Review

State of the Art Technologies for High Yield Heterologous Expression and Production of Oxidoreductase Enzymes: Glucose Oxidase, Cellobiose Dehydrogenase, Horseradish Peroxidase, and Laccases in Yeasts P. pastoris and S. cerevisiae

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Review

State of the Art Technologies for High Yield Heterologous Expression and Production of Oxidoreductase Enzymes: Glucose Oxidase, Cellobiose Dehydrogenase, Horseradish Peroxidase, and Laccases in Yeasts *P. pastoris* and *S. cerevisiae*

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Abstract: Oxidoreductase (OXR) enzymes are in high demand for biocatalytic applications in food industry and cosmetics: glucose oxidase (GOx), cellobiose dehydrogenase (CBDH), bioremediation: horseradish peroxidase (HRP), laccase (LAC), and medicine for biosensors and miniature biofuel cells (GOx, CBDH, LAC, HRP). They can be used in a soluble form and/or within the yeast cell walls expressed as chimeras on the surface of yeast cells (YSD), such as *P. pastoris* and *S. cerevisiae*. However, most of the current studies suffer from either low yield for soluble enzyme expression or low enzyme activity when expressed as chimeric proteins using YSD. This is always the case in studies dealing with heterologous expression of oxidoreductase enzymes, since there is requirement not only for multiple OXR genes integrations into the yeast genome (super transformations), and codon optimization, but also very careful design of fermentation media composition, and fermentation conditions during expression due to need for adding transition metals (copper, iron), and metabolic precursors of FAD and heme. Therefore, scientists are still trying to find optimal formula using above mentioned approaches, and most recently also using protein engineering and directed evolution for additional increase in the yield of recombinant enzyme production. In this review article we will cover all the current state of the art technologies and most recent advances in the field that yielded high expression level for some of these enzymes in specially designed expression/fermentation systems. We will also tackle and discuss new possibilities for further increase in fermentation yield by the cutting-edge technologies such are directed evolution, protein and strain engineering, high-throughput screening methods based on *in vitro* compartmentalization, flow cytometry and microfluidics.

Keywords: recombinant; oxidoreductase; expression; yeasts; directed evolution; high-throughput screening; flow cytometry; in vitro compartmentalization

1. Introduction

Enzymes are biocatalysts with complex structures and specific catalytic mechanisms that determine their distinctive properties, such as high catalytic activity and selectivity on specific substrates. According to the BRENDA database, oxidation-reduction reactions constitute at least thirty percent of all enzymatic reactions; given that fact, oxidoreductase (OXR) occupies a special place among biocatalysts.[1–3] These enzymes catalyze the transfer of electrons from an electron donor to an electron acceptor molecule. Different cofactors, such as heme, flavin, and metal ions, are necessary for OXR catalytic activity.[4] That usually complicates their expression. The abundance of these enzymes is versatile, and the source of OXR defines their biological functions. These enzymes act as efficient biocatalysts in various processes and fields of biotechnology and have a wide range of applications in the degradation of xenobiotic compounds, the design of biosensors for environmental or medical purposes, the food and textile industry, and other fields.[5]

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1.1. Glucose oxidase (GOx)

Glucose oxidase (EC 1.1.3.4) is an enzyme belonging to the OXR group. This flavoprotein uses molecular oxygen as an electron acceptor in a two-step reaction to catalyze the oxidation of β-D-glucose to D-glucono-δ-lactone and H_2O_2. In the first half of the reaction, namely reduction, GOx catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone, which is then hydrolyzed to gluconic acid. During the first phase cofactor of GOx, flavine adenine dinucleotide (FAD) is reduced to $FADH_2$. In the second phase, the oxidative half-reaction, oxygen reoxidizes the reduced GOx to produce H_2O_2, and $FADH_2$ oxidizes to FAD. [6] In 1999, the structure of glucose oxidase from *Aspergillus* was confirmed, which determined that GOx consists of two uniform subunits and that both subunits contain two separate domains: one is not covalently bound with FAD, and the second attaches to the substrate. Structurally, the first domain is mainly a β-sheet, and the second domain consists of four α-helices and an antiparallel β-sheet.[7,8] GOx is an enzyme that it is considered an "ideal enzyme". It is often called an oxidase "Ferrari" because it has high activity, stability and specificity and can be used in various biotechnological, medical, and industrial applications. [9,10]

1.2. Cellobiose dehydrogenase (CDH)

Cellobiose dehydrogenase (CDH; EC1.1.99.18; CAZy AA3_1) belongs to the OXR enzyme family.[11] Many species of wood-decaying fungi produce this glycosylated enzyme involved in lignocellulose degradation.[12] CDH is usually monomeric and consists of two domains, a C-terminal cytochrome-binding fragment (CYT) and a catalytic flavin-containing dehydrogenase domain (DH), which are connected by a linear papain-sensitive linker peptide.[13] The catalytic mechanism of CDH is comprised of oxidative and reductive half-reaction. The substrate is converted into appropriate lactone during oxidative half-reaction while FAD is reduced to FADH2. Subsequently, electrons are transferred to the electron acceptor.[14] Intensive investigation has been carried out in the last decade on this enzyme because of its possible application in many fields like biosensors, biofuel cells, bioremediation, and clinical application.[11,15]

