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PRODUCTION OF THE LACCASE BY PLEUROTUS OSTREATUS:A REVIEW

ESHA DWIVEDI[1*], LALIT KUMAR SINGH[2]

[1,2]Department of Biochemical Engineering, School of Chemical Technology, Harcourt Butler Technical University Kanpur, India

ABSTRACT

The laccase is an important enzyme for the industry. The strain Pleurotus ostreatus which is a white rot fungus produces the maximum amount of the laccase. The supernatant of the fungus Pleurotus ostreatus detected the ligninolytic activity of the laccase.

Keywords:

Laccase,fermentation,lignolytic enzyme,lignin

1)INTRODUCTION:

1.1 Structure of Laccase:

Laccase, a lignolytic enzyme is basically multi-copper protein and its EC no. is 1.10.3.2, belongs to oxidoreductase. The culture taken was solid and the coconut shell was employed as a substrate. The addition of the yeast extract followed by adding the copper salts, the amount of the laccase was produced. The ability of laccases to catalyse the reactions without additional cofactors, they are also useful in manufacturing of biosensors for the detection of morphine, codeine and catechol amines and for electro-immunoassay. Poly aromatic hydrocarbons (PAHs) were also found to degrade by laccases. Other applications include: the degradation of the herbicides, insecticides and the reduction of 2, 4, and 6- trinitrotoluene (TNT).

Laccases (benzenediol) catalyzes the reduction of O_2 to water and oxidizes substrate like di-phenol. Yoshida (1883) initiated the studies on Laccase [1]. The study was started from the 'sap of the Japanese lacquer tree 'Rhus vernicifera [1] The mechanism of laccase action is suggested as, single-electron (e^-) oxidation of various substrate molecules such as di-phenols, methoxy-substituted mono-phenols and aromatic as well as aliphatic amines associated with the 4-electron (e^-)reduction of O_2, molecular oxygen to water [2]. Laccase is broadly distributed in the nature. It is reported in microorganisms and in plants/ insects also. The purposeful laccases are found from fungal origin [3].

The most laccases are found and investigated on the basis of the lignin degradation. Basidiomycetes, mainly white rot fungi viz. Pleurotus ostreatus and Trametes versicolor have explored by the researchers because of their ability to mineralize lignin by releasing the oxidative enzymes, such as the peroxidases and the laccases. However, its biological function is still not clear [4].

This exclusive ability can be used to differentiate laccases action from the other enzymes like PPO (polyphenol oxidases) categories e.g. Monophenol monooxygenases or Tyrosinases (E.C.1.14.18.1), Catechol oxidases (E.C.1.10.3.1) [5]. Characterization of Laccases maybe done on the basis of types of 'copper prosthetic groups' which shows difference in their light absorbance capacity with different electron paramagnetic resonance signal [6]. First kind is T1 (Type-1) copper, with absorbance (A_{max}) at 600 nm and it is the first oxidation site. The blue color of the enzyme appeared due to the absorption of the light Cu-cysteine covalent bond of the T1 copper. The other types are T2 (Type-2) and the T3 (Type-3) copper form a tri-nuclear group, responsible for the reduction of O_2. Though the T2 is electron paramagnetic resonance active but it showed low visible light absorption. The maximum absorbance by T3 copper was found at the 330 nm band [7]. Witayakran et al 2009 showed the two mechanisms as per the enzymatic properties of the laccase Cu-centers. In the first mechanism the substrate is reducing and the e^- is accepted by the T1 copper and is thus reduced. Then the transfer of the e^- from to the tri-nuclear T2 or T3 cluster. At the final stage the molecular oxygen is reduced to the water. In the second possible mechanism of the 4-e^- reduction of the O_2 molecule to the H_2O at the Cu-sites of laccase [8].

The industrial oxidation reactions are the major reasons for various environmental problems. This leads to researchers to make use of the alternate biological systems. The biological reactions using enzymes are explored to circumvent various environmental threats like the undesirable side reactions and the generation of the hazardous/toxic chemicals [9]. The 4-hydroxy-3, 5-dimethoxy-benzaldehyde azine (Syringaldazine) is explored and found suitable as the laccase explicit substrate [10]. Special attention in the use of laccases has been increased in recent times because of their possible use in the modification/bioremediation of pollutants/toxics and phenolic compounds [11]. The thrust areas of laccase utilization which are the most extensively studied by the researches include textile dyes bleaching, pulp de-lignification [12], effluent clarification/detoxification, modification to detergents and biopolymer for improved applications [13]. The active site of the enzyme laccase with relative orientation and distances of the Cu atoms is shown in Fig. 1.1.

