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DEVELOPMENT OF LIQUID STATE FERMENTATION PROCESS FOR THE PRODUCTION OF LIGNINOLYTIC ENZYMES BY CORIOLUS VERSICOLOR IBL-04 USING LIGNIN CONTAINING SUBSTRATE

Erum Moqueem[1], M. Asgher[1], Naveed Munir[2,3*], Imtiaz Mahmood Tahir[3] and Muhammad Riaz[4]

Abstract: Increasing popularity of white rot fungi (WRF) in the field of biotechnology and its application in various industries formed the rationale of designing the study. The primary objective was to find the optimized culture conditions under Liquid state fermentation (LSF) process usingindigenous strain of white rot fungi, Coriolus versicolor IBL-04. Corn stover, rice straw, corncobs, wheat straw, sugarcane Bagasse and banana stalk as lignin containing substrates were used for the production of ligninolytic enzymes by LSF. The medium containing the sugarcane Bagasse as substrate showed the maximum activity of lignolytic enzymes including MnP (377± 2.7 IU/ mL), LiP (289± 1.5IU/mL) and Laccase (381 ± 4.2IU/mL) on 5th day of incubation at 35°C. During the second phase of present study, various fermentation parameters both physicals and nutritionals were optimized to enhance the activities of ligninases. It was noted that media of pH
4.5 with 3 mL inoculums size, 2g of substrate, molasses as C source, beef extract as N source at the C: N ratio of 20:1, MnSO4 among various mediators and CuSO4 among various metal ions showed significantly ($p<0.05$) high activities of MnP (641±7.6), LiP (488±5.9) and Laccase (675±9.1) fatty acids (omega-3 and omega-6) present in seed mixture.

Keywords: Lignin, White rot fungus, ligninases, fermentation, optimization

1 Department of Biochemistry, University of Agriculture, Faisalabad-Pakistan
2 Department of Biochemistry, Govt. College University, Faisalabad- Pakistan
3 College of Allied Health Professional, Government College University, Faisalabad-Pakistan
4 Department of Allied Health Sciences, Sarghoda Medical College, University of Sarghoda,Sarghoda-Pakistan
Corresponding author email: naveedmunir215@gmail.com

INTRODUCTION

As biological raw material for renewable energy and industrial products wood has been used for millennia by human societies and it is among one most abundant world's raw material. The physical and chemical characteristics as well as constituents of energy of wood are primary determined by the secondary cell wall composition and structure (1). Ligno-cellulosic material including hemicellulose, cellulose and lignin made the plant cell wall and lignocellulosic material constituted approximately 95% of plant biomass. Cellulose made major part of lignocellulosic material in plants about 45% while hemicellulose and lignin consisted approximately 25-30% and 25% respectively (2). Lignin as organic material present in the secondary plant wall and middle lamella of higher plants is the supreme on earth next to cellulose (3). Lignin is a structurally complex aromatic heteropolymer (4), optically inactive with water non soluble property having three dimensional amorphous heteropolymer and highly branched structure. As lignin heteropolymer which is substituted of phenylpropane units that is a benzene ring with a tail of three carbons linked by various types of linkages (5) and this is generated on the production of free radicals, synthesized during the peroxidase-mediated dehydrogenation of three phenyl propionic alcohols: the coniferyl alcohol, hydroxycinnamyl alcohols (or monolignols) and sinapyl alcohol, and with characteristically low concenteration of p-coumaryl alcohol (6). In result of the polymerization a heterogeneous structure in which basic structural units are joined by C-C and aryl-ether linkages, with arylglycerol-β-aryl ether (β-O-4) being the principal structure (7).

Degradation of lignin is well-thought-out the rate-limiting steps in recycling of carbon on the earth because the degradation of this structurally complex aromatic biopolymer is very difficult and only a few microbes are able to decompose this complex structure (8). Lignolytic enzymes including manganese peroxidase (MnP), lignin peroxidase (LiP) and Laccase are extracellular, unspecific and oxidative enzymes, and specifically catalyze the depolymerization of lignin because these have potentially able to release the highly unstable products and further undergo many different oxidative reactions. The initial step involved in the degradation of lignin may be the cleavage of Cα- Cβ side chain bonds in lignin structure (2).

