent claims of the '656 patent also recite the use of an inducible "AOX1" promoter element.

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*.

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*.

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

27. Consistent with the prior art I discuss in this Declaration, the '492 and '656 patents recognize that "[m]ethylotrophic yeast such as *Pichia pastoris* are commonly used for expression of recombinant proteins." EX-1001, 1:30-33; EX-

1002, 1:31-34. The specification “describes the use of *P. pastoris* strains that overexpress the transcriptional activator, Mxr1, from the AOX1 promoter to increase expression of transgenes that also are expressed from the AOX1 promoter, which significantly improves the recombinant production of one or more proteins.” EX-1001, 1:37-42; EX-1002, 1:38-43.

28. The '492 and '656 patents state that “expression of Mxr1 from the AOX1 promoter creates a positive feedback loop that allows for expression of other transgenes from the AOX1 promoter in the absence of methanol ... when repressing carbon sources are depleted.” EX-1001, 1:42-46, 3:50-57; EX-1002, 1:43-47, 3:50-57. The specification also notes that “[e]xpression of Mxr1 results in a significant increase in the amount of protein produced.” EX-1001, 1:46-48; EX-1002, 1:47-49. As I discuss further below, this disclosure in the patents is consistent with the prior art’s recognition of Mxr1p activity.

## **B. Person of Skill in the Art for the '492 and '656 Patents**

29. Based on my review of the '492 and '656 patents, the patents are directed to the art of recombinant protein production. In my opinion, a person of ordinary skill in this art (a “POSITA”) would have a Bachelor of Science degree in biochemistry, chemistry, biological sciences, or an equivalent area of study. A POSITA would also have at least three years of graduate study or professional experience with recombinant techniques for protein production or general genetic

modification experience.

30. I meet or exceed the qualifications of a POSITA for the '492 and '656 patents.

31. In rendering my opinions in this Declaration, I have applied the perspective of a POSITA for the '492 and '656 patents in 2015.

## **VI. LITERATURE**

32. In addition to my general familiarity with the technical literature concerning recombinant protein expression in *P. pastoris*, I have reviewed the literature listed at the front of this Declaration. In this section, I comment on selected publications that are of particular relevance to my opinions.

### **A. Lin-Cereghino (2006)**

33. I am the first author of a paper titled *Mxr1p, A Key Regulator of the Methanol Utilization Pathway and Peroxisomal Genes in Pichia pastoris*, published in 2006 in Volume 26, Number 3 of the journal *Molecular and Cellular Biology*, pages 883-97 ("Lin-Cereghino (2006)") (EX-1006). I am informed and understand that the Lin-Cereghino (2006) reference is considered prior art to the '492 and '656 patents under 35 U.S.C. § 102(a)(1) as it was published in 2006, before the 2015 priority date of the '492 and '656 patents.

34. The Lin-Cereghino (2006) article discloses work performed by myself and my colleagues. I was involved in drafting Lin-Cereghino (2006) and am personally familiar with its contents.

35. As I discuss further in this Declaration, methanol is an important energy source for a few eukaryotic species, including methylotrophic yeast such as *Pichia pastoris*. The Lin-Cereghino (2006) article describes experiments conducted “to understand, at the molecular level, how *P. pastoris* coordinately regulates expression of ... genes necessary for methanol utilization.” EX-1006(Lin-Cereghino (2006)), 884. In particular, “[t]o elucidate the molecular mechanisms responsible for methanol regulation,” my colleagues and I “identif[ied] genes required for the expression of *AOXI*,” an enzyme that acts early in the methanol metabolic pathway. EX-1006(Lin-Cereghino (2006)), 883. Of these, the *MXRI* gene, which encodes the Mxr1 protein (Mxr1p), was necessary for *P. pastoris* to grow on methanol. EX-1006(Lin-Cereghino (2006)), 883. Mxr1p also translocates to the nucleus, where its transcription-regulating activity occurs, when cells are exposed to methanol or glycerol. EX-1006(Lin-Cereghino (2006)), 883.

36. These experiments led to the conclusion that, “[w]ithout *MXRI* function, *P. pastoris* cells do not induce the transcription of many genes involved in methanol metabolism, with *AOXI* being the most dramatically affected.” EX-1006, 894. Thus, Lin-Cereghino (2006) represents the first report of the Mxr1p

transcription factor and its importance in regulating *P. pastoris*'s methanol-dependent growth.

37. When my colleagues and I expressed Mxr1p under control of P<sub>AOX1</sub> (e.g., yeast transformed with the pGC215 expression construct, described in the Lin-Cereghino (2006) paper at page 885), we observed that one particular yeast strain “did not grow on methanol and did not induce Mxr1p or other methanol pathway components in response to methanol.” EX-1006, 893. We stated that, “[a]pparently, high levels of Mxr1p are toxic to *P. pastoris*.” EX-1006, 893. However, we did not perform any additional experiments to test or confirm this hypothesis.

38. A POSITA would recognize that further investigation would be needed to determine what caused this strain not to grow, including designing and performing experiments using appropriate controls. For example, one way to test would be to clone Mxr1p under P<sub>AOX1</sub> control in a different vector to eliminate potential concerns with that cloning experiment or vector. Another way to evaluate would be to quantify potential toxicity to *P. pastoris* cells as a function of Mxr1p levels in these cells (e.g., expressed under control of other promoters).