1.2 Microorganisms:

The laccase enzyme produced by the various 'white-rot fungi'(WRF) such as *Phellinus pini*, *Ceriporiopsis subvermispora*, *Phlebia* sp., and *Pleurotus ostreatus* are found to degrade lignin more easily and preferentially over the cellulose and hemicellulose biopolymers of the plants. On the other hand many other WRF, such as *T.versicolor*, *H. annosum*and *I. lacteus,* demonstrated an outline of concurrent decay of the other cell wall components. Diamantidis et al. 2000 reported the first prokaryotic laccase produced by the bacterium *Azospirillum lipoferum* found in soil [5]. The laccase thus found was a hetero-trimer enzyme analogous to a catalytic molecule with one or two larger chains. The extensively studied bacterial laccase till date are *Bacillus subtilis* and *Streptomyces griseus* [14].

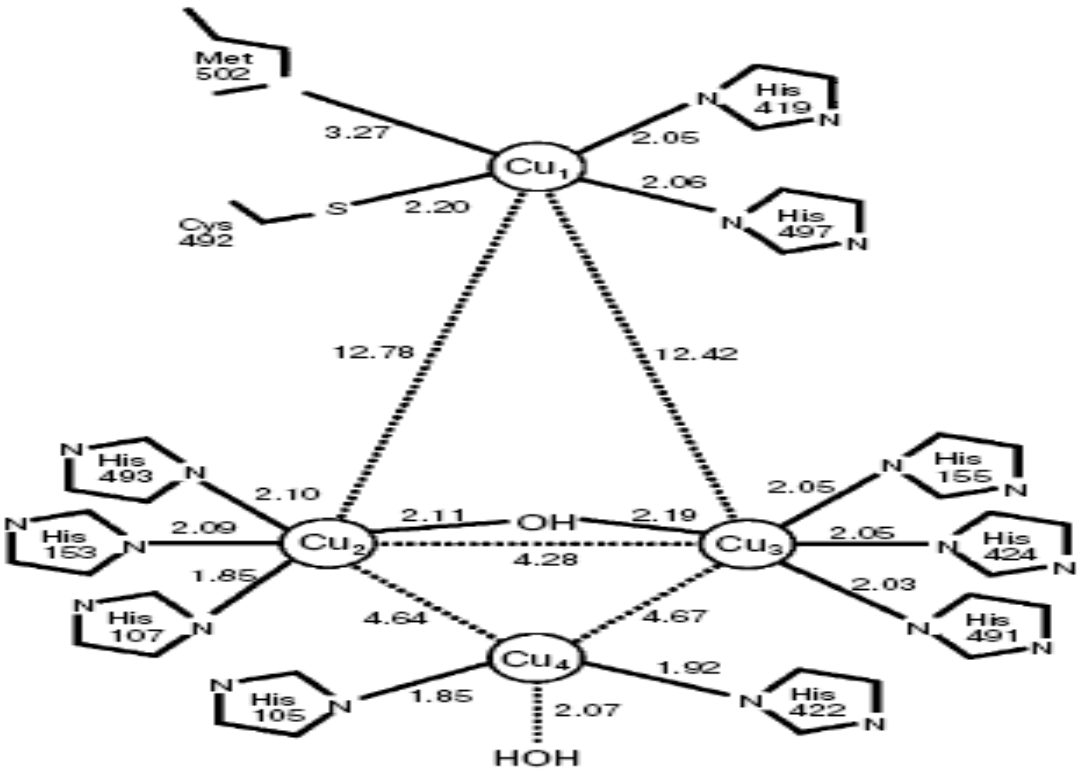

Fig1.1 The relative orientation and distances of the Cu atoms of active site of laccase.

Laccases are used mainly to degrade lignin, a plant heteropolymer made up of many phenolic units. However industrial application of the laccases include: in making of the biofuel cell (in the form of oxygen cathode), bio-bleaching of Kraft pulp, de-colorization of synthetic dyes, organic synthesis, laundary cleaning, bioremediation, biosensors, labeling in immunoassays, drug analysis, clarification of juices and wines, design of laccase fungicidal and bactericidal preparations [15].The development on laccases research has been increased in last decade, focusing mainly on their catalytic adaptability and oxidative reactivity in the absence of any other reactive compounds like peroxidases. Furthermore, the opportunity to clone laccases and to increase the range of oxidative reaction via redox-mediators reactions give substantial biotechnological applications. Laccases are mostly extracellular in nature. These come in the category of 'Glycoproteins', also these are multinuclear enzymes [16]. The molecular weight of laccases ranges from 60 to 80 kDa [16]. The laccase molecule is reported as an active holo-enzyme and found in a di-meric

or tetra-meric form. It is typically containing 4-Cu atoms / monomer which are attached to the 3-redox sites viz. T1, T2, and T3 Cu. The mono-nuclear Cu centre contains one T1 Cu atom that is trigonally coordinated to two histidine molecules and a cysteine molecule. The tri-nuclear clusters contain one T2Cu atom and a pair of T3 Cu atom. The T2 Cu is organized by two and the T3 Cu atoms by 6 conserved Histidine molecules. The T1 and T2 Cu atoms are reported as paramagnetic and can be identified in 'Electron Paramagnetic Resonance' (EPR) spectrum. The T3 Cu is anti-ferromagnetically coupled by bridging a hydroxide. Laccase catalysis comprises three major steps “Type 1(T1) Cu reduction by the reducing substrate, internal electron transfer from Type1 Cu to Type2 (T2) and Type3 (T3) Cu tri-nuclear cluster at a distance of 12.5 A and O_2 reduction to water at T2 and T3 Cu centre”. Laccases are basically a green catalyst and they produce H_2O as the only by-product in the presence of air. Therefore, laccases are known as “ecofriendly” enzyme. The growth of WRF, White rot fungi is shown in Fig. 1.2.