White-rot fungi (WRF) are a group of fungus and encompass a heterogeneous collection of several hundreds of species of basidiomycetes. Basidiomycetes destroy the white rots and have significant ability to depolymerize the lignin present in the secondary wall of woody plants as well as are able to degrade broad range of recalcitrant organic pollutants (9). The degradation of lignin by WRF has been broadly studied, and it is well reported that lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) are the enzymes responsible for the decomposition of plant lignin (9-10).

Trametes (Coriolus) versicolor is one of the WRF having ability to decompose the lignin in woody plants and so considered as valued fungus in biotechnology for the degradation of lignin as well as to decompose other phenolic compounds. It was found that the capability of this fungus to degrading the lignin is due to its ability to secrete extracellular oxidative enzymes which potentially initiate the decomposition of complex lignin structure which is the highly complex aromatic polymer with non-hydrolysable property (11).

MATERIAL AND METHOD:

PLACE OF WORK:

All the research work was conducted in IBL (Industrial Biotechnology Laboratory), Department of Biochemistry, University of Agriculture Faisalabad, Pakistan.

Fermentative organism:

The pure culture of indigenous strain C. versicolor IBL-04 available in Industrial Biotechnology Laboratory (IBL), Department of Biochemistry, University of Agriculture, Faisalabad (UAF) was used as fermentative organism for ligninases production using LSF.

Lignocellulosic-wastes Collection and Pretreatment:

Lignocellulosic substrates including wheat straw, rice straw, sugarcane bagasse, banana stalk, corn cobs and corn stover were used as substrates for the production of ligninase enzymes by C. versicolor IBL-04. These substrates were collected from different local places of Faisalabad like rice straw and wheat straw were

obtained from Student Research Farms, UAF, Pakistan. Banana stalks and sugar cane bagasse were provided by local fruit market, Ghulam Muhammad Abad, Faisalabad and Crescent Sugar Mills, Faisalabad, respectively. Corn cobs and corn stover were available in CPC-Rafhan maize products, Faisalabad, Pakistan. After the collection of lignocellulosic substrates, they were cut into tiny pieces, dried in Sun, oven and milled to powder form of mesh size 40 mm and tightly packed to preserve from moisture.

LIQUID STATE FERMENTATION/ SCREENING:

Homogenous Coriolus versicolor IBL-04 spore suspension was prepared (3-5 days) using Kirk's basal nutrient medium. 1 % Glucose sterilized solution with millipore filter as supplement and 10 mL/L trace elements solution contain (CuSO4, Na2MoO4, MnSO4. H2O, ZnSO4.
H2O, Fe2(SO4)3 were added (12). Spore size of 1x106 to 1x108 spores/ mL was used for the production of ligninases (13). For fermentation, triplicate experiments were performed and flasks were inoculated for 1 to 10 days at 30°C under continuous shaking condition containing lignocellulosic residues. Samples were harvested after every 24hour for successive 10 days to select the best substrate. In the second phase of study, for optimization of LSF production process Completely Randomized Design (CRD) was used. After selecting the most suitable substrate for enzyme synthesis the different levels of independent variables such as pH (3.0, 3.5, 4.0,
4.5 and 5.0); temperature (25ºC, 30ºC, 35ºC, 40ºC and 45ºC); carbon (Glycerol, Starch, Glucose, Molasses and Wheat bran) and nitrogen (Yeast Extract, Urea, Corn Steep Liquor, Beef Extract and Peptone) sources; carbon: nitrogen ratio (10:1, 15:1, 20:1, 25:1,30:1); mediators (Oxalate, Veratryl alcohol (VA), H2O2, MnSO4, ABTS (2, 2'-azinobis-3 ethylbenzthiazoline-6-sulphonate); and metal ions (Cu2+, Ca2+, Fe2+, Zn2+, and K2+) were optimized to achieve the maximum yield of ligninases at 3mL inoculums size and2g of substrate.

Duncan's Multiple Range Test (DMR) was used to compare analysis of variance (ANOVA) and treatment means to evaluate statistically. The data values presented in tables are means ± Standard error (Mean ± S.E).

