

Pichia pastoris – recombinant enzyme producent for environment treatment – review

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Abstract: Since environmental pollution is increasing, scientists try to find a sustainable way for its clean up and for environment protection. Due to increasing knowledge of genetics and recombinant technologies, recombinant enzymes have been increasingly applied for these purposes. This article deals with the possibilities of environmental treatment with different types of enzymes produced by *P. pastoris*. Environment is polluted mostly with pesticides, wastewaters, phenol compounds, plastics, toxic compounds, wastes from medical treatment, etc. All these compounds have to be eliminated considering the deteriorating biodiversity, human health, and condition of plants. Enzymes are an environmentally friendly way of such treatment.

Keywords: *Pichia pastoris*, recombinant enzymes, environment, pollution

Introduction

In the last century, a massive expansion of oil industry and different industries have negatively affected our environment. The situation got serious a long time ago and that is why scientists already devised some ways to stop damaging and even partially restore environment. First significant effort was the concept of chemical catalysis which dealt with catalytical reduction of contaminants derived from petroleum. The idea started in the 1960s and was in its heyday in the 1990s due to widespread application of chemical catalysis in industry (Alcade et al., 2006). Nowadays, long-term use of chemical catalysis is questioned mostly because of its health risks, and waste and toxic compounds production. Even though chemical catalysis has advantages in reducing chemicals footprint, a “cleaner technique”, biocatalysis, had to be introduced. Its concept was introduced in the 1990s and it is still interesting as an environmentally friendly technique. Basically, it is an application of microorganisms, or their enzymes, in the production of renewable and clean products replacing chemical catalysts mainly due to their negative environmental impact or energy and raw material requirements. Biocatalysis requires mild reaction conditions and it offers high activity products and chemo-, regio- and stereoselectivity (Sheldon & van Rantwijk, 2004). The same enzymes and microorganisms can also be used for the remediation of toxic wastes. Biocatalysis is very closely connected with recombinant techniques (Alcade et al., 2006). Combined with remediation efforts, microorganisms producing recombinant enzymes for environment remediation. Also, bio-industry wastes are studied as carbon source for

some microorganisms. Attention, especially these days, is focused on crude glycerol.

Pichia pastoris

One of the most widely used microorganism in recombinant technologies is *Pichia pastoris*. This methylotrophic yeast belongs to the family of *Saccharomycetaceae* (Satyanarayana & Kunze, 2009) and its genome was sequenced in 2009, which opened the door to further research (Schutter et al., 2009). Previously, in the 1970s, *P. pastoris* was used as “single cell protein” (SCP) producer for animal feeding. At that time, there was an effort to produce SCP from cheap carbon sources, e.g. methanol. *P. pastoris* later became the most cited methylotrophic yeast, but due to oil crisis, this interest decreased. Ten years later, the company which started to use *P. pastoris* for SCP – Philips Petroleum – together with the Salk Institute of Biotechnology prepared a strain for heterologous expression for academic and industrial purposes (Cos et al., 2006). Since the production of a functional heterologous protein expression is closely related to the metabolism of the production microorganism, *P. pastoris* offers several advantages in this respect. It is suitable for the expression of milligrams up to grams of protein per liter of fermentation broth – for basic laboratory research but also for industrial production. The advantages include its easy production in fermenters, where it is possible to regulate parameters affecting protein productivity, such as pH, aeration, the amount of carbon source supplied and induction of protein production. Compared to other producers, *P. pastoris* does not need complex growth media and the culture conditions are easily ensured. Yeasts are easily manipulated on the genetic level and their protein synthesis is similar to

that of higher organisms. Therefore, some proteins that cannot be efficiently expressed in bacteria, *Saccharomyces cerevisiae* or baculoviruses are often produced in functionally active forms in *P. pastoris*. These yeasts are beneficial for several reasons: easy genetic manipulation, functional complementation cloning, high-frequency DNA transformation, high intra- and extracellular protein production, and post-translational modifications of proteins. The yeast's post-translation modifications include disulfide bridge formation, glycosylation, and phosphorylation. During the cultivation, high cell densities are achieved using minimal media, which means production of proteins can reach high yields. Also, vectors with recombinant genes are integrated into the yeast chromosome which makes them genetically stable even in continuous and large-scale fermentation processes. At the same time, recombinant *P. pastoris* produces low levels of native proteins facilitating the purification of recombinant proteins. Powerful and available genetic techniques, together with the overall economics of the process, make *P. pastoris* a suitable expression system for the production of homologous proteins (Macauley-Patrick et al., 2005).

Cultivation of *P. pastoris* consists of two steps. The first is the growth of *P. pastoris* on glycerol to reach high cell density. Then, after glycerol depletion, methanol is added to the medium and protein production begins (Markošová et al., 2015).