Fig 1.2: Photograph of White rot fungi.

Laccases are very much used for the biotransformation of various toxic molecules. These include aromatic compounds e.g. dyes and other aromatic toxic chemicals for waste water. Laccase Mediator System (LMS) has been used as ‘Lignozyme’ process for bio-bleaching of pulp, to activate the fiber-bound lignin during manufacturing of the composites, giving good mechanical properties without the use toxic synthetic adhesives. Poly aromatic hydrocarbons (PAHs) were also found to degrade by laccases. Other applications include: the degradation of herbicides, insecticides and the reduction of 2, 4, and 6- trinitrotoluene (TNT). The laccases also found important role in Cosmetic and dermatological preparations where proteins for skin lightening have been developed.

1.3 Production

A white-rot fungi *Pleurotus ostreatus* is extensively studied. It was found that this strain produces extracellular enzymes viz. laccase and manganese peroxidase. It does not produce lignin peroxidase. For production of laccases, various agro industrial residues/waste such as orange peel, sugarcane bagasse, corn stalk, tea, and peanut shells have been explored as substrate. The fermentation models were suggested for laccase production. The effects of agro industrial waste, copper induction, and agitation on the fermentation kinetics were studied. There is some literature available on laccase production from bacteria also. The fermentation method demonstrated, used conventional fermentation technique or statistical methods using experimental designs. Enhancement of laccase production in fungi has been investigated by many researchers in respect of fermentation media. The major difference between bacterial laccases and fungal laccases is based on the degree of thermo-stability of the enzyme. The laccase from *S. lavendulae* showed high thermo-resistance and the laccase from *B. subtilis* has a half-life of inactivation at 80^0C of about 4 h and 2 h. *Thermus thermophilus* produced thermophilic laccase which has the optimal reaction temperature of 92^0C [11].

The microbial strain selected for the laccase production was *Pleurotus ostreatus*, which is well documented in the existing literature for its ability to produce good amount of laccase.

2. REVIEW OF LITERATURE

Laccases also known as eco-friendly catalyst is a group of oxidative enzymes. These enzymes belong to oxidoreductases category of enzymes. These enzymes have significant potential to reduce or replace chemical load to the environment. Wide range of applications of laccases is available such as industrial waste water treatment generated by various industries like textile, paper and pulp. Laccase are also used in various food processing industries and bioremediation processes. Due to their wide substrate acceptability, laccases have enormous biotechnological prospective and applications include pulp delignification, textile effluent detoxification and dye-bleaching. Laccases are used in the removal of aromatic alcohols (phenolics) from product like wine and bio-transformation of medicinal compounds like antibiotics and steroids. Also, the enzymatic properties of laccases have an important role on the development of bio-sensors for detection of important pollutants and other clinically important metabolites. Lignin is found in plants and known as cementing material that binds cellulose and hemicellulosic fractions of lignocellulosic material. The composition of various plant material (lignocelluloses) is given in Table 2.1. These lignocellulosic materials that can be utilized for the laccase production.

Table 2.1: Various lignocellulosic materials with their composition.

Lignocellulosic Materials	Cellulosic fraction (%)	Hemicellulosic fraction (%)	Lignin fraction (%)	References
Hardwood stems	40-55	24-40	18-25	[17]
Softwood stems	45-50	25-35	25-35	[17]
Barley bran	23	27	21	[18]
Corn cobs	32-45	35	15-20	[19]
Grasses	25-40	25-40	10-30	[17]
Wheat straw	31	29	18	[19]
Sweet sorghum	23	14	11	[20]
Newspaper	40-55	25-40	18-30	[21]
Sugarcane Bagasse	50	25	25	[22]
Switch grass	37	29	19	[23]
Rice straw	43.5	22	17.2	[24]