2.3. ANALYTICAL METHODS:

2.3.1. Enzymes Activities:

After stipulated period of time, the culture flasks were harvested. Whatman No. 1 filter papers were used to filter the contents of fermented flasks andfiltrates were further processed by centrifugation at 1000 x g for 10 min. The supernatants were assayed for ligninase enzymes, i.e. Tien and Kirk (1984) method was used to determine the activity of lignin peroxidase (LiP) using H2O2 dependent oxidation of veratryl alcohol to veratraldehyde at 25 °C (12). Wariishi et al. (1992) method was used to measure the activity of Manganese peroxidase (MnP) based on the manganic-malonate complex oxidation at 25°C using 270 nm wavelength (14). Extracellular laccase was measured spectrophotometerically by monitoring oxidation of 2, 2 azinobis 3-ethylbenzthiazoline 6-sulphonate (ABTS) and sodium malonate as buffer at 420 nm was used (15).

RESULTS:

Screening of Lignocellulosic Substrates:

For the screening of best lignocellulosic substrates Coriolus versicolor IBL-04 was cultured in medium containing 2gm lignocellulosic substrate dissolved in 100mL Kirks basal salt solution for the production of ligninase enzymes using LSF. After every 24 hours harvested flasks were analyzed for enzymes production for consecutive 10 days. Maximum production of MnP (377 ± 2.7 IU/mL), LiP (289 ± 1.5IU/mL) and Laccase (381
± 4.2IU/mL) on 5th day of incubation in medium containing sugarcane bagasse as substrate was observed (Table 1).

Table 1 Ligninase enzymes activities produced by Coriolus versicolor IBL-04 on different lignocellulosic substrates under LSF.

Table 1: Ligninase enzymes activities produced by Coriolus versicolor IBL-04 on different lignocellulosic substrates under LSF

Substrates (2gm/100ml)	Enzymes	Enzymes Activities (U/mL)						
		Incubation Time (Days)						
		1	2	3	4	5	6	7
Rice Straw	MnP	98 ± 2.8	90 ± 2.5	117 ± 1.5	166 ± 4.7	212 ± 2.8	220 ± 3.3	239 ± 3.4
	LiP	65 ± 1.5	75 ± 1.9	87 ± 1.1	96 ± 1.4	87 ± 2.2	94 ± 2.5	102 ± 2.2
	Lac	91 ± 1.7	98 ± 1.2	115 ± 1.3	145 ± 1.5	198 ± 2.4	266 ± 1.9	250 ± 1.8
Wheat Straw	MnP	77 ± 1.6	91 ± 2.1	98 ± 2.4	120 ± 2.8	134 ± 3.3	187 ± 3.4	221 ± 2.8
	LiP	57 ± 2.5	87 ± 1.2	51 ± 2.4	120 ± 3.1	140 ± 1.4	174 ± 1.5	196 ± 3.4
	Lac	101 ± 1.1	128 ± 1.8	172 ± 2.3	201 ± 2.3	241 ± 2.6	304 ± 3.4	359 ± 3.4
Sugarcane Bagasse	MnP	112 ± 2.0	188 ± 1.7	225 ± 2.6	202 ± 2.8	**377 ± 2.7**	345 ± 2.5	308 ± 1.8
	LiP	88 ± 0.7	149 ± 1.4	217 ± 1.6	223 ± 2.4	**289 ± 1.5**	276 ± 2.1	218 ± 2.8
	Lac	115 ± 1.3	188 ± 1.2	193 ± 2.4	266 ± 1.9	**381 ± 4.2**	312 ± 2.6	343 ± 2.4
Banana Stalk	MnP	92 ± 2.4	95 ± 2.6	112 ± 1.7	143 ± 1.6	175 ± 1.8	217 ± 3.1	199 ± 2.2
	LiP	88 ± 1.1	95 ± 1.5	110 ± 1.6	132 ± 3.2	188 ± 2.8	206 ± 2.8	212 ± 2.4
	Lac	109 ± 1.9	151 ± 1.6	121 ± 1.6	142 ± 2.1	196 ± 2.5	265 ± 2.6	214 ± 3.3
Corn Stover	MnP	91 ± 2.1	102 ± 1.9	127 ± 2.3	143 ± 1.4	157 ± 1.5	181 ± 3.1	134 ± 1.2
	LiP	67 ± 2.5	92 ± 1.6	97 ± 1.5	111 ± 1.6	125 ± 1.2	185 ± 1.2	150 ± 3.2
	Lac	93 ± 1.1	102 ± 1.8	172 ± 2.6	132 ± 3.3	145 ± 1.5	199 ± 2.1	175 ± 3.1
Corn Cobs	MnP	172 ± 1.5	143 ± 2.6	199 ± 2.2	238 ± 2.5	266 ± 2.9	249 ± 1.3	217 ± 1.5
	LiP	98 ± 1.2	121 ± 1.9	138 ± 4.1	123 ± 1.3	129 ± 2.2	174 ± 1.1	144 ± 2.1
	Lac	149 ± 1.4	161 ± 1.1	241 ± 2.2	303 ± 4.7	350 ± 2.6	380 ± 4.4	222 ± 2.8