1.3. Horseradish peroxidase (HRP)

Horseradish peroxidase (EC 1.11.1.7) belongs to the group of OXR. This glycoprotein, with 44 kDa, contains at least 15 distinct isoenzyme forms, the most common and thus most studied of which is isoenzyme C1A. [16] It was determined that the tertiary structure of HRP includes two domains formed by ten α-helices, four disulfide bonds, two Ca2+ -ions per molecule, and the prosthetic heme group. [17–19] The reaction between H_2O_2 and Fe(III) in the active center is the first phase of the catalytic cycle. As a result of this phase, a high oxidation state intermediate consisting of Fe(IV) oxoferryl center and porphyrin-based cation radical, called Compound I, is generated. Compound II, which represents Fe(IV) oxoferryl species, is generated in the first one-electron reduction step and reduced in the second step by producing an enzyme resting state. The excess hydrogen peroxide reacts with the resting state enzyme and, as a product, is obtained compound III. [20] Due to the increasing possibilities for the applications of this enzyme in biotechnology and other branches of industry, solving the problem of recombinant production of this enzyme represents a significant challenge in science.

1.4. Laccase (LAC)

Laccases (benzenediol: oxygen oxidoreductase; p-diphenol oxidase EC 1.10.3.2) belong to a large family of multicopper oxidases. Like all multicopper oxidases, LAC also possess relatively uncomplicated 3D structure, mainly comprised of beta sheets and thurns, including a small cupredoxin-like domain. They are glycoproteins with four Cu atoms per monomer. These four Cu atoms form the catalytic core, which helps the enzyme catalyze the redox reaction. LAC couple the four single electron oxidations of a reducing substrate to the four-electron reduction cleavage of the dioxygen bond using four Cu atoms.[21] LAC are one of the oldest and most widespread enzymes in nature, and they can be found in fungi, bacteria, plants, and animals, and their function depends on

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biological source. The reactions performed by LAC include the rupture of alkyl-aryl bonds, the oxidation of benzyl alcohol, and the rupture of aromatic rings that generate a wide variety of oxidized phenolic compounds. In addition, in vitro studies have shown that LAC are capable of polymerization, depolymerization, methylation, and demethylation reactions, as well as oxidation of o- and p-diphenols, aminophenols, polyphenols, polyamines, aryl-amines, and several other phenolic compounds.[22–25] The scope of laccase-catalyzed reactions can be expanded using mediators. The wide substrate spectrum and molecular oxygen as a final electron acceptor with the water molecule as the only by-product make LAC "eco-friendly" and attractive for various biotechnological industries. These biocatalysts have several bioremediation and biodegradation applications in numerous industries (food, cosmetics, nanobiotechnological, textile, woodworking, pulp/paper).[21,26,27]

Production of oxidoreductases from native sources cannot meet the high market demand due to low yields and incompatibility of the standard industrial fermentation processes with the conditions required for the growth of many microorganisms.[6] Recombinant technologies can be used to achieve higher yields of these enzymes. The diversity and scaling-up possibilities of heterologous protein expression opened new commercial opportunities for their industrial uses. [6]

The main goal of this review article is a detailed overview of the most recent trials of heterologous expression of GOx, CDH, HRP, and LAC, and the main principles and problems drawn from these trials. Discuss of expression systems based on yeast to OXR will be given since they offer a compromise between simplicity and high protein expression yield offered by procaryotic expression systems (*E. coli* and *B. subtilis*) and posttranslational modifications offered by mammalian expression systems (too complex and low protein yield) often needed for eukaryotic proteins.

In the end, some of the state-of-the-art technologies that could be used for increasing the expression of these OXRs, like directed evolution, strain engineering, protein engineering, high-throughput screening, flow cytometry, microfluidics and *in vitro* compartmentalization, will be given, and possible future research directions.

2. Heterologous expression

Various expression systems exist for recombinant protein production in bacteria, yeasts, insects, and mammalian cells. All of them have their drawbacks and advantages. Yeasts are interesting and versatile hosts caused by benefits such as growth speed, simple genetic manipulations, secretory expression, posttranslational modification, scalable fermentation, high biomass concentrations, and safe, pathogen-free production.[28,29] There are two large yeast expression systems: methylotroph and non-methylotroph. Typical examples of non-methylotroph and methylotroph yeasts are *Saccharomyces cerevisiae* and *Pichia pastoris*.

2.1. Saccharomyces cerevisiae

Saccharomyces cerevisiae, known as baker yeast, was the first eukaryotic organism with a completely sequenced genome.[30] As said before, *S. cerevisiae* belongs to non-methylotroph yeast. It was initially developed as a replacement host for producing a recombinant protein that could not be expressed in bacterial cells.[28,31] Native resistance to low pH, high osmolality, and numerous inhibitors allow low-cost and facile fermentation procedures with high biomass concentrations under aerobic and anaerobic conditions.[32] Plentiful genetic tools are developed for expression in *S. cerevisiae,* such as recombinant protein expression controlled by strong constitutive promoters like TEF1 and GAP or inducible promoters like galactose-inducible GAL1.[33] Unfortunately, this expression host has some disadvantages; these are connected with protein secretion, especially hyperglycosylation [34] and proteolytic degradation of expressed proteins. From a biotechnological point of view, this expression system is “generally recognized as safe” (GRAS) because it is nonpathogenic and has a history in the food and pharmaceutical industry.[28]

With the intention for a higher expression level, a few approaches, such as fermentation conditions, optimization of the codon, strong promotors and terminators, and multi-copy expression vector, Table 1. [28,35,36]

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Table 1. Overview of the fermentation experiments and parameters used to optimize recombinant GOx, CDH, LAC, and HRP expression in *S. cerevisiae* with fermentation yield of the enzyme.