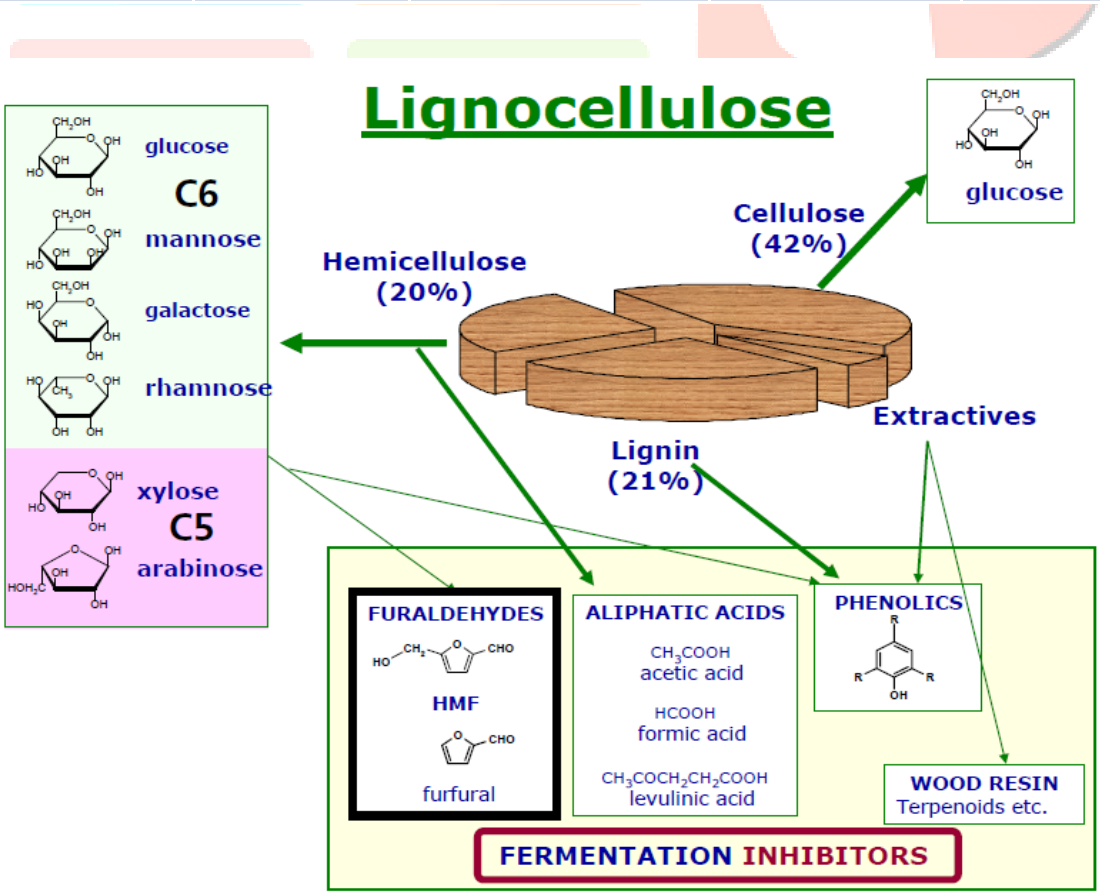

Fig.2.1 lignocellulosic biomaterials: their composition and inhibitors generated inhibitors during hydrolysis

The lignocellulosic biomaterials used for generation of various useful monomers like glucose, xylose etc. undergone harsh conditions of chemical and physical conditions, which leads to generation of toxic chemicals. Various monomers generated during the hydrolysis of lignocellulosic biomaterials is shown in Fig. 2.1. These toxics leads to inhibit the growth of microorganisms during fermentation. These toxics includes the monomers generated from the degradation of lignin i.e. phenolic or aromatic cyclic compounds. Thus, to remove these chemicals from the hydrolysate is essentially required before going for the fermentation. Laccases are known as biological degrader of lignin.

2.1 Lignin

Lignin is stable polymer of various monomeric aromatic compounds. The structure of Lignin is very complex and it is heterogeneous in nature. Different monomeric compounds are connected via C-C bonds and ether-bonds. The complex structure of lignin is shown in Fig. 2.2. Due to the similarity between the lignin structure and the chemical structure of several dye compounds, use of WRF (white-rot fungi) and enzymes produced by them for the decomposition of dye compounds have been investigated (Zhou and Zimmermann 1993).