39. A POSITA also would understand that other factors could have affected the growth of the pGC215-containing *P. pastoris* cells, such as the level of methanol in the culturing environment or contamination with other toxic agents. EX-1032(Guarna), 279 (“A challenge associated with methanol feeding is that the

methanol concentration must be maintained within a relatively narrow range. We have observed that recombinant protein production decreases when the methanol concentration exceeds ca. 1.0% (v/v) ....”); EX-1033(Katakura), 482, 485-86 (similar); EX-1034(Hong), 439 (“[A]ccurate regulation of the methanol concentration in *P. pastoris* cultures is necessary not only to maintain the induction of genes under the control of the *AOX1* promoter but also to prevent accumulation of methanol to levels that are toxic to the cells.”); EX-1035(Trinh), 438 (methanol concentrations “above 0.4% inhibit growth and production” in *P. pastoris*).

40. Indeed, as noted below in Sections VI.C-D and summarized in Vogl, later studies of Mxr1p overexpression in *P. pastoris* (Takagi) reported that *P. pastoris* yeast strains expressing Mxr1p under another strong promoter ( $P_{GAP}$ ) regulated  $P_{AOX1}$ -driven expression of phytase in cells grown in glucose-based medium and achieved favorable protein production under these conditions. Similar results were reported by Tsutsumi. Section VI.B; EX-1024(Tsutsumi), [0137]-[0145] (reporting the results of methanol-grown *P. pastoris* expressing Mxr1p under  $P_{GAP}$  and a cutinase gene under  $P_{DAS}$  variants).

41. In light of these findings, which are discussed in more detail below, a POSITA would not be deterred from attempting to improve heterologous protein expression in *P. pastoris* by overexpressing Mxr1p under strong promoters, including but not limited to  $P_{AOX1}$ , to which Mxr1p can bind and regulate.

**B. U.S. Patent Publication No. 2011/0129874 (Tsutsumi)**

42. I have reviewed U.S. Patent Publication No. 2011/0129874 to Tsutsumi and Takagi, which was published on June 2, 2011 (“Tsutsumi”) (EX-1024). I am informed and understand that the Tsutsumi reference is considered prior art to the ’492 patent under 35 U.S.C. § 102(a)(1) as it was published in 2011, before the 2015 priority date of the ’492 patent.

43. Tsutsumi teaches “a method according to the invention for producing a polypeptide of interest.” In one embodiment of this method, “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), [0075]. Similarly, the claims of Tsutsumi recite:

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.

44. Tsutsumi discloses an example of producing a recombinant polypeptide (cutinase) in *P. pastoris* using Mxr1. To do so, Tsutsumi teaches including a plasmid expressing Mxr1p under the control of P<sub>GAP</sub> (pGMxr), which results in “Mxr1 Over-

Expression” in *P. pastoris* cells. As disclosed in Tsutsumi, this approach leads to “improved” expression of the recombinant cutinase protein in *P. pastoris*. EX-1024(Tsutsumi), [0137]-[0145].

#### Example 5

##### Expression of *Humicola Insolens* Cutinase from Modified DAS Promoter with Prm1 and/or Mxr1 Over-Expression

The aim of this experiment was to check whether the expression of a protein of interest, exemplified by the *Humicola insolence* 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*....

pGMxr comprises the expression cassette (SEQ ID NO: 45) having the GAP promoter in position 1-483, Mxr1 CDS in position 493-3957, terminator in position 4028-4368....

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

45. Notably, according to Tsutsumi, “[t]he obtained data indicate an improved expression of the test protein when Mxr protein was over-expressed.” EX-

1024(Tsutsumi), [0145]. These expression data are tabulated in the chart below. As shown for the pNori58-RSII0007 and pNori58-RSII0014 plasmids expressing cutinase, co-expression with pGMxr leads to higher levels of cutinase expression than the expression plasmids alone.

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

### C. U.S. Patent No. 8,143,023 (Takagi)

46. I have reviewed U.S. Patent No. 8,143,023 to Takagi et al., titled “Method for Methanol Independent Induction from Methanol Inducible Promoters in *Pichia*”, which issued as a patent on March 27, 2012 (“Takagi”) (EX-1007). I am informed and understand that the Takagi reference is considered prior art to the ’492 patent under 35 U.S.C. § 102(a)(1) as it was published in 2012, before the 2015 priority date of the ’492 patent.

47. Among other things, the Takagi patent discloses manufacturing plasmids containing the *MxrI* gene using familiar recombinant genetic engineering methods (EX-1007(Takagi), 17:30-18:52):

Example 4[:] Constitutive Expression of Mxr1

Mxr1, a gene from *Pichia pastoris* reported to be a positive regulator for methanol induction (G. P. Lin-Cereghino et al.; MOLECULAR AND CELLULAR BIOLOGY, Feb. 2006, p. 883-897 Vol. 26, No. 3), was isolated from genomic DNA of *Pichia pastoris* by PCR using the primers Mxr-F and Mxr-R....

The resulting plasmid pGMxr carries the Mxr1 gene under GAP promoter control and a zeocin resistance gene as the transformation marker.

48. Takagi teaches that constitutive expression of Mxr1p can increase the yield of recombinant phytase in *P. pastoris*: “The effect of constitutive expression of the Mxr 1 gene was observed as increased phytase activity in YPD medium in the transformants after introduction of pGMxr.” EX-1007(Takagi), 18:23-26.