Source	Oxidoreductase	Host strain; Vector	Promoter	Inducer	Signal sequence	Additional information	Enzyme yield	Ref.
GOx								
A. niger		2805; YEp352	GAL1	1% galactose	ss of α-factor	NR	32[a] U/mL	[37]
A. niger		2805; Yep352	Hybrid ADH2-GPD	2% glucose	ss of α-factor	1.5 % EtOH	260[a] U/mL	[37]
A. oryzae		2805	GAL-10	NR	α-amylase signal sequence	30 ^{O}C, 150 rpm, feed-back controlled fed-batch	NR	[38]
CDH								
T. clypeatus	GAFV01008428.1	BY4742; pFL61	PGK	No	No	NR type of cultivation; Czapek medium, 3 days;	0.039[b] U/mg	[39]
M. thermophilum	Wild type	BJ5465; pJRoC30	GAL1	2% galactose	Native	Deep-well plate (500 µL of medium); 30 ^{O}C; 5 days;	50[b] U/L	[40]
M. thermophilum	Wild type	BJ5465; pJRoC30	GAL1	2% galactose	Ss of α factor	Deep-well plate (500 µL of medium); 30 ^{O}C; 5 days;	16[b] U/L	[40]
P. chrysosporium	U46081.1	InvSC1; pYES2	GAL1	Galactose	Native	Shake flask; 30 ^{O}C; 16 h	NR	[41]
HRP								
Horseradish	Wild type	SIP-Ost1 (Δ44-70); modified pESC-URA	TDH3		pre-Ost1	Fermentor 5 L (batch fermentation)	13506[c] U/L	[42]
Horseradish	HRP 3-17E12	BJ5465; pYEX-S1	PGK1	No	NR	Expression time 25 h	about 250[c] U/L	[43]
LAC								
Trametes sp. C30	Clac1, 2, 3	W303-1A; YIp351	PGK1	No	Ss of SUC2 gene product	Fermentor 3 L; 1 mM $CuSO_4$; 28 ^{O}C; 3 days	1200[d] U/L	[44]
M. thermophila	MtL	BJ5465; pJRoC3	NR	NR	NR	Shake flask 2.8 L; 0.005 mM $CuSO_4$ 30 ^{O}C; 1 day	0.6[d] U/L	[45]
M. thermophila	T2 mutant	BJ5465; pJRoC3	NR	NR	NR	Shake flask 2.8 L; 0.005 mM $CuSO_4$; 30 ^{O}C; 1 day	102[d] U/L	[45]
T. versicolor	Cvl3	BY2777; pYES2	GAL1	4% Galactose	Native	Shake flask 0.3 L; 0.5 mM $CuSO_4$; 20 ^{O}C; 6 days	45[e] U/L	[46]
L. edodes	Lcc4	FGY217; pBG13	GAL1	4% Galactose	Native	Fermentor 4 L; 0.5 mM $CuSO_4$; 20 ^{O}C; 7 days	10[e] U/L	[47]

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A. pediades	ApL	BJ5465; pJRoC30	GAL1	2.2% Galactose	α_{9H2} signal peptide	Shake flask 0.1 L; 0.4 mM $CuSO_4$; 20 ^{0}C; 4 days	280[e] U/L	[48]
M. thermophila	T2 mutant	BW31a; pVT-100U	ADH1	No	Native	Shake flask 0.25 L; 0.6 mM $CuSO_4$; 30 ^{0}C; 1 day; 0.8% alanine	6.52[e] U/L	[49]
T. versicolor	Lcc1	BW31a; pVT-100U	ADH1	No	Native	Shake flask 0.25 L; 0.6 mM $CuSO_4$; 30 ^{O}C; 1 day; 0.8% alanine	0.45[e] U/L	[49]
T. trogii	Lcc1	BW31a; pVT-100U	ADH1	No	Native	Shake flask 0.25 L; 0.6 mM $CuSO_4$; 20 ^{O}C; 14 days; 0.8% alanine	14.12[e] U/L	[49]

NR not reported, *Ref.* reference, *ss* secretion signal, [a] GOx activity was determined by using o-dianisidine. [b] CDH activity was determined by using 2,6-dichlorophenolindophenol (DCIP). [c] HRP activity was determined by using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid (ABTS). [d] LAC activity was determined by using syringaldazine (SGZ). [e] LAC activity was determined by using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid (ABTS).