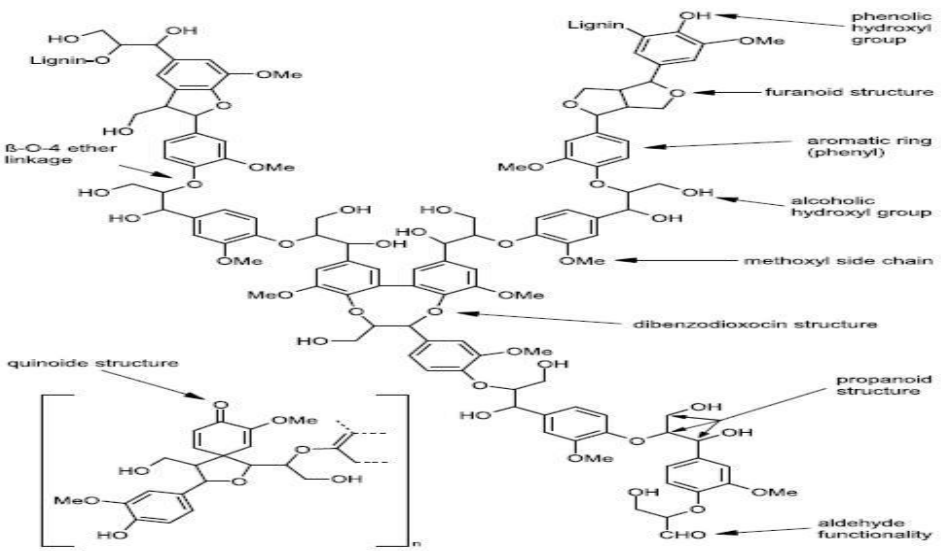

Fig. 2.2 Structural illustration of lignin biopolymer found in lignocellulosic biomaterials [25]

The catalytic reaction (direct and indirect oxidation) of laccase is shown in Fig. 2.3.

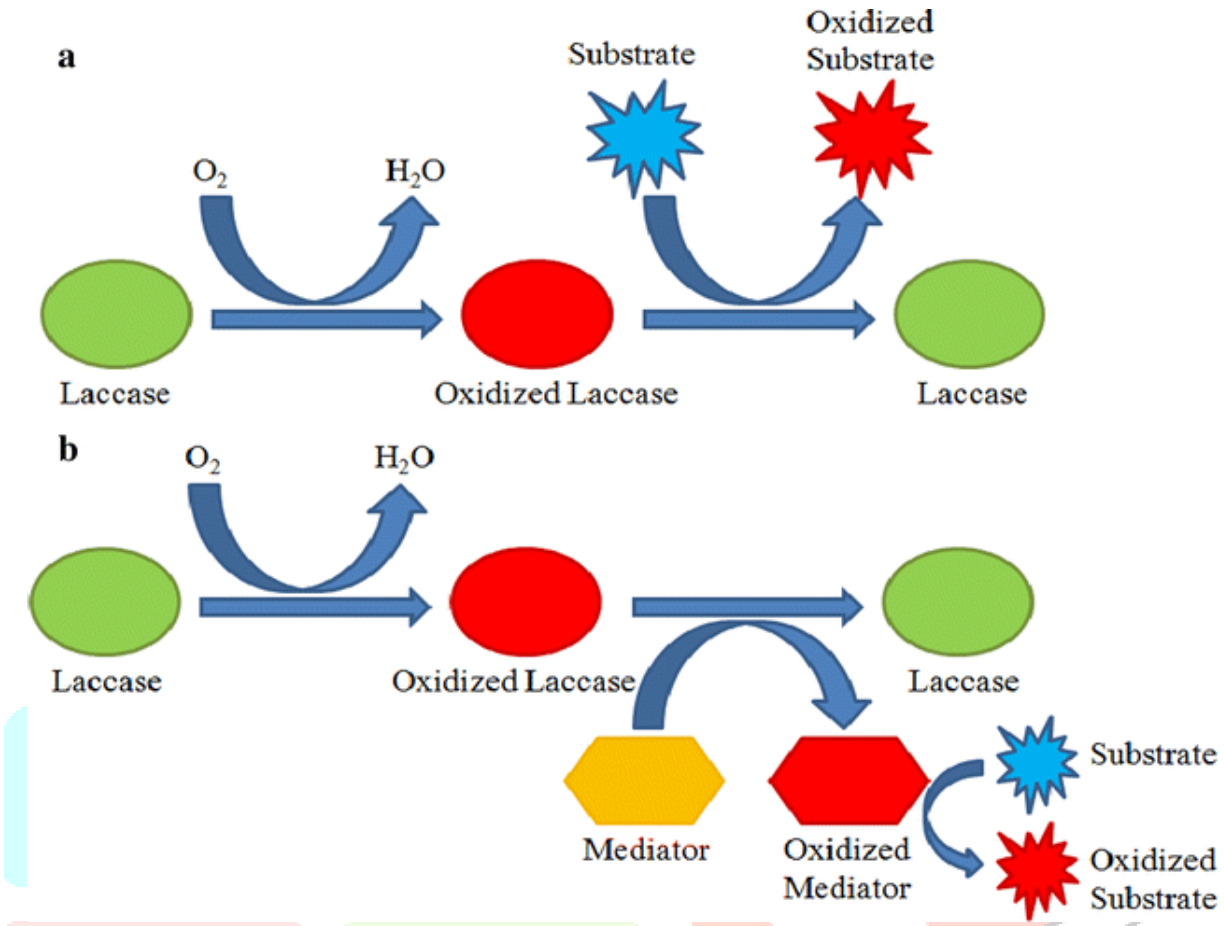

Fig.2.3. Graphical representation of(a) Direct oxidation: and (b) In-direct oxidation by laccase

2.2 Micro-organisms:

Fungal laccases

The laccases produced by fungi are identified so far are particularly from WRF *basidiomycetes.* These organisms have good property of lignin degradation. Various fungus strains used for laccase production is given in Table 2.2.