**D. Vogl (2013)**

49. I have reviewed an article authored by Vogl and Glieder, titled *Regulation of Pichia pastoris promoters and its consequences for protein production*, published in 2013 in volume 30 of New Biotechnology (pages 385-404 ) (“Vogl”) (EX-1005). I am informed and understand that the Vogl reference is considered prior art to the '492 patent under 35 U.S.C. § 102(a)(1) as it was published in 2013, before the 2015 priority date of the '492 patent.

50. Vogl is a review article summarizing research on different promoters and culture conditions affect recombinant protein production in *P. pastoris*. Like

other references I discuss in this Declaration, Vogl recognizes that “methylophilic yeast *Pichia pastoris* is a widely used host for heterologous protein production.” EX-1005(Vogl), 385. Vogl reviews the use of both “classic” and “new” promoters from *P. pastoris* in order to achieve recombinant protein production. EX-1005(Vogl), 385. In particular, Vogl teaches that the promoter for the alcohol oxidase 1 (*AOX1*) gene, P<sub>AOX1</sub>, is particularly advantageous, because it is a strong promoter (it induces expression of *AOX1* at relatively high levels) and its activity can be tightly controlled (EX-1005(Vogl), 386):

As P<sub>AOX1</sub> is a remarkably strong promoter and at the same time directly controllable by simply changing the inexpensive carbon source [24], 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<sub>AOX1</sub> .... Concerning controlled bioreactor cultivation, the tight regulation of P<sub>AOX1</sub> is used to enable cell growth to high densities on a repressing carbon source without heterologous protein expression hampering the cellular metabolism.

51. Vogl also summarizes various studies of P<sub>AOX1</sub>, including the discovery of P<sub>AOX1</sub>'s transcriptional activator Mxr1p first reported in Lin-Cereghino (2006).

Despite the favorable properties of P<sub>AOX1</sub> and the basic understanding of its regulatory modes, only in recent years light has been shed on the molecular basis of P<sub>AOX1</sub> transcriptional regulation, thereby enabling new tools and concepts for *P. pastoris* based gene expression. A series

of *cis*-acting DNA sequences and also the first two transcriptional activators Mxr1p and Prm1p were identified.

EX-1005(Vogl), 387. Vogl reviews investigations of Mxr1p and its effect on the methanol utilization pathway. EX-1005(Vogl), 387. In particular, Vogl discusses reports of differences in methods of expressing Mxr1p in *P. pastoris* and the observed effects on those cells (EX-1005(Vogl), 391):

[C]onstitutive expression of *MXR1* under control of  $P_{GAP}$  was also investigated under growth on glucose. This resulted in a 7.6-fold increased activity of the phytase expressed from  $P_{AOXI}$  compared to the non *MXR1* coexpressing strain. Again no comparison to methanol induction or comparative biomass production was performed. The 7.6-fold increase might still account only for a low absolute expression level, as  $P_{AOXI}$  is increased ~1000-fold from glucose to methanol [27]. Furthermore, the results of the *MXR1* coexpression seem to oppose previous findings by Lin-Cereghino et al., where overexpression of *MXR1* under control of  $P_{AOXI}$  caused a growth defect on methanol and inhibited expression of MUT genes [28]. Additionally, Mxr1p was shown to localize to the cytoplasm when grown on glucose and to migrate only upon shift to gluconeogenic carbon sources to the nucleus. This raises the question how constitutively overexpressed Mxr1p reaches the nucleus during growth on glucose.

52. In light of my earlier Lin-Cereghino (2006) work discussed above, and the findings and results obtained by Takagi and others when overexpressing Mxr1 under the control of other strong promoters, Vogl expressly invites “further

investigation,” which would motivate a POSITA to conduct further experiments overexpressing Mxr1 from P<sub>AOX1</sub> and other strong promoters to regulate expression of heterologous proteins under the control of methanol-inducible promoters to which Mxr1p binds. EX-1005(Vogl), 391. I discuss the findings further below in Section VII.B.2.

## VII. RECOMBINANT PROTEIN EXPRESSION IN *PICHTA PASTORIS*

### A. Overview of Recombinant Protein Expression

53. Producing proteins in a recombinant expression system generally requires three steps. The **first** step is insertion of a gene sequence encoding the protein of interest (which may be referred to as a “heterologous” gene sequence) into a circular “plasmid” or “vector,” forming an “expression construct” or “expression vector.” This approach is sometimes referred to as “cloning.” Below is an example of work from my wife, Joan Lin-Cereghino, regarding the preparation of expression vectors for use in *P. pastoris*.

Most expression vectors have an expression cassette composed of a 0.9-kb fragment from *AOX1* composed of the 5' promoter sequences and a second short *AOX1*-derived fragment with sequences required for transcription termination [19]. Between the promoter and terminator sequences is a site or multiple cloning site (MCS) for insertion of the foreign coding sequence. In the native *AOX1* gene, the alcohol oxidase open reading frame (ORF) is preceded by an unusually long 5' untranslated region (116 nt) [8]. Generally, the best expression results

are obtained when the first ATG of the heterologous coding sequence is inserted as close as possible to the position of the *AOX1* ATG.