The beginning of the methanol pathway (Fig. 1) in *P. pastoris* is in special organelles – peroxisomes and continues in cytoplasm. First step, where formaldehyde and hydrogen peroxide are formed, is catalyzed by alcoholoxidase (AOX). For methanol utilization, dihydroxyacetone synthase is necessary for formaldehyde assimilation in peroxisomes. A part of formaldehyde leaves the peroxisomes and is further oxidized by cytoplasmic dehydrogenases

to formate and carbon dioxide. These reactions are an important source of energy for the cell growth (Cereghino & Cregg, 2000). Genetically modified *P. pastoris* has, under AOX promoter control, located the gene encoding the homologous protein of interest. This promoter is induced only by methanol, which means methanol is the carbon source as well as the inductor. Since formaldehyde is formed during its utilization, methanol has to be added carefully due to its toxic effect (Markošová et al., 2015).

As mentioned above, *P. pastoris* initial substrate is usually glycerol, which is mainly used as pure compound. However, several publications show that *P. pastoris* is also able to grow on industrial crude glycerol (Anastácio et al., 2014). It is a by-product of biodiesel production and in the last decades started to be an environmental burden (Bartocci et al., 2018). The possibility of utilizing crude glycerol is only one way in which *P. pastoris* can participate in the reduction of environmental pollution. It has also great potential in the production of proteins, which can help the environment directly. Genetic engineering tools and commercial *P. pastoris* strains availability are also important advantages of this yeast application in research and industry (Fisher & Glieder, 2019).

Recombinant products of *Pichia pastoris* and their use in the environment

Antimicrobial peptides

Currently, the massive use of pesticides in the agro-sector causes significant pollution. Pesticides have been used worldwide and for many years to prevent pathogen diseases of plants. On the other hand, pesticide residues accumulate in soil and from there they can reach drinking water supplies. In the 1990s, a study by U.S. Geological Survey

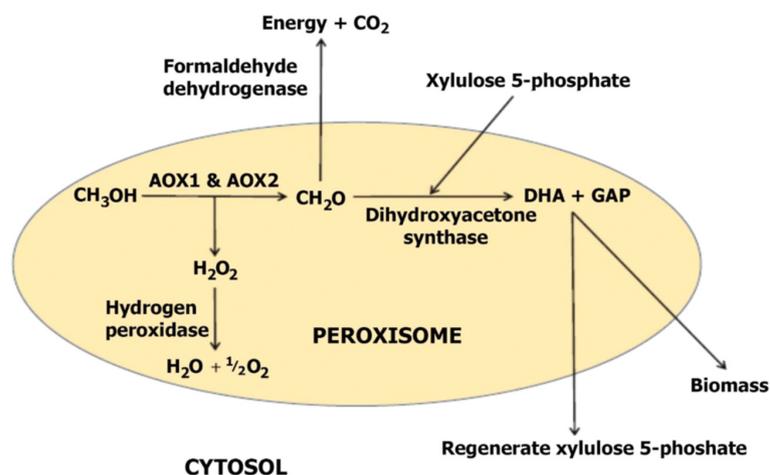


Fig. 1. Methanol pathway of *P. pastoris* (Ali et al., 2020).

(USGS) stated that 90 % of fish samples and water from all streams were contaminated by one or several pesticides. According to USGS, pesticides from every major chemical class were found in ground water and its pollution is a worldwide problem (Aktar et al., 2009). From water, pesticides can easily enter any organism. There is evidence that they cause cancer, respiratory problems, neurologic deficiency, birth defects, miscarriages or dermatologic difficulties (Bassil et al., 2007) (McCauley et al., 2006). Moreover, biodiversity is damaged since many animals are endangered by them (Mahmood et al., 2006). Pathogens have become resistant to pesticides, and new and more environmentally safe agents are needed.

One of the antimicrobial peptides is pexiganan, which has lethal effect on many types of undesirable microorganisms. It attacks the bacterial membrane resulting in pores on their surface and thus causing their death (Lazarev & Govorun, 2010). This peptide is recombinantly produced by *P. pastoris* because it has low sensitivity to almost no antimicrobial effect in eukaryotic cells. Thanks to that, large quantities can be produced without damaging the host microorganism. For example, production of recombinant peptide via *E. coli* reached 30 mg/L (Sun et al., 2018) while via *P. pastoris* it was 52 mg/L. Furthermore, to avoid *E. coli* inhibition, inactivation sequence is added to the peptide gene which has to be cleaved using thermal, enzymatic or chemical methods leading to higher production costs and partial loss of produced peptide. Recombinant peptide produced in *P. pastoris* was tested on eight plant pathogens causing significant economic losses in agriculture industry and on two human pathogens (Tab. 1). Pexiganan showed 500 times stronger antimicrobial activity than copper oxychloride

which is a common chemical pesticide against plant pathogens. Peptide has high long-term stability and temperature resistance. According to calculations, production of 52 mg of recombinant peptide costs about 10 \$, while synthetic production of crude pexiganan is 20–30 times more expensive and in case of 98 % purity, it is even 120 times more expensive (Neshani et al., 2019), (www.entonbio.com; www.synpeptide.com).