Table 2.2: Important fungal strains used for production of laccases.

S. No.	Fungi	Type of fermentation	Laccase Activity (U/L)	Reference
1	*P.cinnabarinus*	SmF	280	[26]
2	*T.pubescens*	SmF	333,000	[27]
3	*N.crassa*	SmF	10,000	[28]
4	*T.versicolor*	SSF	229	[29]
5	*T.versicolor*	SSF	3500	[29]
6	*T.hirsuta*	SSF	18,715	[29]

Bacterial Laccases

Laccases found in bacterial species are intracellular and as peri-plasmic protoplast [6]. The first bacterial laccase was found in *Azospirillum lipoferum*: Bacterium associated with plant root.

Table 2.3 Important bacterial strains used for production of laccases.

S. no.	Name of organism	Substrate used in enzyme assay	Reference
1	*Aquisalibacillus elongatus*	DMP	[30]
2	*Bacillus subtilis* MTCC 1039	Guaiacol	[31]
3	*Bacillus sp.* WT	ABTS, SGZ	[32]
4	*P. extremorientalis* BU118	DMP	[33]
5	*Streptomyces bikiniensis* CSC12	SGZ	[34]
6	*Bacillus subtilis* MTCC 2414	Guaiacol	[35]
7	*Bacillus cereus TSS1*	Guaiacol	[36]
8	*Bacillus tequilensis* SN4 MTCC 11828	DMP	[37]
9	*Bacillus safenis* DSKK5	NR	[11]
10	*G. thermocatenulatus* MS5	ABTS	[38]
11	*Pseudomonas aeruginosa*	ABTS	[39]
12	*Streptomycetes sp.*	ABTS	[40]
13	*Streptomycetes cyaneus*	ABTS	[41]
14	*Bacillus subtilis* WPI	ABTS	[42]

2.3 Fermentation techniques

Laccase production may be done by using SSF or SmF. In the absence of free water microorganisms grow on the solid substrates. In SmF the microorganisms are cultivated in liquid media. In SSF, the appropriate moisture content is essentially required for microbial growth. The fermentation techniques used for production of enzymes are show in Fig.2.4.

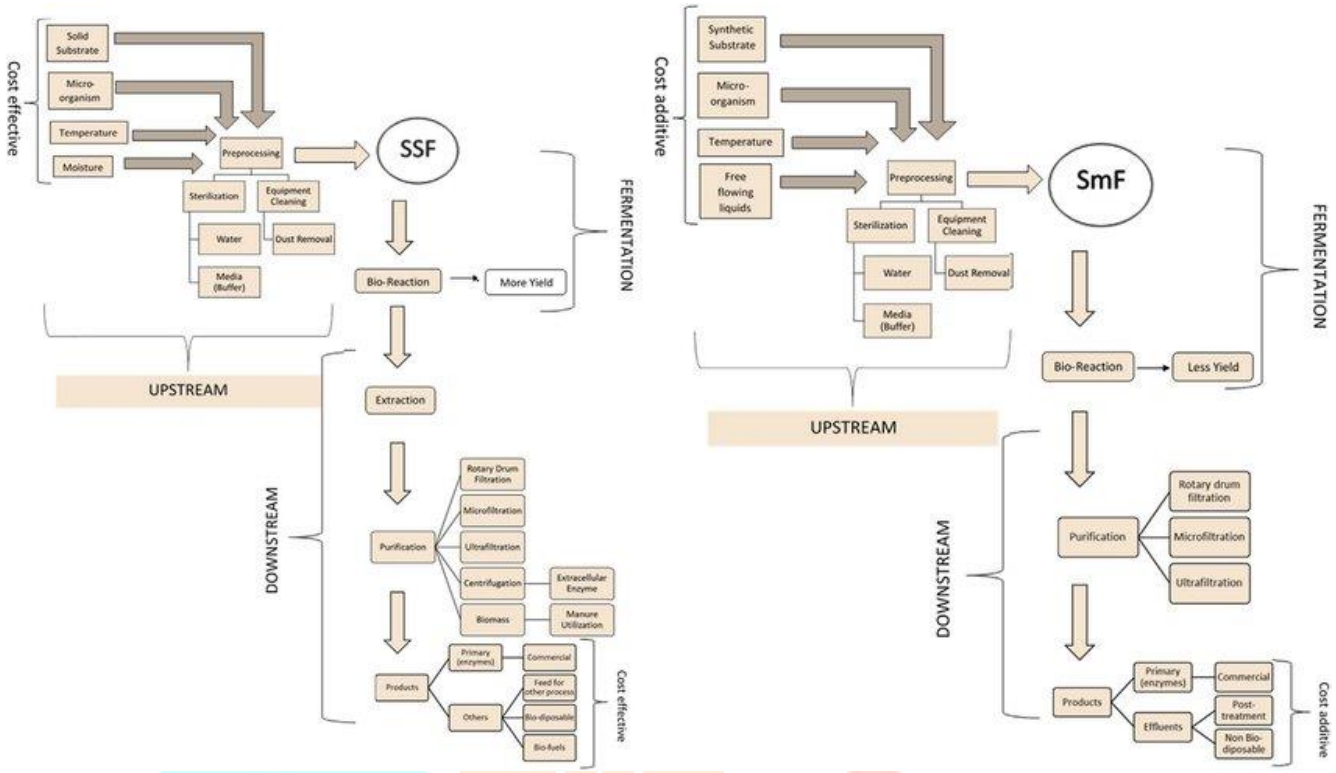

Fig.2.4 Fermentation techniques used for enzyme production.