EX-1015(Lin-Cereghino (2000)), 48. A schematic of an exemplary expression vector from this publication (EX-1015(Lin-Cereghino (2000)), Fig. 3) is reproduced below:

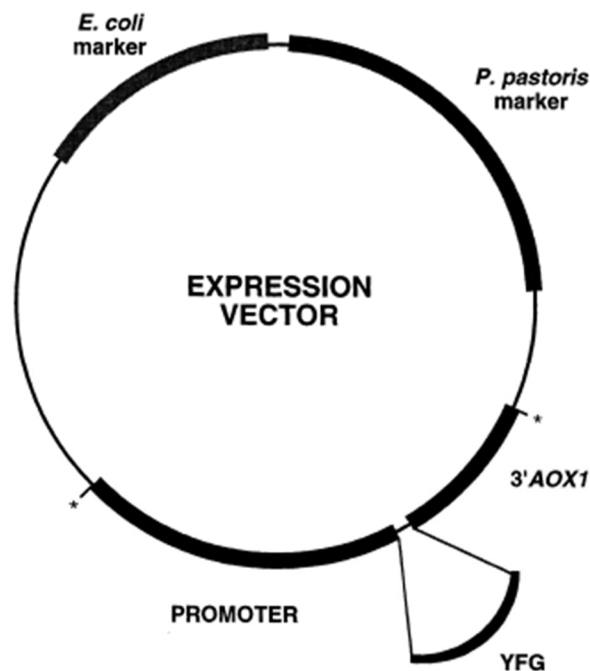


Fig. 3. General diagram of a *P. pastoris* expression vector. YFG, 'Your Favorite Gene;' \*, sites for cassette amplification.

54. The **second** step towards producing recombinant proteins is transfection of the expression construct into the host cell, where it is stably integrated into the host's genome. EX-1015(Lin-Cereghino (2000)), 50 ("Expression vectors are integrated into the *P. pastoris* genome to maximize the stability of expression strains."). Typically, the **third** step is evaluation of transfected cells for expression of the protein of interest and preparation of stable cell lines based on the evaluation

and selection of these cells for further use or study. EX-1029(Ahmad), 5310 (“The majority of transformants will have a single copy of the expression vector integrated into the genome, and numerous clones will have to be screened to find high-copy transformants.”).

55. Inside the host cell, promoter sequences for a gene sequence—both native and recombinant gene sequences—regulate the expression of that sequence. In the genome, a promoter is typically a non-coding region located upstream of the sequence encoding a gene. EX-1042(Alberts), 306. Promoter sequences provide specific binding sites for “transcription factors”, which regulate transcription of the gene sequence of interest. EX-1005(Vogl), 386; EX-1042(Alberts), 310 (“[T]ranscription factors help to position the RNA polymerase correctly at the promoter [and] aid in ... allow[ing] transcription to begin ....”). In binding to the promoters, transcription factors can act either as activators or repressors of transcription. EX-1005(Vogl), 386. The host cell’s transcription machinery similarly recognizes promoters on an expression construct that are associated with the gene of interest.

56. In order to activate gene expression, for example, transcription factors can bind promoters and recruit the RNA polymerase, which transcribes the sequence of interest into a transcript of messenger RNA (mRNA). The transcript is subsequently translated into a polypeptide sequence, which may be folded, modified,

and trafficked within or outside of the cell. *E.g.*, EX-1005(Vogl), 386 (“Expression of the respective gene of interest (GOI) for recombinant protein production includes transcription, translation, folding and possible posttranslational modification as well as the correct targeting by the heterologous host organism.”).

57. Control of recombinant protein expression inside a host cell such as *P. pastoris* is therefore dependent on the promoter(s) associated with the sequence of interest. *E.g.*, EX-1005(Vogl), 386 (“[S]trong and controllable promoters are a crucial tool for efficient heterologous protein production.”). As I discuss further below, promoters can be either “constitutive” or “inducible.” When a promoter is constitutively active, the associated gene sequence is constantly transcribed. In contrast, gene sequences associated with inducible promoters are not expressed until the promoter activity is “induced.”

58. In assembling an expression construct, familiar genetic engineering techniques can be used to link different promoters to a gene sequence of interest. In one of my prior art publications, for example, my co-authors and I describe creation of a “promoter library” based on modifications of the  $P_{AOX1}$  sequence that could be used in order to “fine-tune” the expression of different heterologous proteins. EX-1017(Hartner (2008)), 1 (“In addition to improving yields and quality of heterologous protein production, the new  $P_{AOX1}$  synthetic promoter library constitutes a basic toolbox to fine-tune gene expression in metabolic engineering and

sequential induction of protein expression in synthetic biology.”). When the promoter is coupled to the sequence of interest in an expression construct, expression of the sequence of interest may be driven in a similar way as the native gene associated with the promoter in the host genome.

**B. *P. pastoris* as an Expression System**

59. *Pichia pastoris* has been used for decades in industrial settings; initially, as a protein source for high-protein animal feed. EX-1015(Lin-Cereghino (2000)), 46. By the 1980s, scientists recognized *P. pastoris*’s usefulness as a system to produce foreign (heterologous) proteins. EX-1015(Lin-Cereghino (2000)), 46; EX-1029(Ahmad), 5301 (“The methylotrophic yeast *Pichia pastoris* ... has become a substantial workhorse for biotechnology, especially for heterologous protein production.” (citation omitted)).

60. Indeed, by 2015, *P. pastoris* had “become the premier example of yeast species used for the production of recombinant proteins.” EX-1030(Haon), 2. The art recognized multiple advantages of the *P. pastoris* expression system for use in producing proteins via recombinant technology, particularly eukaryotic proteins, which typically require correct three-dimensional folding and post-translational modifications in order to function:

Advantages of this yeast include the use of efficient and tightly regulated promoters, higher expression levels for lower cost than insect and mammalian cells. Compared to prokaryotic expression, it provides

better protein folding, allows post-translational modifications (N- and O-glycosylation, methylation, and acylation), and is able to secrete high levels of recombinant proteins in the culture medium.