Pathogens elimination can also be done using killer toxin produced by the yeast *Tetrapisispora phaffii*. Killer toxin, known as Kptk, has β -1,3glucanase and killer activity which induces ultrastructural modifications to the cell wall of the yeast of genera *Kloeckera/Hanseniaspora* and *Zygosaccharomyces* causing spoilage of grapes and losses in wine and sweet beverage production. Recombinant killer toxin was produced by *P. pastoris* and reached final concentration of 23 mg/L which is significantly more than the amount produced by native *Tetrapisispora phaffii*. Moreover, compared to native Kpkt, recombinant Kpkt shows a wider spectrum of activities, e.g. toxic effect on *Dekkera bruxellensis*, spoilage yeast in wine making and biofuel industry (Chessa et al., 2017).

Pesticides

Enzymes are also applied for pesticides monitoring and their degradation. One of them is organophosphorus hydrolase from *Pseudomonas pseudoalcaligenes* (Shen et al., 2016) which can be used for biodegradation of residues from organophosphorus pesticides or their detection (Mulchandani et al., 1998). These pesticides are synthetic esters, amides, or thiol derivatives of phosphonic, phosphoric or phosphonothioic acid. They are widely used in agriculture and domestic cultivation. Organophosphorus pesticides were

Tab. 1. Minimum inhibitory concentration of recombinant pexiganan and copper oxychloride needed for lethal effect on pathogen (Neshani et al., 2019).

		Minimum inhibitory concentration (∞ g/ml)	
		Pexiganan-based biopesticide solution	Cooper oxychloride 35 % WP
	<i>Pseudomonas syringae pv. syringae</i>	0.81	625
	<i>Ralstonia solanacearum</i>	0.4	1250
	<i>Pantoea stewartii</i>	0.4	312.5
Plant pathogens	<i>Xanthomonas arboricola pv. pruni</i>	0.81	625
	<i>Erwinia amylovora</i>	0.4	2500
	<i>Pectobacterium carotovorum</i>	0.4	625
	<i>Agrobacterium tumefaciens</i>	0.4	1250
	<i>Brenneria nigrifluens</i>	0.4	312.5
Human pathogens	<i>Staphylococcus aureus ATCC 25923</i>	8	-
	<i>Escherichia coli ATCC 25922</i>	16	-

detected in soil and drinking water long after their application and they belong to the most toxic pesticides causing long-term damage to human health since they inhibit the enzyme acetylcholinesterase which is essential for the function of central nervous system (Songa & Okonkwo, 2016). Decontamination methods for these pesticides are often toxic, corrosive, allergic and not environmentally friendly and thus it is essential to hydrolyze them, preferably with enzymes (Farnoosh & Latifi, 2014). Organophosphorus hydrolase was produced on purified glycerol with later methanol induction by *P. pastoris*. Recombinant organophosphorus hydrolase had good biodegradation properties and expression reached high level (7.8 g/L of protein); this production can provide an effective way of dealing with organophosphorus pesticides. Activity of this enzyme was measured by hydrolysis of substrate methyl parathion, a popular organophosphorus pesticide widely used for crop protection, its activity reached 21.6 $\mu\text{mol/h/mg}$ of wet weight (Shen et al., 2016). To compare, organophosphorus hydrolase produced in *Moraxella sp.* Hydrolysing reached the activity of only 0.6 $\mu\text{mol/h/mg}$ of dry weight using the same substrate (Shimazu et al., 2001).

Wastewater, phenol compounds and lignin treatment

Historically, the most well-known enzymes used for bioremediation of wastewater and soils are wood-destroying fungal enzymes involved in lignin degradation (laccase, lignin peroxidase, manganese peroxidase). Water and soils are widely polluted with chemical compounds like phenol, aromatic amines, synthetic dyes, antibiotics, hydrocarbons, chlorine etc., which have negative effect on the biodiversity or plant and human health. Since industry is very developed and these pollutants are still used, there is no other option than to eliminate them. The use of oxidative enzymes for their biodegradation is environmentally friendly, low cost and more effective compared to physical and chemical methods which have many disadvantages such as hazardous by-products or limited degradation activity (Alcade et al., 2006). Besides biodegradation, the mentioned enzymes can also be used in other industrial sectors. First and the most studied enzyme is laccase, which can be found in bacteria, fungi, higher plants and even insects. Laccase finds its application in the following industries: food, paper, pulp, textile, cosmetics, and it is used for bioremediation, removal of endocrine disruptors and biodegradation of phenolic pollutants. Laccases are also able to oxidize non-phenolic lignin-related compounds. An important environment application of laccase is decolorization of dyes (Shraddha et al., 2011). More than 100,000 dyes are commercially available, most