There are many reports available for laccase production under Submerged fermentation technique. Reports reveled the various scales using various microorganisms with free and immobilized microbial cells. The role of induces for laccase production was also studied. The maximum production of laccase was found [27] (740,000 U/l) by *T. pubescens*. The submerged fermentation was carried out in 20-L stirred bioreactor. Cu^{++} was used as inducer. In the other research, [43] reported maximum laccase production (16,000 U/l) using free cells.

2.4 Laccase production under SmF and SSF:

The maximum laccase activity obtained by SSF or SmF using Inducer along with production conditions and characteristics are mentioned in Table 2.3 and in Table 2.4 while using white rot fungi and bacterial strains srespectively. .

Table 2.3. Laccase activities obtained by the cultivation of different white-rot fungi under SmF or SSF at flask and reactor scale.

S. No.	Fungal strain	Bioreactor	Type of fermentation	Inducer	Max Laccase activity [U/L]	Reference
1	*P.cinnabarinus*	Packed bed reactor (10L)	SmF (immobilized on nylon cubes)	10 mM VA	280	[26]
2	*N.crassa*	Capillary membrane	immobilized on membrane supports	1 M cyclo-heximide	10,000	[28]
3	*P.chrysosporium*	RDR	SSF(immobilized on nylon cubes)	0.05% Tween 80 and 2mM VA	56	[45]
4	*T.pubescens*	20-L STR (150 rpm)	SmF, free cells	2 mM Cu^{+2}	61,900	[27]
5	*C. hirsutus*	10-L Jar fermentor (160 rpm)	SmF, free cells, semi-continuous	0.25 g/L Cu^{+2}	83,830 (1st fermentor) 80,730 (2n fermentor)	[46]
6	*P.sanguineus*	15- L Biostat C (250 rpm)	SmF, free cells	16 mM VA	8131	[47]
7	*P.flavidoalba*	Bioflo III (70 rpm)	SmF, free cells	OMW	72	[48]
8	*P.sanguineus*	2-L Biostat C	SmF	16 mM VA	460	[47]
9	*T.multicolor*	STR	SmF	-	-	[49]
10	*T. pubescens*	20-L STR (100 rpm)	SmF, free cells	2 mM Cu^{+2}	333,000	[27]
11	*Irpex lacteus*	Packed-bed (27 mL)	SmF, immobilized on PW	-	-	[50]
12	*I. lacteus*	Packed-bed (27 mL) 3-L STR (2 L; 250 rpm)	immobilized on PUF SmF, free cells	-	-	[50]
13	*Panus tigrinus*	20-L RDR	SSF (maize stalks)	OMW	1309	[51]
14	*Pleurotus ostreatus*	Bench-top fermenter (3 L; 200 rpm)	SmF, free cells	OMW	65	[52]

Table 2.4 Production conditions and characteristics of bacterial laccase from different microorganisms

S. no.	Name of organism	Substrate used in enzyme assay	Temp. Optima of activity	Temp. stability	pH optima of activity	Reference
1	*A. elongatus*	DMP	40	[80%/25-55 LC/6 h	8.0	[30]
2	*Bacillus subtilis MTCC 1039*	Guaiacol	30	100%/30 LC/60 h	5.0	[31]
3	*Bacillus sp. WT*	ABTS /SGZ	37	[100%/70 LC/ 90 min	8.0	[32]
4	*P. extremorientalis BU118*	DMP	40–50	NR	8.0	[33]
5	*S. Bikiniensis CSC12*	SGZ	6-7	NR	50–60	[34]
6	*B. subtilis MTCC 2414*	Guaiacol	30–40	NR	7.0	[35]
7	*B. subtilis MTCC 2414*	Guaiacol	35	NR	9.0	[35]
8	*B. cereus TSS1*	Guaiacol	37	NR	7.0	[36]
9	*P. luridastrain LR5.1*	ABTS	32	NR	NR	[37]
10	*B. tequilensis* SN4 MTCC 11828	DMP	85	100% /65 LC/24 h	8.0	[39]
11	*B. safenis* DSKK5	NR	37	NR	6.2	[40]
12	*Pseudomonas aeruginosa*	ABTS	37	NR	4.5	[41]
13	*B. subtilis* WPI	ABTS	25	NR	NR	[42]

2.5. Application of fungal laccase

Some of the important industrial applications are shown in Fig. 2.5. Also some other industrial applications are mentioned in Table 2.5. The limitations of laccase research work done till date with possible solutions are given in Fig. 2.6.