EX-1030(Haon), 2.

A major advantage of *P. pastoris* over bacterial expression systems is that the yeast has the potential to perform many of the post-translational modifications typically associated with higher eukaryotes, such as processing of signal sequences (both pre and prepro type), folding, disulfide bridge formation, certain types of lipid addition, and O- and N-linked glycosylation.

EX-1015(Lin-Cereghino (2000)), 52.

61. By 2015, the field had developed tools to improve expression in *P. pastoris*. EX-1029(Ahmad), Abstract (“The last few years have been particularly fruitful because of numerous activities in improving the expression of such complex proteins with a focus on either protein engineering or on engineering the protein expression host *P. pastoris*.”). POSITAs at that time had many options in designing expression constructs for use in *P. pastoris*, including the choice of promoters to use in the vectors.

When devising strategies for cloning and expression of heterologous proteins in *P. pastoris* some points need to be considered from the start, that is, the choice of promoter–terminator combinations, suitable selection markers and application of vector systems for either intracellular or secreted expression including selection of proper

secretion signals (Fig. 1). The choice of the proper expression vector and complementary host strain are a most important prerequisite for successful recombinant protein expression.

EX-1029(Ahmad), 5302; *see generally* EX-1021(Sreekrishna) (reviewing methods and techniques for “optimizing” protein expression in *P. pastoris*).

62. *P. pastoris* “utilize[s] methanol as a source of carbon and energy”—i.e., it is a “methylophilic” yeast species. EX-1015(Lin-Cereghino (2000)), 46. As noted on the use of *P. pastoris*, “[t]he conceptual basis for the *P. pastoris* expression system stems from the observation that some of the enzymes required for methanol metabolism are present at substantial levels only when cells are grown on methanol.” EX-1015(Lin-Cereghino (2000)), 46.

63. As the art recognized, *P. pastoris* natively contains certain promoter sequences which are tightly regulated by the presence of methanol (“methanol inducible” promoters), and which are advantageous for purposes of expressing proteins recombinantly in that yeast: “[o]ne of the most favorable properties of *P. pastoris* and the other methylophilic yeasts are the strong methanol inducible promoters present in these organisms.” EX-1005(Vogl), 386. In particular, “[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.” EX-1005(Vogl), 386. “This tight regulation is exerted on

the transcriptional level, making MUT promoters ideal tools for heterologous protein production.” EX-1005(Vogl), 386.

64. Indeed, “most of the published knowledge about the regulation of *P. pastoris* promoters is focused on the most commonly used classic *P. pastoris* promoters.” EX-1005(Vogl), 386. The “promoter[] of the MUT pathway gene alcohol oxidase 1” ( $P_{AOX1}$ ) (EX-1005(Vogl), 386), which I discuss further below, is particularly significant for recombinant expression in *P. pastoris*.

65. In addition to  $P_{AOX1}$ , other *P. pastoris* promoters were well studied and available as part of a “toolkit” for a POSITA to use in expressing proteins recombinantly in this yeast.

Although the *AOX1* promoter has been successfully used to express numerous foreign genes, there are circumstances in which this promoter may not be suitable. For example, the use of methanol to induce gene expression may not be appropriate for the production of food products since methane, a petroleum-related compound, is one source of methanol. Also, methanol is a potential fire hazard, especially in quantities needed for large-scale fermentations. Therefore, promoters that are not induced by methanol are attractive for expression of certain genes. Alternative promoters to the *AOX1* promoter are the *P. pastoris* *GAP*, *FLD1*, *PEX8*, and *YPT1* promoters.

(EX-1015(Lin-Cereghino (2000)), 48.

**1. The *P. pastoris* *AOX1* Promoter ( $P_{AOX1}$ )**

66. As the product of a MUT gene (*AOX1*), the *P. pastoris* alcohol oxidase enzyme is involved in metabolizing methanol. EX-1005(Vogl), 386 (“Alcohol oxidase (AOX) catalyzes the first step in the metabolism of methanol, the conversion of methanol to formaldehyde.”). In *P. pastoris*, expression of the alcohol oxidase gene *AOX1* is natively regulated by its promoter ( $P_{AOX1}$ ).

67. The art long recognized the usefulness of the  $P_{AOX1}$  sequence for use in producing proteins recombinantly in *P. pastoris*. Indeed, by 2015,  $P_{AOX1}$  was “the most commonly used promoter for heterologous protein expression in *P. pastoris*.” EX-1005(Vogl), 386. For example, in another publication it is reported that “strong, regulated expression” of proteins under control of  $P_{AOX1}$  “effected surprisingly high levels of foreign protein expression.” EX-1015(Lin-Cereghino (2000)), 46. Others have reported similar efficacy. EX-1032(Guarna), 279 (“*P. pastoris* is also capable of expressing heterologous genes to high levels under control of the strong and tightly regulated alcohol oxidase 1 (AOX1) promoter.”). Additionally, prior to 2015, the art taught methods of modifying this promoter in order to “fine tune” recombinant protein expression, including work described in my publications. See generally EX-1017(Hartner (2008)) (describing production of a “promoter library” based on  $P_{AOX1}$ ).