2.1.1. GOx

In 1992, Baetselier et al. confirmed that expressing up to 1.5 g/L with a suitable vector-host system was possible. [50] In this study, they were using expression vectors pαGO1 and pSGO2, which contain expression cassette of the regulated hybrid promoter ADH2-GAPDH, the α-factor or GOx signal sequence for secretion, the mature GOx cDNA and the GAPDH terminator. The yeast strain GRF181 transformants of these plasmids were cultured on a YP medium with 8% sucrose.[50] The bidirectional inducible promoters can control recombinant expression in S. cerevisiae, galactose dehydrogenase 1 and 10 (GAL1 and GAL10). For the expression induced by the GAL1 promoter, 1% galactose concentration was the most successful and was obtained at 32 U/ml.[37] It was also noted that indirect control of ethanol production in feed-back controlled fed-batch processes could be accomplished via feeding carbon sources. Galactose feeding was utilized to control the pH of the culture, decrease ethanol production, and improve the quantity of GOx produced.[38]

2.1.2. CDH

Termitomyces clypeatus CDH intracellular expression in *S. cerevisiae* was achieved by Banerjee et al. [39]. They obtained a relatively low but detectable degree of the expressed protein, 0.039 U/mg, with cellobiose as an electron donor. [39] Previous research showed that secretion signal influences the level of cellobiose dehydrogenase expression. Hence, the native secretory leader sequence gave three times higher expression than a signal sequence of α-factor. [40] Also, in research conducted earlier in our laboratory, the ability of *S. cerevisiae* to produce cellobiose dehydrogenase was confirmed, but yields of expressed enzymes were not high. [41] Most prior research has shown that *S. cerevisiae* is not a suitable host for high-yield cellobiose dehydrogenase production. Still, it is a great host choice for protein engineering techniques due to its higher transformation efficiency.

1.1.3. HRP

Zhao et al. obtained 5093 U/L of HRP by strain engineering, such as selecting an appropriate cis-regulatory module (CRM) and substituting a core promoter sequence (CPS) and terminator. The yield is significantly increased by replacing the α-factor signal peptide with another signal peptide for transport of HRP in the endoplasmic reticulum.[42]

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2.1.4. LAC

Functional LAC expression in *S. cerevisiae* was described in the early nineties. Afterward, articles about the optimization of heterologous LAC expression were published.[51] Cassland et al. recognized several important factors affecting LAC expression: the cultivation temperature, the gene selected, and the selected *S. cerevisiae* strain.[44,52] Previous research showed that heterologous production of LAC can be upgraded using protein engineering methods, so mutated *M. thermophila* LAC showed 170-fold higher activity than wild type.[45] In the 2013. group of authors demonstrated the possibility of secretory production of initially intracellular LAC and considered aeration an important factor affecting LAC heterologous production.[47] Iimura et al. confirmed that aeration is an important factor besides inducer and copper concentration, so LAC yield was nearly two times higher when the culture device was used. Aeration was accelerated (45 U/L in baffled flask and 80 U/L when the culture device was used).[53] A recent study examined a few factors affecting LAC expression in *S. cerevisiae*. It concluded that expression depends on gene source, used construct, temperature, pH value of cultivation media, and copper concentration. This research has provided evidence for adding alanine to maintain the pH value of cultivation media because that greatly impacts laccase expression. Also, they proved that lowering the temperature during expression from 30 to 20 ^{0}C yielded more than double laccase activity in the identical cultivation period.[49] Several studies suggest that secretion signal could greatly impact LAC expression in *S. cerevisiae*, so Aza et al. successfully used an improved α_{9H2} signal sequence in their work.[48,54]

2.2. Pichia pastoris

Pichia pastoris has gained an important role in recombinant protein production in the past several decades. *P. pastoris* can use methanol as a sole carbon source, given the fact it belongs to methylotroph yeasts. Undemanding genetic manipulation, high cell culture density, capability of secretion of recombinant proteins into the cell culture medium, unexacting purification of secreted proteins, eukaryotic post-translational modification, and stability of genetic constructs make this host convenient for recombinant expression of protein.[55]-[56] Promoters that control the heterologous expression of proteins could be constitutive (glyceraldehyde-3-phosphate dehydrogenase, PGAP) or inducible (alcohol oxidase, AOX1, and AOX2).[57]Considering the existence of PAOX1 and PAOX2 in this host genome, there are three possible phenotypes of *P. pastoris*, specifically, Mut+(wild-type methanol utilization; both of the alcohol oxidase enzymes, AOX 1 and AOX2 are functional), Muts (slow methanol utilization; disrupted AOX1, functional AOX2) and Mut- (no methanol utilization; both AOX1 and AOX2 are disrupted). [30] According to Walsh and Walsh (2022), *P. pastoris* takes a superior position regarding *S. cerevisiae* in the field of recombinant protein production; the reason for that probably lies in the tightly regulated expression of both the intracellular and extracellular recombinant protein, achieving high cell density caused by the aerobic process of respiration, similarity glycosylation to mammals' cells. [29][58] *P. pastoris* is "generally recognized as safe" (GRAS) by the Food and Drug Administration (FDA) and suitable for diverse biotechnological applications.

Several strategies can be applied to achieve a high yield of recombinant proteins. The strategies cover codon optimization, choice of suitable host strains and expression vectors, gene copy number, insertion of the gene of interest under the control of the strong promoter and appropriate signal sequence, optimization of fermentation conditions (temperature, incubation period, agitation, carbon source, concentration of inducer), Table 2. [29,59,60]

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Table 2. Overview of the fermentation experiments and parameters used for optimization of recombinant GOx, CDH, LAC and HRP expression in *P. pastoris* with fermentation yield of the enzyme.