of which are very difficult to eliminate from water due to their complicated structure and synthetic origin. They are specially designed to resist fading after exposure to light, water and oxidizing agents. Due to their high stability, wastewater treatment techniques to decompose them include electrochemical destruction, membrane filtration, electroflotation, ozonation, ion exchange or adsorption (Nigam et al., 2000). The use of enzymes is a more simple, clean, and effective technique leaving less waste. One of the examples of enzymatic treatment is detoxification and reduction of color and aromatic compounds of black liquor – wastewater from pulp production. Detoxification was performed with *Trametes versicolor* producing laccase. This microorganism was also used for complete decolorization of Amaranth, Congo Red, Reactive Black 5, Tropaeolin O, and Reactive Blue 15 colors (Ramsay & Nguyen, 2002). In bioremediation, laccase also achieves excellent results; laccase from *T. villosa* removed 100 % of 2,4-dichlorophenol from soil (Ahn et al., 2002). Recently laccases have been applied in nanobiotechnology, since they are able to catalyze electron transfer reactions with no additional cofactors (Shraddha et al., 2011). Laccase and other ligninolytic enzymes are produced during the secondary metabolism of fungi under nitrogen or other nutrient limited conditions when they are secreted in the medium extracellularly. Most widely known species for laccase production are *Basidiomycetes* and *Saprotrophic* fungi and the production usually proceeds via solid-state or submerged fermentation, which have some disadvantages like broth viscosity, oxygen and mass transfer problems (Moreira et al., 2003). Laccase production via solid-state and submerged fermentation does not reach the maximum yield and it is not possible to produce large volumes of highly active enzyme at affordable cost (Shraddha et al., 2011).

Although enzymes are widely used, due to the slow growth of fungi and the mentioned disadvantages, their recombinant production by *Pichia* is a more effective way of production (Xu et al., 2017). As an example, recombinant laccases from *Pleurotus ostreatus* produced by *P. pastoris* were capable of efficient removal of nitrophenols, chlorophenols and sulfonamide antibiotics (Fig. 2), dangerous agents with high toxicity and carcinogenic effect. Their residues were found mainly in wastewater. The best degradation effect was achieved in case of sulfonamide antibiotics (sulfadiazine: 98.1 %, sulfamethazine: 97.5 % and sulfamethoxazole: 97.8 %). Degradation of nitrophenols was also successful (2-nitrophenol: 59.21 %, 3-nitrophenol: 47.91 % and 4-nitrophenol: 60.24 %). The worst results were obtained for chlorophenols (4-chloro-

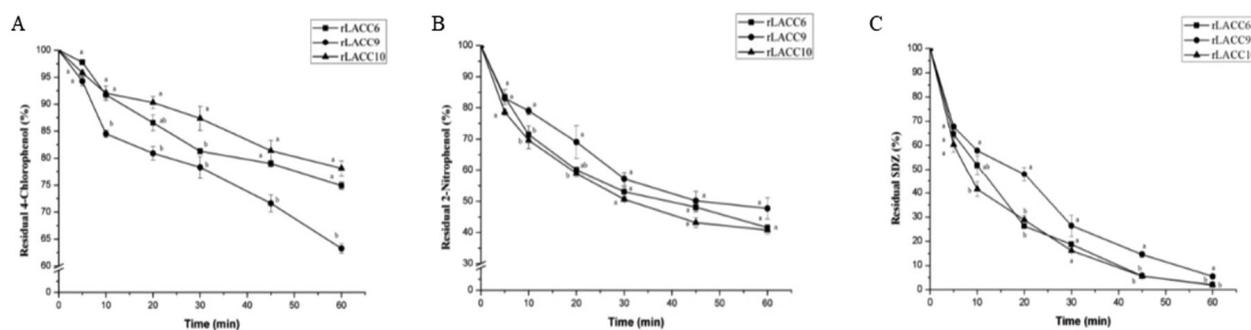


Fig. 2. Degradation of 4-chlorophenol (A), 2-nitrophenol (B) and sulfadiazine (C) by three recombinant laccases produced by *P. pastoris* (Zhou et al., 2018).

phenol: 47.9 %, 2,4-dichlorophenol: 28.9 % and 2,6-dichlorophenol: 35.1 %) (Zhou, et al., 2018).

In comparison, laccase from *T. versicolor* provided 97 % degradation of 4-nitrophenol (Levil et al., 2016) and laccase from *T. pubescens* ensured 41.1 % degradation of 2,4-dichlorophenol (Gaitan, et al., 2011). However, this result may be affected by the cooperation of multiple enzymes in fungi. Also the reaction mixture for laccase from *T. pubescens* and *T. versicolor* contained 15 mg/L and 139 mg/L of the pollutant while recombinant laccase showed the concentration of 100 mg/L. Degradation ability of recombinant laccase was observed only for 1 hour and in case of native laccase from *T. pubescens* and *T. versicolor*, a 4 and 72 hour experiments were done, respectively. Based on these results, it can be concluded that recombinant laccase has better degradation ability the native one (Zhou et al., 2018).