68.  $P_{AOX1}$  is considered a methanol-inducible promoter. Early studies of

P<sub>AOX1</sub> found that this promoter was activated when *P. pastoris* was exposed to methanol. EX-1015(Lin-Cereghino (2000)), 46-47; EX-1018(De Schutter), 564 (“The commonly used methanol-inducible promoters in *P. pastoris*—[including P<sub>AOX1</sub>]—drive the production of enzymes needed for methanol assimilation and therefore produce extremely high levels of these transcripts upon switching the carbon source to methanol.”). Later work described methods of expressing *AOX1* via methanol-independent means. EX-1007(Takagi), 2:63-3:8 (“[C]ontrolled expression of a single positive factor, encoded by the *Prm 1* gene from *Pichia pastoris* ... can be sufficient in order to induce transcription from several methanol inducible promoters without the need for methanol .... The results obtained have shown that it is possible to induce the *AOX1* ... promoter[] simply by controlling the expression of the *prm 1* gene and without the presence of methanol in the growth medium.”).

69. In contrast to methanol-containing culture conditions, culturing *P. pastoris* in carbon sources such as glucose or glycerol “represses” P<sub>AOX1</sub> transcription, meaning that relatively low levels of protein are expressed under this promoter’s control. When glucose or glycerol is depleted or at low concentrations, this repression is reduced (“derepressed”) and P<sub>AOX1</sub>’s activity increases, albeit at significantly lower levels than when methanol is the sole carbon source for *P. pastoris*. As reported in one of my publications, for example:

Being tightly repressed by glucose, its [ $P_{AOX1}$ ] transcription is highly induced upon shift to methanol as sole carbon source. Other carbon sources which repress transcription from the *AOX1* gene are glycerol and ethanol, while very low derepression of transcription occurs upon carbon starvation.

EX-1017(Hartner (2008)), 1 (footnotes omitted).  $P_{AOX1}$  regulation has been characterized as having “three regulatory states” (EX-1005 (Vogl), 387):

Catabolite repression suppresses expression as long as a repressing carbon source is present. Even if methanol is fed, the promoter is still repressed. As soon as the repressing carbon source is depleted or at low concentration in fed-batch processes, the promoter reaches the state of derepression. In *P. pastoris*, under these conditions the native *AOX1* promoter is still only slightly derepressed (2–4% of induced levels) and only methanol addition leads to full induction, which is the third state of regulation.

70. As a POSITA would recognize, the use of methanol-inducible promoters such as  $P_{AOX1}$  is advantageous because these promoters provide tight control over the expression of the gene sequence of interest, particularly if increasing levels of the resulting protein product may result in toxicity to the *P. pastoris* cells. As recognized by various researchers:

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. For nontoxic proteins, which do not hamper cell growth, a strong constitutive expression system can help to minimize cultivation efforts and lead to a higher space/time yield.

EX-1005(Vogl), 392.

The use of tightly regulated promoters such as the alcohol oxidase (*AOX1*) promoter holds advantages for overexpression of proteins. By uncoupling the growth from the production phase, biomass is accumulated prior to protein expression. Therefore, cells are not stressed by the accumulation of recombinant protein during growth phase, and even the production of proteins that are toxic to *P. pastoris* is possible.

EX-1029(Ahmad), 5302. Thus, when toxicity of the recombinant protein is a concern, *P. pastoris* cells can be initially grown and expanded in a non-inducing carbon source such as glucose/glycerol in order to allow the cells to proliferate more effectively. Once the cells have expanded, methanol can be introduced to the culturing medium as the carbon source, which can greatly increase  $P_{AOX1}$  activity. However, after the glucose and glycerol are consumed, but before the addition of methanol, the  $P_{AOX1}$  activity begins to slowly rise.

71. Culturing in a “repressing” carbon source, or under “derepressing”

conditions, can also lead to a favorable protein yield. Bawa and colleagues showed that, when *P. pastoris* is cultured in a fermenter (bioreactor) in the presence of glycerol or glucose, P<sub>AOX1</sub> exhibited activity prior to methanol induction (to fully activate this promoter), which Bawa et al. characterize as “leaky” promoter activity.

Functional hA<sub>2a</sub>R [protein] was detected in the pre-induction phases of a 1L bioreactor cultivation of glycerol-grown *P. pastoris*. In a separate experiment, a glycerol-grown *P. pastoris* strain secreted soluble GFP prior to methanol addition. When glucose, which has been shown to repress *AOX* expression, was the pre-induction carbon source, hA<sub>2a</sub>R and GFP were still produced in the pre-induction phases. Both hA<sub>2a</sub>R and GFP were also produced in methanol-free cultivations; functional protein yields were maintained or increased after depletion of the carbon source.

EX-1036(Bawa), Abstract, 9.

72. Bawa et al. teaches the importance of optimizing culture conditions in order to achieve the desired protein expression in *P. pastoris*. EX-1036(Bawa), Abstract (“We conclude that in order to maximize total yields, pre-induction phase cultivation conditions should be optimized, and that increased specific productivity may result in decreased biomass yields.”), 2 (“This highlights the importance of understanding the influence of pre-induction phase cultivation conditions on both recombinant protein yields and *P. pastoris* biomass yields.”). Based on Bawa’s observations, for example, a POSITA would understand that first growing *P.*

*pastoris* in glucose and later switching to methanol could desirably affect protein production.