Fig. 2.5 Important industrial applications of laccases**.**

Laccase Producing Organism	**Application**	**Reference**
Trametesversicolor	Filtration aid	[54]
Trametesversicolor	Wine- stabilization	[55]
Myceliophthorathermophilia	Dough- conditioner	[56]
Rhizoctoniapraticola	Phenolic compound removal	[57]
Trametesversicolor; Rhizoctoniapraticola	Soil decontamination	[57]
Coriolopsisgallica	Beer factory waste water	[58]
Trametes sp.	Distillery waste water	[58]
Trametesversicolor; Pleurotusostreatus	Olive Mill wastewaters	[58]
Trameteshirsuta	Dough Conditioner	[59]

Table 2.5: Applications of laccases in various industries.

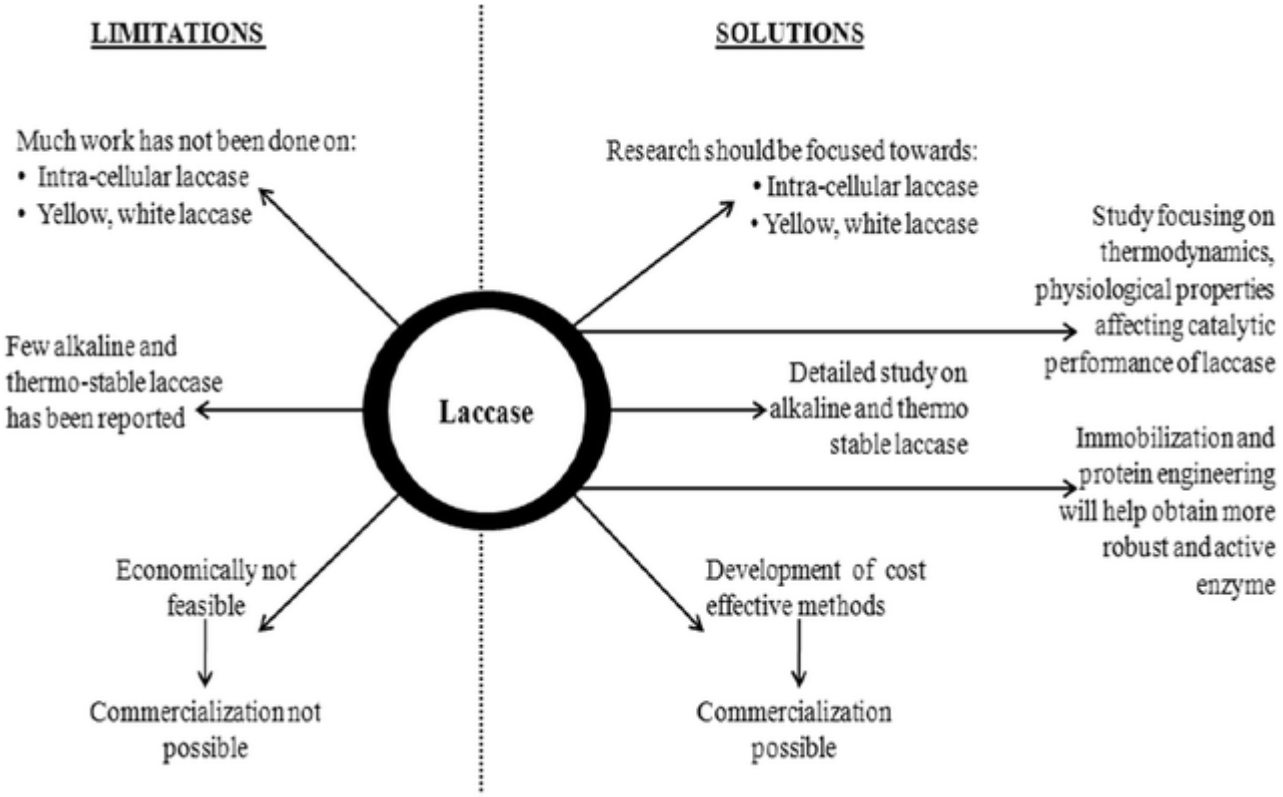

Fig. 2.6 Limitations and probable solutions for laccase production and utilization.

maintained with liquid medium and inoculums addition.

3)CONCLUSIONS:

The coconut shell as lignin containing substrate was successfully used to produce laccase under SSF. The constitution and the configuration of the culture, carbon to nitrogen ratio determines the production of the laccase. The laccase stabilization was improved in the co-cultures. Further research programs should focus on large-scale laccase production and applications. Future work on identification of inhibitors and knowledge of their behavior will allow us to develop better strategies to maintain long term enzyme stability.

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