Different types of laccase from the wood-destroying fungi *T. troglia* was also successfully expressed in *P. pastoris* (Colao et al., 2006). The recombinant enzyme exhibited similar specific activity (232 U/mg) and the same properties as the native one. Its decolorizing ability was tested on synthetic dyes including azo dyes, triarylmethane, indigo carmine and the anthraquinonic dye alizarin red S. Relative decolorization (almost 100 %) was the highest in case of blue indigo and since this is the most important dye in blue jeans manufacturing, it is an important advantage of recombinant laccase. Moreover, production of recombinant laccase achieved concentrations (129,6 mg/L) which, according to the authors, exceeds that obtained from *T. troglia* cultures, which also produce a number of laccase isoforms and are difficult to separate since they have similar chemico-physical properties. This does not happen in case of *P. pastoris* production (Colao et al., 2003).

Another ligninolytic enzyme is manganese peroxidase catalyzing the depolymerization of plant lignin. Manganese peroxidase is one of the most common lignin degradation enzymes and has great

potential in agriculture for its ability to degrade cellulose, hemicelluloses, and lignin. It was first isolated in 1985 from the wood-destroying fungus *Phanerochaete chrysosporium* (Paszczynski et al., 1985). It is the most abundant lignolytic enzyme in nature. Currently, it has been isolated and characterized from several fungi and its properties are the subject of extensive research. Many studies suggest manganese peroxidase to effectively degrade azo dyes. In the field of environmental protection, it is also used for the degradation of organic and toxic substances such as polycyclic aromatic hydrocarbons, chlorophenols, industrial dyes and nitroaromatics (Xu et al., 2017). Polycyclic aromatic hydrocarbons are derived from industrial activities causing health risks due to their carcinogenic and mutagenic potential. Manganese peroxidase from *Bjerkandera* sp. degraded them (specifically anthracene, dibenzothiophene and pyrene) to a large extent and in a short period of time, 7 hours for anthracene, and 24 hours for dibenzothiophene and pyrene (Eibes et al., 2006). Manganese peroxidase from *Pleurotus ostreatus* detoxicated aflatoxin, which is a mycotoxin with carcinogenic, mutagenic, hepatogenic and immunosuppressive properties (Yehia, 2014). Enzyme from *Phanerochaete chrysosporium* was successful in removing tetracyclines and oxytetracyclines. The results show that manganese peroxidase degraded 72.5 % of tetracyclines and 84,3 % of oxytetracyclines (Wen et al., 2010). Manganese peroxidase crude enzyme extract of *Nematoloma frowardii* and *Stropharia rugosoannulata* proved to convert several aminonitrotoluenes such as 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene and 2,6-diamino-4-nitrotoluene (Scheibner & Hofrichter, 1998). All mentioned application showed great potential in the removal of impurities from the environment. However, natural production of this enzyme is disadvantageous due to the slow growth of fungi, accumulation of extracellular polysaccharides, or production of compounds with similar chromatographic properties. Therefore, recom-

binant microorganisms have been studied for its production (Xu et al., 2017).

Manganese peroxidase from *Ganoderma lucidum* produced by *P. pastoris* was tested for phenol and synthetic azo dyes degradation (Xu et al., 2017). It was proved that during photocatalytic degradation of azo dyes, several aromatic intermediates are produced (Fig. 3). They are harmful to the nature and human health (Tanaka et al., 2000).

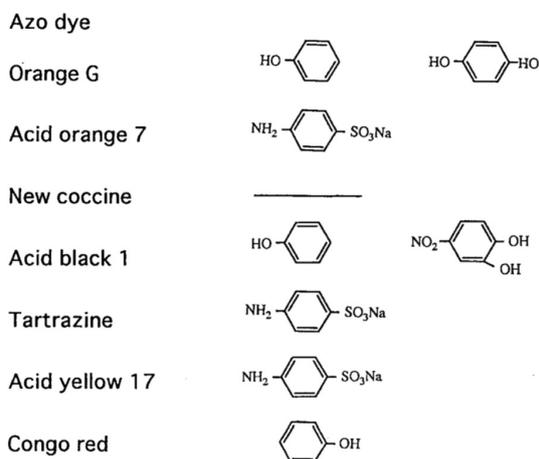


Fig. 3. Intermediates (phenolic compounds, aromatic amines) in photocatalytic degradation of azo dyes (Tanaka et al., 2000).

It is an advantage that manganese peroxidase can degrade both phenol and synthetic dyes. There are several studies confirming that manganese peroxidase decolorizes wastewaters from factories. However, expression on high level has to be considered before commercial use. In many isolates it is usually too low for industrial application but *P. pastoris* could meet these requirements. Compared with the natural host, *P. pastoris* increases expression levels by 10-, 100- or even 1000-fold. Recombinant enzyme from *P. pastoris* was produced with specific activity of 524,61 U/L and final yield of 126 mg/L. Activity of manganese peroxidase was confirmed through its ability to degrade phenol which was monitored chromatographically. In case of the tested synthetic dyes, the results are easily visualized (Fig. 4). In Figure (c) are dyes before degradation and Figure (d) shows dyes after applying crude protein extract of recombinant *P. pastoris* containing manganese peroxidase. Decolorization of all dyes reached 70 % (Xu et al., 2017).