Source	Oxidoreductase	Host strain; Vector	Promoter	Inducer	Signal sequence	Additional information	Enzyme yield	Ref.
GOx								
A. niger	GOx accc30161	SMD1168; pGAPZαA	GAP	NR	ss of α-factor	30 °C; pH 6	107.18[a] U/mL	[61]
A. niger	GOxM	SMD1168; pPIC3.5	AOX1	1% MeOH		30 °C; 3 days, 220rpm	26.93[a] U/mL	[62]
A. niger ATCC 9029	-	GS115; pPIC9	AOX1	1% MeOH		28 °C; 225 rpm;	NR	[63]
A. niger	M12 mutant	KM71H; pPICZαA	AOX	0.5% MeOH	Proalpha sequence	Nine days of fermentation	17.5[b] U/mL	[64]
CDH								
M. thermophilum	N700S mutant	X33; pPICZαA	AOX1	0.5% MeOH	Ss of α-factor & propeptide	Fermentor 7 L; 30 °C; 5 days;	1800[c] U/L	[40]
P. cinnabarinus	Wild type	X33; pPICZαA	AOX1	3% MeOH	Ss of α-factor	Fermentor 1 L; 4 days;	7800[c] U/L	[65]
N. crassa strain FGSC 2489	NC-cdh1	X33; pPICZαB	AOX1	1% MeOH	Ss of α-factor	Shake flask 0.25 L; 30 °C; 1 day	7451[c] U/L	[66]
P. chrysosporium	Mutant	KM71H; pPICZαA	AOX1	0.5% MeOH	Ss of α-factor	Shake flask; 28 °C; 6 days	950[c] U/L	[66]
HRP								
Horseradish	wild type	X-33; pPICZαB	AOX1	0.5% MeOH	ss α-factor	30 °C; BMGY medium supplemented with 1% casamino acids; BMMY medium supplemented with 1.0 mM vitamin B1, 1.0 mM δ-ALA and trace element mix;the highest yield in 80-90 h post-induction;	377[d] U/mg	[43]
Horseradish	mutant HRP 2-13A10	X-33; pPICZαB	AOX1	0.5% MeOH	ss α-factor	same as for wild-type	2053[d] U/mg	[43]
Horseradish	mutant HRP 3-17E12	X-33; pPICZαB	AOX1	0.5% MeOH	ss α-factor	same as for wild-type	1049[d] U/mg	[43]
Horseradish	A2A isoenzyme	X-33; pPICZαC	AOX1	0.5% MeOH	α-MF-pre-pro signal peptide	BMMY medium supplemented with 1% casamino acids and 1% sorbitol	25.63[a] U/mg	[67]

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	Horseradish	HRP-SpG	PpFWK3; pPpT4_alpha_S		MeOH	nr	136 h of methanol induction	113[d] mg/L	[68]
LAC									
	T. versicolor	Lcc1	SMD 1168; pHIL-D2	AOX1	0.5% MeOH		Shake flasks 1 L; 0.1 mM $CuSO_4$; 20[0]C; 3 days of induction	11500[f] U/L	[69]
	T. versicolor	Lcc1	SMD 1168; pHIL-D2	AOX1	0.5% MeOH		BioFlo III fermentor; 0.1 mM $CuSO_4$; 20[0]C; 8.5 days	140[f] U/L	[69]
	T. versicolor	Lcc1	GS115; pPIC3.5	AOX1	1% MeOH		Shake flasks (0.1 L; 0.2 mM $CuSO_4$; 22[0]C ; Initial pH 6; 0.8% alanine:	23.9[f] U/L	[70]
	T. versicolor	LccA	X33; pPICZαB	AOX	0.6%	ss of α-factor	Shake flask (0.05 L of medium); 0.5 mM $CuSO_4$; 28[0]C; 16 days; Initial pH 7;	11.972[f] U/L	[71]
	T. versicolor	LccA	X33; X33; pPICZαB	AOX	0.6%	ss of α-factor	5 L fermenter; 0.5 mM $CuSO_4$; 28[0]C; 4.2 days; Initial pH 7;	18.123[f] U/L	[71]
	C. gallica	LcCg	X33; pPICZB	AOX	1%	Modified α-factor preproleader	Fernbach flask; 0.5 mM $CuSO_4$; 28[0]C; 12 days; Initial pH 6; 0.8% alanine	250[e] U/L	[72]
	Trameters sp. 48424	Lac48424-1	GS115; pPIC3.5K	AOX	0.5%	Native	Shake flasks; 0.3 mM $CuSO_4$; 20[0]C; 7 days; Initial pH 6; 0.8% alanine	104.45[f] U/L	[73]
	C. cinerea	Lcc9	GS115; pPIC9K	AOX	0.5%	Native	Shake flasks 0.5 L; 0.3 mM $CuSO_4$; 28[0]C; 10 days; Initial pH 6.5; 0.8% alanine	3138 ± 62[f] U/L	[74]
	C. cinerea	Lcc9	X33; pPICZαA	AOX	0.5% MeOH	ss of α-factor	Shake flasks 0.25 L; 0.3 mM $CuSO_4$; 20[0]C; 7 days; 0.8% alanine	9.3[f] μkat/L	[75]
	C. cinerea	Lcc9	X33; pGAPZαA	GAP	*regulation by 0.5% glucose	ss of α-factor	Shake flasks 0.25 L; 0.3 mM $CuSO_4$; 20[0]C; 4 days; 0.8% alanine	12.8[f] μkat/L	[75]
	P. ostreatus	rPOXA 1B	X33; pGAPZαA	GAP	*regulation by 0.5% glucose	ss of α-factor	Bioreactor 10 L; 1mM CuSO4; 2% peptone; 1.5% yeast extract; 170h; geometry of flask	3159.93 [f] U/L	[76]