Table 2 Manganese peroxidase (MnP), Lignin peroxidase (LiP) and Laccase (Lac) activities producedby Coriolus versicolor IBL-04 with varying pH and temperature values

pH	Enzymes	Enzyme Activities (U/mL)				
		Temperatures (°C)				
		20	25	30	35	40
3.0	MnP	117 ± 2.3	212 ± 3.2	220 ± 2.6	214 ± 2.9	101 ± 2.2
	LiP	87 ± 1.1	87 ± 1.6	102 ± 1.6	96 ± 1.3	112 ± 1.6
	Lac	115 ± 1.1	198 ± 4.6	250 ± 2.6	202 ± 1.9	144 ± 1.8
3.5	MnP	120 ± 2.6	187 ± 1.9	166 ± 1.9	108 ± 1.9	88 ± 3.4
	LiP	51 ± 1.6	120 ± 2.6	140 ± 2.5	174 ± 2.6	69 ± 1.8
	Lac	201 ± 2.2	172 ± 4.6	241 ± 2.2	255 ± 2.8	201 ± 2.1
4.0	MnP	188 ± 2.4	225 ± 3.2	202 ± 2.2	223 ± 4.2	135 ± 1.8
	LiP	149 ± 2.9	217 ± 2.8	223 ± 2.5	149 ± 1.3	121 ± 2.1
	Lac	115 ± 2.6	193 ± 2.9	266 ± 2.7	293 ± 2.2	133 ± 1.8
4.5	MnP	102 ± 1.6	157 ± 2.1	198 ± 1.7	**377 ± 3.1**	143 ± 1.9
	LiP	92 ± 1.2	125 ± 2.1	102 ± 3.4	**289 ± 3.2**	125 ± 2.8

	Lac	172 ± 3.4	199 ± 4.1	102 ± 1.9	**380 ± 3.3**	172 ± 1.9
5.0	MnP	238 ± 1.9	266 ± 2.8	249 ± 3.3	201 ± 2.6	119 ± 1.1
	LiP	123 ± 3.2	129 ± 1.9	174 ± 2.3	112 ± 1.9	92 ± 1.6
	Lac	303 ± 2.6	350 ± 4.6	222 ± 3.2	152 ± 1.8	158 ± 2.3

Table 3: Manganese peroxidase (MnP), Lignin peroxidase (LiP) and Laccase (Lac) produced by Coriolus versicolor IBL-04 with varying carbon and nitrogen sources