Manganese peroxidase is also important in pulp and paper industry. Pulp mills discharge a wide range of chlorinated and non-chlorinated organic compounds because they use molecular chlorine for bleaching. Chlorinated compounds include chlorinated phenolic derivatives and chlorinated

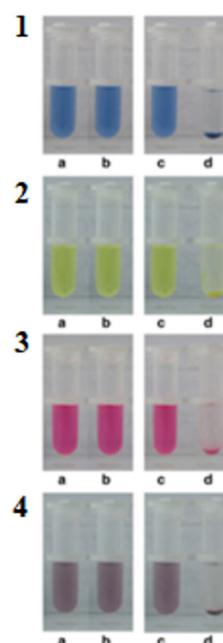


Fig. 4. Visual representation of dyes degradation: Drimamer Blue CL-BR (1), Yellow X-8GN (2), Drimaren Red K-4BI (3) a Disperse Navy Blue HGL (4). On the left side: crude protein extract of non-recombinant *P. pastoris* used as a control at the beginning (a) and the end of dye degradation (b). On the right side: (c, d) is crude protein extract of recombinant *P. pastoris* containing manganese peroxidase at the beginning (c) and the end of dye degradation (d) (Xu et al., 2017).

dioxins and furans, and they are known to accumulate in the tissue of fish (Owens et al., 1994). Public has forced pulp and paper mills to reduce chlorine-based chemicals and use more environmentally friendly bleaching techniques based on enzymes like manganese peroxidase. Results from *P. pastoris* manganese peroxidase fermentation showed that recombinant enzyme can degrade lignin and it is very effective when combined with alkaline extraction in delignification and brightening. Kappa number of the resulting pulp was reduced by 61 %, to 8.6 (ideal value should be below 6.5 (Viikari et al., 1968)) and brightness increased by 26 points (Xu et al., 2010).

Another enzyme involved in lignin degradation is lignin peroxidase, which oxidizes non-phenolic substrates. Lignin is an aromatic polymer which protects plants from microbial attacks. Lignin degradation in nature is interesting because it is an essential part of the carbon cycle and it is unusually biochemically stable (Hammel et al., 1993). Lignin as a waste is generated as a by-product from pulping or ethanol production. Due to its complex structure, it is an environmental burden and its degradation is essential (Li & Takkellapati, 2018).

Lignin peroxidase was expressed in various hosts. One of them was *Escherichia coli*. Even though the enzyme production was fast and provided high yields, it was limited by the low yield of subsequent *in vitro* refolding. This was necessary due to the lack of haem incorporation inside the cells and formation of inclusion bodies. Lignin peroxidase was also expressed in baculovirus with low yield of active peroxidase and high production cost. The yield in *Aspergillus niger* was also comparatively low. On the other hand, production of recombinant lignin peroxidase in *P. pastoris* was successful and it proved that multi-copy transformation of plasmid increases the final yield. Activity of the enzyme reached 15 U/L after a 12 hours induction (Wei & Xianghua, 2009).

From peroxidases, horseradish peroxidase is another interesting enzyme to be used for the treatment of the following pollutants and wastes: aromatic amines, bromophenols, chloromethylphenols, methoxyphenols, chlorophenols, methylphenols, phenol, bleach plant effluent, cotton mill effluent and coal conversion wastewater (Aitken, 1993). Nowadays, great progress has been made using directed evolution inside *P. pastoris*. The enzyme showed a 2.3-fold higher specific activity towards the pollutant guaiacol and a 5.4-fold higher activity towards ABTS [2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)]. ABTS is a chemical compound used to determine the enzyme kinetics or enzyme-linked immunosorbent assay. It is used as a substrate for horseradish peroxidase to identify its kinetics. Production of horseradish peroxidase was also realized in *S. cerevisiae*, but the enzyme from *P. pastoris* had a 5.6-fold higher total activity and a three-fold higher cell density (Morawski et al., 2000).

Acrylonitrile and acrylamide are widely used in petroleum industry and are the main pollutants in generated wastewaters. Their high toxicity and carcinogenicity are very dangerous, but nitrile hydratase showed bioconversion activity for these compounds. It was expressed in *P. pastoris* but, unfortunately, it was unstable and low activity was achieved. To solve this problem and enhance the activity of the enzyme, directed evolution can be used combined with another *P. pastoris* strain (Shi et al., 2004).