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2.2.1. GOx

The yield of recombinant protein production in *P. pastoris* depends on several factors, such as gene optimization and synthesis, plasmid construction, host strain, and fermentation conditions. [77]

Optimization of culture conditions represents one of the techniques to overcome the problem of low yield—the composition of medium plays a significant role in the production of recombinant protein. Establishing optimal reaction conditions such as pH and temperature is one of the critical steps for a higher yield of recombinant expression. As reported by Qui et al. (2016), the highest activity of recombinantly produced GOx was obtained when the expression was carried out at 30 °C, and the pH of the medium was 6. [61,62]

Methanol is used as a source of energy and carbon for recombinant protein production in *P. pastoris* as an expression system. As a result, methanol content in the culture medium is a significant variable while producing GOx. [63,64]

Apart from the mentioned fermentation conditions, codon optimization can influence protein expression. It has been shown that replacing low-usage codons (<15%) with high-usage codons can increase expression yield. For example, high-level expression of *P. notatum* F4 GOx was enabled by the distribution of G+C codons and removal of AT-rich regions, and a newly designed gene was named god-m. [78]

The construction of multi-copy or high-copy transformants, which increase the gene dosage for expression GOx in *P. pastoris*, represents good strategies for enlarging the yield of production enzymes. Recent works have demonstrated that to construct recombinant strains containing multiple copies of the GOx gene, it is first necessary to build vectors containing two or three tandem copies of the GOx expression cassette, termed pPICZHisα2GOX and pPICZHisα-3GOX. The generation of vectors containing more than three copies of the GOx expression cassette was unsuccessful, probably due to vector size limitations. The best results are obtained for the secretory expression of GOx in a 3-copy strain. [79]

Research showed that the yield of expression of GOx can be regulated by overexpression of the Hac1p, unfolded protein response regulator of chaperones. Yu et al. finally achieved an enzyme activity level of around 2125.3 U/mL in fermenter culture with basic process changes. [79].

2.2.2. CDH

P. pastoris is established as a standard expression system for CDH. Bey et al. reported that cultivation mode impacts CDH expression; cultivation in a shake flask and a bioreactor produced 1176 U/L and 7800 U/L, respectively. [65] A higher yield of CDH is expected when multiple copy transformants are selected. [65] According to prior research, the duration of CDH expression depends on CDH origin. [40,66]

2.2.3. HRP

HRP expression in *P. pastoris* was done using the construct pPICZαB-HRP and, as an inducer, used methanol. For better yields of that recombinant production were added 1.0 mM vitamin B1, 1.0 mM δ-ALA and 0.5 ml/l trace element mix (0.5 g/l MgCl2, 30 g/l $FeCl_2 \cdot 6H_2O$, 1 g/l $ZnCL_2 \cdot 4H_2O$, 0.2 g/l $CoCl_2 \cdot 6H_2O$, 1 g/l $Na_2MoO_4 \cdot 2H_2O$, 0.5 g/l $CaCl_2 \cdot 2H_2O$, 1 g/l $CuCl_2$ and 0.2 g/l H_2BO_3) in growth medium at the point of induction. [43] One such carbon source is 1% sorbitol, given to the medium. Casamino acids are used to stop protein breakdown. [67] Specific productivity (qp) of the recombinant *P. pastoris* strain was increased 5.5-fold via dynamic feeding strategy, where the setpoint for the specific substrate uptake rate (qs) was increased stepwise until a predetermined maximum

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(qsmax), in contrast to a traditional feed-forward strategy. [80] [81] *P. pastoris* successfully secreted a specific fusion construct of HRP and SpG in bioreactor cultivations with 110 mg/L yields. Recombinant HRP yields from *P. pastoris* that have been previously reported are normally in the 10 mg/L range. [68]