Using glucose as a pre-induction carbon source, specific productivity could be increased at the expense of improvements in biomass yield, with methanol having a positive impact on these cells.

EX-1036(Bawa), 10.

## 2. Mxr1p and Its Relationship to P<sub>AOX1</sub>

73. In *P. pastoris*, a zinc-finger transcription factor known as methanol expression regulator 1 (“Mxr1”) or Mxr1 protein (“Mxr1p”), “is a key regulator of methanol induced genes” such as *AOX1*. EX-1005(Vogl), 390. My colleagues and I identified Mxr1p and published on it in 2006. EX-1006(Lin-Cereghino (2006)).

74. Mxr1p binds to and activates various promoter sequences—for example, the promoters associated with the genes dihydroxyacetone synthase (*DHAS*) and peroxin 8 (*PEX8*). EX-1023(Kranthi (2010)), Abstract, 710-11 (reporting “new insights into the molecular determinants of target gene specificity of Mxr1p” based on promoter mapping, in particular that Mxr1p preferably binds to DNA sequences containing the CYCCNY motif); EX-1006(Lin-Cereghino (2006), Abstract (“We conclude that Mxr1p is a transcription factor that is necessary for the activation of many genes in response to methanol.”).

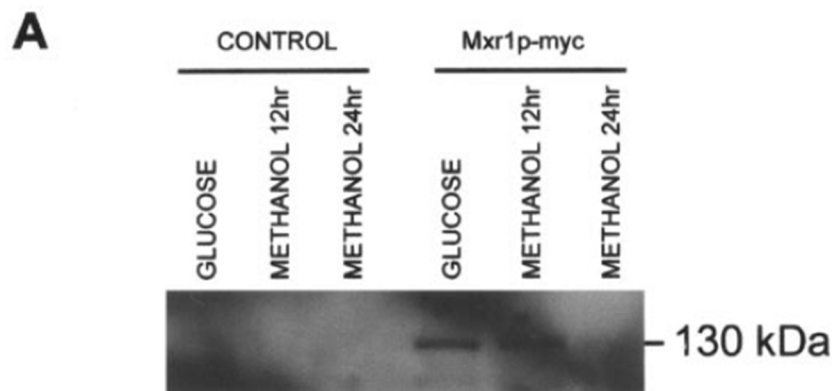
75. One of Mxr1p’s well-known targets is P<sub>AOX1</sub>. EX-1006(Lin-Cereghino (2006)), 894 (“Without *MXR1* function, *P. pastoris* cells do not induce the

transcription of many genes involved in methanol metabolism, with *AOX1* being the most dramatically affected.”).

76. As noted above in Section VII.B.1,  $P_{AOX1}$  is a strong promoter and drives expression of associated gene sequences at a high level. A POSITA would be motivated to express Mxr1p in *P. pastoris* in order to enhance production of recombinant proteins resulting from Mxr1p- $P_{AOX1}$  and other inducible promoter binding, and would reasonably expect success in doing so due to Mxr1p’s well-characterized ability to activate  $P_{AOX1}$  and other inducible promoters. More specifically, as explained above, when the  $P_{AOX1}$  (or other inducible promoters) sequence is included in an expression construct in order to drive expression (transcription) of a gene sequence of interest, Mxr1p binding can activate transcription. *E.g.*, EX-1006(Lin-Cereghino (2006)). Because Mxr1p is known to bind and activate  $P_{AOX1}$  and other inducible promoters, a POSITA would reasonably expect that increasing levels of Mxr1p in a *P. pastoris* cell would lead to increased expression of sequences associated with  $P_{AOX1}$  (or sequences associated with other promoters activated by Mxr1p) compared with a condition in which Mxr1p levels are not increased.

77. Additionally, when Mxr1p is itself expressed under control of  $P_{AOX1}$ , this expression would be predicted to further drive its own expression (by increasing Mxr1p- $P_{AOX1}$  binding as Mxr1p levels increase), creating a positive feedback

system. As shown below, Lin-Cereghino (2006) teaches that overexpression of Mxr1p in *P. pastoris* cells under the control of a promoter derived from a *P. pastoris* gene (*PEX8*) led to “relatively high levels” of Mxr1p in those cells. EX-1006(Lin-Cereghino (2006)), 893.



Because the *PEX8* promoter, like the *AOX1* promoter, has Mxr1p binding sites and can be regulated by Mxr1p, this result supports a POSITA’s understanding that overexpressing Mxr1p under the control of  $P_{AOX1}$  would create a positive feedback expression system. Further, in light of the state of the art and multiple publications before 2015, a POSITA would have been motivated to express Mxr1p in *P. pastoris* under control of  $P_{AOX1}$  specifically and would have reasonably expected success in doing so.  $P_{AOX1}$  is a well-known strong promoter, is widely used to express proteins in *P. pastoris*, and can be used alternatively to  $P_{PEX8}$ , depending on the needs of the expression system. EX-1005(Vogl), 386, 393. Also, a POSITA would have the benefit of all the research prior to May 11, 2015 on Mxr1p’s effect on protein

expression, including the Takagi and Tsutsumi patent publications I discuss above, which teach that expressing Mxr1p in *P. pastoris* under the control of other promoters is useful to produce recombinant proteins under control of P<sub>AOX1</sub>, P<sub>DAS</sub> or other inducible promoters.