Pulp bleaching and conversion of agrowaste

In addition to manganese peroxidase, xylanase also provided excellent bleaching results. Moreover, xylanase is commercially important due to its ability to convert lignocellulosic material and agrowaste enabling the production of high value products such as furfural, xylitol, biofuels, and artificial low-calorie sweeteners. Xylanolytic en-

zymes find their application in animal feed as they improve their digestibility by breaking down arabinoxylans and reduce the raw material viscosity. As it has already been stated, xylanase is also an effective enzyme for wood pulp bleaching and structure modification of pulp fibers (Barnoud et al., 1986). Together with manganese, peroxidase increased pulp delignification (Xu et al., 2010). Xylanase treatment is important for baking, where it breaks down hemicelluloses in wheat flour and water insoluble hemicellulose is converted to soluble forms, leaving the dough softer and easier to knead. Fruit juices are also treated with xylanase to reduce their viscosity and improve organoleptic characteristics. Natural sources of xylanases are bacteria and fungi, while those from fungi have higher activity. On the other hand, fungi also secrete cellulase, which is undesirable for some industrial applications, especially during paper pulping and bleaching. Xylanases are produced using submerged or solid-state processes. Solid-state fermentation suffers due to some limitations such as lower process control or low capacity (Malhotra & Chapadgaonkar, 2018). Since xylanase is a very interesting enzyme with wide use, its recombinant production has been studied. Xylanase was expressed in *P. pastoris* reaching very high yields (1.2 g/L). The final yield of the enzyme is important for its further application in industry, which makes *P. pastoris* suitable for industrial production of high quantities of enzymes (Mellitzer et al., 2012). *P. pastoris* is not only suitable due to its high enzyme yields but also due to post-translation modifications like glycosylation enzyme which show better properties. Higher thermostability of glycosylated enzyme from *P. pastoris* compared to those from *E. coli* were confirmed (Fonseca-Maldonado et al., 2013). Recombinant xylanase was produced in *P. pastoris* by directed evolution which yielded a strain with 1,3-fold higher xylanase production compared with the parent strain (Ma et al., 2019). This result excellently demonstrates how directed evolution can improve the produced enzyme quality.

Degradation of bioplastics waste

The problem with plastic waste is probably the most widespread and well-known. That is why biodegradable plastics produced from renewable agricultural sources are a promising alternative. Polyhydroxyalkanoates have commercial potential as biodegradable thermoplastics; their biodegradation and mineralization are attributed to polyhydroxyalkanoate depolymerase from *Thermobifida* sp. The enzyme was expressed in *P. pastoris* and it catalyzed the degradation of poly-[(R)-3-hydroxy-

butyrate] (PHB) films, which was accelerated in (R)-3-hydroxyvalerate copolymers with the maximum degradation rate of $882 \text{ ng} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$. Such prepared enzymes can be used in standard processing of bioplastics waste (Phithakrotchanakoon et al., 2009).

Pichia recombinant products for alternative medical treatment

Overuse of drugs is also an environmental problem because their residues are found in water or animals. It means that replacement of pathogens' control methods with e.g. enzymes or antigens has to be prioritized. Strains of *P. pastoris* were used to produce AHL-lactonase which can be used as a cure of *Aeromonas hydrophila* infection in fish. This serious pathogen causes hemorrhagic septicemia which is controlled by antibiotics and chemicals. Excessive use of chemicals for the treatment of this disease leads to their accumulation in the food chain and thus they can enter human body. In addition, frequent use of antibiotics results in "superbugs" (strains resistant to antibiotics). Recombinant production was successful and the results indicate the possibility of mass low-cost production of AHL-lactonase (Chen et al., 2010). Another veterinary problem is caused by *Boophilus microplus* which is a cattle tick causing problems with their reproduction. Control methods of this ectoparasite include the use of chemicals which are costly and cause environmental and residual problems. Vaccination is a way of protection against tick infestations. Vaccine Gavac™ (Heber Biotec S.A., Cuba), a recombinant Bm86 protein, is produced in *P. pastoris* and used in Latin America. Unlike other expression system (baculovirus, *Aspergillus niger*, *E. coli*) recombinant antigen expressed in *P. pastoris* is acquired in a highly immunogenic, glycosylated, and particulate form (Canales et al., 1997).

Bioremediation of toxic compounds

Cyanide is a very toxic and well-known compound. In plants such as almond, cassava, sorghum or flaxseed, cyanogenic products are widely distributed. Due to β -glucosidase in the intestines of mammals, acutely toxic hydrocyanic acid is produced and therefore detoxification is important for safe feeding of animals with these plants. Popular detoxification method is boiling, roasting, autoclaving, microwaving and extrusion. Also, β -glucosidase can be used to hydrolyze these compounds which releases hydrocyanic acid into air. This problem can be solved by cyanide hydratase which catalyzes the hydrolysis of cyanide to formamide. It is used for bioremediation of cyanide-containing wastes and to absorb hydrocyanic acid formed from the cyanogenic glycosides hydrolysis. Recombinant cyanide

hydratase was produced in *P. pastoris* together with β -glucosidase to develop a novel co-enzymatic method with high degradation activity of cyanogenic glycosides. Enzyme preparation produced by recombinant *P. pastoris* completely decomposes cyanogenic glycosides and catalyzes the hydrolysis of more than 80 % of cyanide (Wu et al., 2012).