Carbon sources (1%)	Enzyme Activities (U/mL)					
	Enzymes	Nitrogen sources (0.2%)				
		Urea	Yeast extract	Peptone	Ammonium sulfate	Beef extract
Glucose	MnP	212 ± 2.2	250 ± 2.3	275 ± 3.1	290 ± 2.8	295 ± 4.3
	LiP	101 ± 1.1	195 ± 5.2	201 ± 4.2	275 ± 4.6	245 ± 2.9
	Lac	198 ± 2.8	202 ± 4.6	215 ± 2.8	250 ± 2.6	310 ± 4.2
Fructose	MnP	215 ± 2.6	238 ± 2.8	225 ± 3.2	188 ± 2.6	299 ± 2.2
	LiP	120 ± 1.8	198 ± 6.4	202 ± 4.6	257 ± 5.7	223 ± 6.3
	Lac	220 ± 3.5	265 ± 5.9	275 ± 2.6	300 ± 6.3	299 ± 2.9
Sucrose	MnP	238 ± 3.4	275 ± 2.5	287 ± 2.8	225 ± 3.5	315 ± 4.1
	LiP	270 ± 2.2	223 ± 3.2	295 ± 5.1	275 ± 5.6	211 ± 6.1
	Lac	310 ± 4.6	320 ± 5.7	333 ± 4.1	345 ± 2.6	350 ± 5.3
Molasses	MnP	299 ± 5.3	302 ± 4.2	243 ± 6.2	343 ± 3.7	420 ± 1.9
	LiP	288 ± 2.9	201 ± 2.6	255 ± 2.6	300 ± 2.6	325 ± 3.8
	Lac	375 ± 5.2	303 ± 5.6	379 ± 4.9	355 ± 5.2	475 ± 5.5
Maltose	MnP	315 ± 4.6	333 ± 4.3	299 ± 2.9	322 ± 4.6	310 ± 5.2
	LiP	212 ± 2.3	222 ± 2.2	265 ± 2.4	273 ± 3.6	250 ± 4.9
	Lac	299 ± 3.3	333 ± 3.8	355 ± 5.1	310 ± 5.9	395 ± 4.9

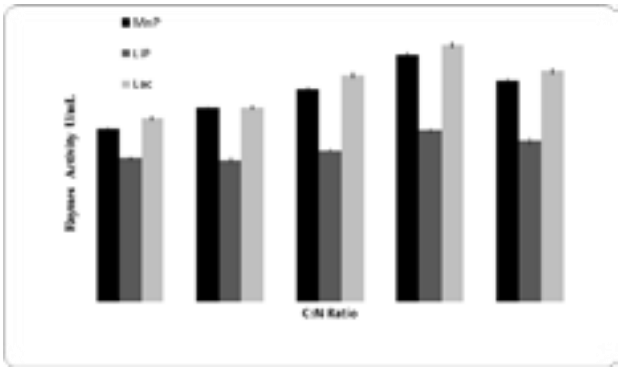

Figure 1: Activities of ligninases produced by *Coriolus versicolor* IBL-04 with varying C: N ratio

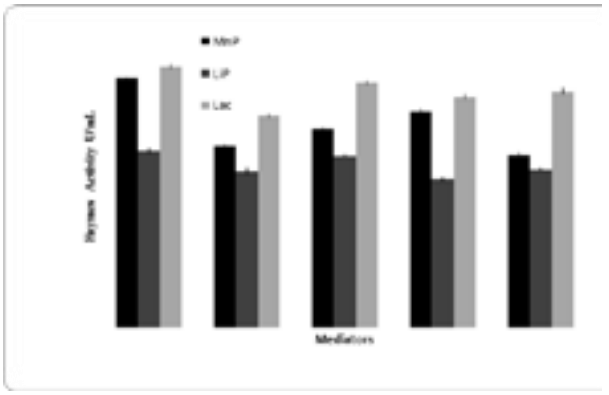

Figure 2: Activities of ligninases produced by *Coriolus versicolor* IBL-04 with varying mediators

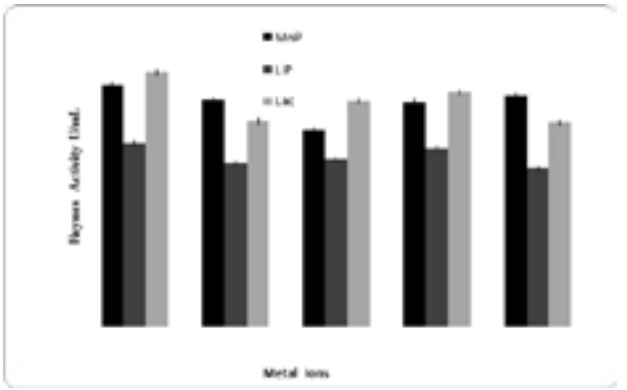

Figure 3: Ligninase enzymes activities produced by *Coriolus versicolor* IBL-04)with different metal ions)1*m*M

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