2.2.4. LAC

Different strategies can be applied to achieve higher yields or higher activity of recombinantly produced LAC in *P. pastoris*. Concrete data is presented in Table. Previous studies have shown that there are two main aspects of optimizing LAC production in *P. pastoris* and yeast in general: control at the level of recombinant gene construction and control of the parameters to obtain optimal cultivation. [82] Prior research recognized temperature, methanol concentrations, pH value during cultivation, and copper concentration as important factors affecting the heterologous LAC expression. Hong et al. concluded that cultivation in shake flasks at low temperatures with a final concentration of methanol 0.5% improved laccase activity; also, they confirmed their observation with cultivation under controlled conditions in the fermenter. [69] As has previously reported in the literature, expression of *T. versicolor* LAC, lcc1 in *P. pastoris* was enhanced if the pH was periodically altered to pH 6.0 during fermentation. [83] O'Callaghan et al. designed a medium for maintaining a constant pH value. They examined the impact of proteolytic and non-proteolytic *P. pastoris* strains and the effect of copper availability on the laccase expression. They did not observe significant differences between proteolytic and non-proteolytic strains, but adding alanine to maintain pH and appropriate copper concentration had a significant role. [70] Other researchers discovered that with optimization of several parameters such as initial pH, the final concentration of methanol as inducing agent, medium volume, initial OD600, copper concentration, and peptone concentration in the medium (pH 7, 0.6% MeOH, 50 mL of medium, 0.5 mM Cu^{2+} and 4% peptone) can obtain 4.4 times higher LAC activity compared to the initial medium. Besides that, they have also suggested that the native LAC secretion signal was superior to the secretion signal of the α-factor. [71] Difficult detection of laccase associated with poor secretion using α-factor secretion signal can be overcome with evolved α-factor preproleader. [72] According to Xu et al., the significance of the laccase expression factors took the following order: concentration of methanol added > initial pH > Cu^{2+} concentration > temperature. [74] A considerable body of literature promotes distinct optimal conditions for heterologous laccase expression. Ardila-Leal et al. statistically improved culture media for rPOXA 1B laccase production, expressed in *Pichia pastoris* containing pGAPZαA-LaccPost-Stop in a 10 L bioreactor. [76] They obtained 3159.93 U/L using ABTS as a substrate.

3. State-of-the-art technologies for increasing recombinant protein expression

In the articles mentioned above, optimization of fermentation was done by changing fermentation media composition, induction time, temperature optimization, etc. Still, there are also trials of increasing the fermentation yield of these enzymes by using state-of-the-art technologies such as precision fermentation, [84] directed evolution, [85] protein, and strain engineering, [86] high-throughput screening methods based on in vitro compartmentalization, [87] flow cytometry, and microfluidics.

3.1. Directed evolution, protein and strain engineering

Strain engineering for protein production can be performed using precision fermentation that combines synthetic biology, genetic engineering, and machine learning approaches. This is based on biofoundries that provide an integrated infrastructure for rapid construction, design and analysing genetically modified organisms. [88] The first step usually involves generating large host organism libraries by diverse genetic modifications like protease knock-out, cassette modifications, etc. Afterwards comes the screening process. Computational approaches such as deep learning based on artificial neural networks and analyzing genome sequences for predicting gene manipulations to enhance recombinant protein production have recently been used. [89–91]. This approach can predict

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the production performance of well-studied organisms like S.cerevisiae. [92], P. pastoris [93] and optimize metabolic flux via altering genes involved in the metabolic network. [94] Therefore, the machine learning approach is proven capable of recommending strain engineering strategies. [94]

Adaptive laboratory evolution is another approach for improving microbial phenotypes in many organisms. [95] In this approach, microbes are cultured in a desired growth environment for an extended period, allowing natural selection to enrich mutant strains. Evolved strains are later characterized and their DNA sequenced to find adaptive mutations that enable phenotypic improvement.

Expression of recombinant proteins in S.cerevisiae, especially oxidoreductases, can be increased using synthetic biology methods by choosing suitable promotors, selectable markers, and plasmids. Further, an increase in enzyme production can also be enhanced by utilizing various secretion factors. For example, it can be increased dramatically by site-directed mutagenesis or directed evolution of secretion peptide recombinant protein production. For instance, protein engineering approaches were carried out by Aza et al. to facilitate the heterologous production of various laccases by S.cerevisiae that included best-evolved signal peptides, new N-glycosylation sites in the enzyme genes, and consensus enzyme design for enhancing protein folding and stability. [96] Authors obtained mutated α-factor preproleader α_{9H2} that enhanced LAC production in the yeast twofold. Using other above-mentioned protein engineering strategies, they obtained 37 mg/L of ascomycete LAC. The same authors in another publication designed improved universal signal peptide α_{OPT} by adding four mutations into the α_{9H2} preproleader sequence. [97]

P. pastoris was by Zhou et all. in 2023 used for the expression of GOx and by using screening of different signal peptides, introducing multiple copies of genes, and engineering vesicle trafficking hyperproducing strain G1Ese co-expressing trafficking components EES and SEC was obtained that could produce up to 7223 U/mL with 30.7 g/L of GOx that is 3.3 fold higher than the highest level reported so far. [98] It is also possible to engineer P. pastoris strain by coexpression of chaperons and protein disulfide isomerase in these yeast cells. [99] To increase secretory expression of heterologous proteins in P. pastoris Duan et all. screened endogenous signal peptides and protein folding factors. Their effects on the expression of three reporter proteins were tested and they were able to identify Msb2 signal peptide and Dan4 signal peptide that increase recombinant protein secretion 8 and 172 fold, respectively compared to alpha-mating pre-pro leader sequence in P.pastoris. [100]