P. pastoris recombinant proteins are not used only for pollutant degradation but also for their detection. One of the commercial *P. pastoris* product is Superior Stock produced by Nitrate Elimination Co. from Michigan (USA). It is a recombinant nitrate reductase used in kits to test nitrates in water, soil, and animal feed. Normally, nitrate is tested through its reduction to nitrite via a copper-cadmium reagent, which is quite a toxic method (Campbell et al., 1997). Thus, an enzymatic analysis has been verified for accuracy, sensitivity and reliability showing minimum impact on people and providing reliable data (www.nitrate.com).

Production on crude glycerol

All mentioned enzymes were produced on purified glycerol in *P. pastoris*. However, there are many studies where crude glycerol was used for enzymes production, e.g. phytase, a very important industrial enzyme used as animal feed additive for swine, poultry, and fish (Xie et al., 2020). Production of recombinant phytase on crude glycerol was upscaled up to a 7.5 l bioreactor. Enzyme activity and biomass concentration reached 137.8 U/ml, and 146 g/L, respectively. Maximum specific growth rate was higher when using crude glycerol than glucose (0.266 h^{-1} vs. 0.203 h^{-1}) as the carbon source; final enzyme activity obtained with glucose achieved 121.6 U/ml (Tang et al., 2009). This proves that crude glycerol does not negatively affect *P. pastoris* growth or recombinant protein expression, which is its advantage over other microorganisms.

Conclusion

High-level expression, growth to high cell densities and post-translation modifications prove that *P. pastoris* is a suitable microorganism for recombinant enzyme production. Given all available data of successfully produced enzymes, *P. pastoris* has also great potential in the environmental sector. Products of *P. pastoris* were able to degrade pesticides or replace them with more environmentally friendly variants, degrade phenol compounds, synthetic dyes, hydrocarbons, antibiotic residues, lignin, nitroaromatics, toluene, bioplastics, and toxic compounds like cyanide. Its products were used also for kraft pulp bleaching, detection of pollutants and as alternative medical treatment

Tab. 2. Summary of enzymes produced by *P. pastoris* with positive effect on environment.

Product	Environmental treatment	Reference
Pexiganan	replacement of pesticides	Neshani et al., 2019
Organophosphorus hydrolase	biodegradation of organophosphorus pesticides	Shen et al., 2016
Kpkt	β -1,3glucanase and killer activity against spoilage yeast in wine and sweet beverage industry	Chessa et al., 2017
Laccase	degradation of synthetic dyes, lignin, nitrophenol, antibiotics and phenolic pollutants	Zhou et al., 2018; Colao et al., 2006
Manganese peroxidase	degradation of lignin, cellulose, hemicellulose, polycyclic aromatic hydrocarbons, chlorophenols, synthetic dyes, nitroaromatics, tetracyclines, oxytetracyclines, bleaching of pulp	Xu et al., 2017; Xu et al., 2010
Lignin peroxidase	degradation of lignin	Wei & Xianghua, 2009
Xylanase	conversion of lignocellulosic material and agrowaste to high-value products, paper pulping and bleaching	Ma et al., 2019
Phytase	helps to digest phytate which prevent phosphorus pollution	Tang et al., 2009
Nitrate reductase	test nitrates in water	www.nitrate.com
Cyanid hydratase	detoxification of cyanogenic glycosides, hydrolysis of cyanide	Wu et al., 2012
Horseradish peroxidase	degradation of aromatic amines, phenolic compounds, coal conversion wastewater, bleach plant effluent	Morawski et al., 2000
Polyhydroxyalkanoat depolymerase	biodegradation of polyhydroxyalkonotes	Phithakrotchanakoon et al., 2009
Bm86 antigen	vaccine against <i>Boophilus microplus</i>	Canales et al., 1997
Keratinase	improvement of nutritional value of feather meal and alleviate the environmental impact associated with its processing or disposal	Porres et al., 2002
AHL-lactonase	control of <i>Aeromonas Hydrophila</i>	Chen et al., 2010
Nitril hydratase	conversion of acrylonitrile and acrylamide	Shi et al., 2004
Azoreductase*	degradation of azo dyes	so far not produced by <i>P. pastoris</i>
Cyanidase*	treatment of cyanide	so far not produced by <i>P. pastoris</i>
Lactoperoxidase*	degradation of chlorophenols	so far not produced by <i>P. pastoris</i>
Polyphenol oxidase*	degradation of phenol compounds	so far not produced by <i>P. pastoris</i>

(Tab. 2). Due to many advantages of recombinant enzyme production, genetically engineered *P. pastoris* could completely replace the native sources of these enzymes. In addition, to enhance its positive environmental impact even more, combination of *P. pastoris* cultivation on crude glycerol, a biodiesel side product, with production of enzymes for environment treatment can be employed.

All these successful attempts point to the potential of *P. pastoris* production of other enzymes suitable for environmental treatment (Tab. 2*).

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