78. A POSITA would be motivated to follow these teachings to create this positive feedback system in order to enhance protein production in *P. pastoris*. For example, a POSITA would recognize that increasing Mxr1p levels in these cells (by activating its own expression by binding P<sub>AOX1</sub>) would further drive the expression of other gene sequences linked to P<sub>AOX1</sub> (the protein of interest).

79. For at least these reasons, in view of the state of the art in May 2015, a POSITA would be motivated to express Mxr1p under control of P<sub>AOX1</sub> in *P. pastoris* cells and would reasonably expect success in doing so.

## **VIII. THE '492 AND '656 PATENTS**

80. The claims and specification of the '492 and '656 patents generally relate to the recombinant production of heme-containing proteins in *P. pastoris*. In my opinion, for reasons explained further in my Declaration, a POSITA working in 2015 would have been motivated to conduct the experiments disclosed in the patents and would have reasonably expected that they would be successful. Additionally, the results obtained from these experiments would not have been surprising or unexpected to a POSITA in view of the prior art's teachings concerning P<sub>AOX1</sub> and

Mxr1p in *P. pastoris*.

81. I have reviewed the Examples and experiments disclosed in the patents. These Examples disclose aspects of recombinant production of heme-containing proteins in *P. pastoris* using techniques which, in my experience, would have been familiar and well-understood to a POSITA before 2015, including cloning and assembly of expression constructs, transformation, medium preparation, preparation and characterization of individual strains of transformed yeast, and fermentation (culturing of yeast cells).

82. As I explain in greater detail above in Section VII.B, by 2015, *P. pastoris* was widely regarded in the field as an especially advantageous system for expressing proteins through recombinant means and had been used for decades for this purpose. A POSITA would reasonably expect, for example, that proteins produced in *P. pastoris* can be folded and post-translationally modified in a similar manner as they would be in other eukaryotic cells (*e.g.*, mammalian cells that natively express the protein). EX-1015(Lin-Cereghino (2000)), 52; EX-1030 (Haon), 2.

83. Of particular relevance to my opinions in this Declaration, correct protein folding would be important to a POSITA seeking to express heme-containing proteins (“hemoproteins”) because this class of proteins binds to heme, a structure that holds an iron atom. EX-1037(Ferreira), 539 (noting that “the protein scaffold

binding the metalloporphyrin modulates the chemistry (redox state and coordination properties) of the metal iron”); EX-1008(Mattoon), 1:13-16 (“An important class of ... proteins produced in genetically engineered cells is represented by proteins carrying a heme prosthetic group.”). As a POSITA would recognize, hemoproteins’ binding to heme could be compromised if the hemoproteins are misfolded.

84. In addition to hemoproteins, the claims of the ’492 and ’656 patents recite production of polypeptides “involved in heme biosynthesis,” which I understand can be various kinds of enzymes. *See, e.g.*, EX-1001, 2:11-23 (“[T]he at least one heterologous nucleic acid encodes one or more polypeptides involved in the biosynthesis of an iron co-factor such as heme (*e.g.*, ALA synthase, ALA dehydratase, porphogilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and/or ferrochelatase).”). Proper folding would also be important to a POSITA seeking to express enzymes, which contain domains (*e.g.*, catalytic domains) that must be correctly folded in order for the enzyme to function. Thus, a POSITA would be strongly motivated to use *P. pastoris*, a methylotrophic *Pichia* yeast species, in order to produce hemoproteins and other molecules involved in their biosynthesis.

85. A POSITA’s motivation to produce hemoproteins (and other kinds of proteins) recombinantly in *P. pastoris* is reflected in a patent publication that names Crane and Sudhamsu as co-inventors:

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*, *Kluyveromyces lactis*, and *Saccharomyces cerevisiae*), soil fungus (e.g., *Trichoderma reesei*), and black mould fungus (e.g., *Aspergillus niger*).

EX1010(Crane), [0043]. Crane teaches that *P. pastoris* is a particularly advantageous and tractable system for this purpose (EX-1010(Crane), [0044]):

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).

To produce the desired hemoprotein, Crane teaches “co-expressing a recombinant heme-binding protein and recombinant ferrochelatase protein, or polypeptide thereof, under conditions effective for complete heme incorporation into the

recombinantly produced heme-binding protein, thereby producing a functional heme-binding protein.” EX-1010(Crane), [0020]. Crane’s methods can be used to produce “various classes of heme-binding proteins” that “are completely functional and are more suitable for research, clinical, and commercial applications.” EX-1010(Crane), [0021], [0024]. From my experience, given the art’s long familiarity with *P. pastoris* as an expression system and the availability of tools for recombinant production in that species, a POSITA would be motivated to express these proteins in *P. pastoris*, and would reasonably expect success in doing so.

86. I am not aware of any evidence of secondary considerations of non-obviousness associated with the claims of the ’492 and ’656 patents and reserve the right to address any such positions that the Patent Owner may assert. I have reviewed the Examples and experiments disclosed in the ’492 and ’656 patents. These Examples disclose aspects of recombinant production of heme-containing proteins in *P. pastoris* using techniques which, in my experience, would have been familiar and well-understood to a POSITA before 2015, including cloning and assembly of expression constructs, transformation, medium preparation, preparation and characterization of individual strains of transformed yeast, and fermentation (culturing of yeast cells).

## IX. CONCLUSION

87. 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.

88. 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.

  
Dr. Geoffrey P. Lin-Cereghino, Ph.D.

January 27, 2023

\_\_\_\_\_  
Date