Ito et al. l., in their recent work, created a terminator catalog by testing 72 sequences of terminators from S. cerevisiae and P. pastoris and found that terminator RNA sequences from S. cerevisiae maintain function when transferred to P. pastoris. [101] They managed to fine-tune protein expression levels in metabolic engineering and synthetic biology in P. pastoris and enhance them 17-fold. In a similar work on RNAi expression tuning, Wang et all. found genes with functions in cellular metabolism, protein modification and degradation, and cell cycle that can significantly influence the expression level of proteins in S. cerevisiae. [102]

One of the main problems with expressing recombinant proteins in Pichia is hyperglycosylation, although usually, glycosylation is necessary for eukaryotic proteins to be correctly folded and expressed. To solve this problem, strain glyco-engineering trials of Pichia were done to prevent hyperglycosylation and enable higher fermentation yield of recombinant peroxidases. [103] Different glycoengineered P. pastoris strains were developed, and the physiology and growth behaviors of Man5GlcNAc2 glycosylating P. pastoris strain in the controlled environment of a bioreactor was characterized using flow cytometry during expression of HRP C1A isoform of the enzyme. The HRP C1A isoform expressed in the novel glycoengineered Pichia strain had similar kinetic characteristics as the one expressed in the wild-type Pichia strain. Still, the thermal stability of the recombinant HRP was decreased due to the reduced glycosylation. Still, the recombinant enzyme formation rate in the novel strain increased from 0.77 U/gh to 1.05 U/gh during fermentation.

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3.2. High-throughput screening methods

3.2.1. Flow cytometry

To follow the influence of various factors on protein production in P. pastoris, it is essential to be able to follow the physiological state of recombinant yeast cells. Hyka et al. quantified factors affecting the physiological state of recombinant P. pastoris Mut+ (methanol utilization-positive) by using a combination of staining with different fluorescent dyes and analyzing by flow cytometry. [104] The authors found that cell vitalities could range from 5% to 95% in high-cell-density cultures with strain-producing HRP, depending on the influence of various stresses such as recombinant protein expression, high cell density, and pH. This quantitative assessment of the individual cells' physiology using flow cytometry enables the implementation of innovative concepts in bioprocess development. This is especially important because the paradigm assumes a uniform cell population and does not differentiate between individual cells whose state can only be followed by single-cell analysis. The conclusion was that only part of the cell population contributes to the recombinant protein production, and the objective should be to maintain productive cells over a long period as long as possible.

Flow cytometry can also be used for following expression and correctly folding active proteins like cytochrome c peroxidase when recombinant protein is fused with a green fluorescent protein (GFP). [105]

3.2.2. Microfluidics

Since the screening phase and early process development based on microtiter plates and flasks still represent a bottleneck due to the high cost and time-consuming procedures, Totaro et al. developed a screening protocol for P. pastoris clone selection based on the multiplexed microfluidic device using 15 μL cultivation chambers that were able to operate in perfusion mode and monitor dissolved oxygen content in the culture in a non-invasive way. [106] Using a microfluidic platform, the authors identified the best producer clone after 12 h from inoculation and confirmed the results by lab-scale fermentation.

Microfluidics combined with flow cytometry was also used for high.-throughput droplet screening and genome sequencing analysis for improved amylase-producing A. oryzae strain. [107] This work, 450000 droplets were screened within two weeks, and a high-producing strain with 6.6 folds increased production was found.

3.2.3. In vitro compartmentalization

In vitro, compartmentalization is often used in protein engineering and can be made in polydisperse format for single-cell experiments, or it can be made in monodisperse format by microfluidics. [108] Microspheres made of soft materials are also used in protein engineering as an alternative to liquid compartments. [109] Both of these compartmentalization methods can be used to improve not only enzyme activity and stability but also production yield during fermentation, and usually, the best way to do so is to perform directed evolution experiments using strains for production.

To optimize recombinant protein production in yeasts (*P. pastoris*), droplet microfluidics can be used to encapsulate (compartmentalize) large genetic libraries of strains within biocompatible gel beads that are engineered to selectively retain any recombinant protein of interest by binding it via His tag usually used for labeling and purification, and afterward staining of secreted protein with fluorescent dyes.[110] This platform can be used broadly for various proteins, including oxidoreductases. As proof of principle, authors found *P. pastoris* strain that 5.7-fold increased recombinant cutinase production after screening more than 10^6 genotypes.

Compartmentalization within double emulsion can also be used to optimize recombinant protein production instead of beads. *In vitro*, compartmentalization within a double emulsion of water-in-oil-in-water was done using microfluidics and fluorinated oil. The fluorescent

immunosensor quench-body detected the secreted recombinant protein (fibroblast growth factor 9), and clones with high protein secretion were detected by fluorimetry. [111] This method also shortens the development period of industrial strains for recombinant protein production.

4. Conclusions

It can be concluded that despite the problems with the expression of OXR in yeasts like *S. cerevisiae* and *P. pastoris* due to the necessity of adding transition metals (copper, iron) and metabolic precursors of FAD and heme during fermentation, there are various approaches to increase expression yield of these enzymes. Some of them are optimizing fermentation conditions, the codon usage, using strong promotors and terminators, and multi-copy expression vectors. As we could see from the literature recently, there are also new possibilities for further increase in fermentation yield that explore cutting-edge technologies such as directed evolution, protein and strain engineering, high-throughput screening methods based on in vitro compartmentalization, flow cytometry, and microfluidics.

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