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Unravelling the capability of *Pyrenophora phaeocomes* S-1 for the production of ligno-hemicellulolytic enzyme cocktail and simultaneous bio-delignification of rice straw for enhanced enzymatic saccharification

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揭开*Pyrenophora phaeocomes* S-1产生木质－半纤维素分解酶混合物的能力
以及稻草秸秆的同步生物脱木质素用于增强的酶促糖化

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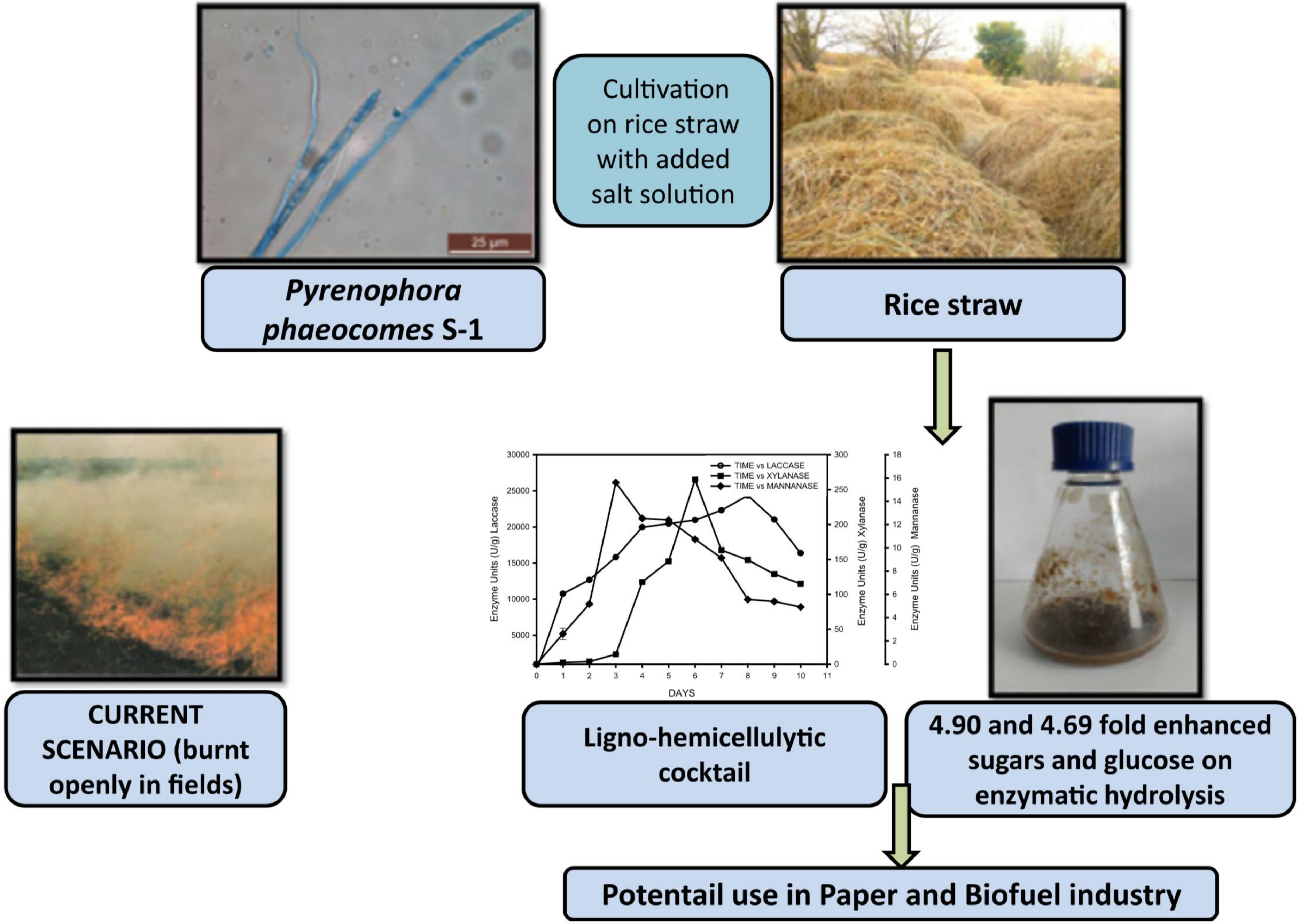

Cultivation on rice straw with added salt solution
25 µm
Pyrenophora phaeocomes S-1
Rice straw
TIME vs LACCASE
TIME vs XYLANASE
TIME vs MANNANASE
Enzyme Units (U/g) Laccase
Enzyme Units (U/g) Xylanase
Enzyme Units (U/g) Mannanase
DAYS
CURRENT SCENARIO (burnt openly in fields)
Ligno-hemicellulytic cocktail
4.90 and 4.69 fold enhanced sugars and glucose on enzymatic hydrolysis
Potentail use in Paper and Biofuel industry

章节

Part 01

Introduction

Introduction

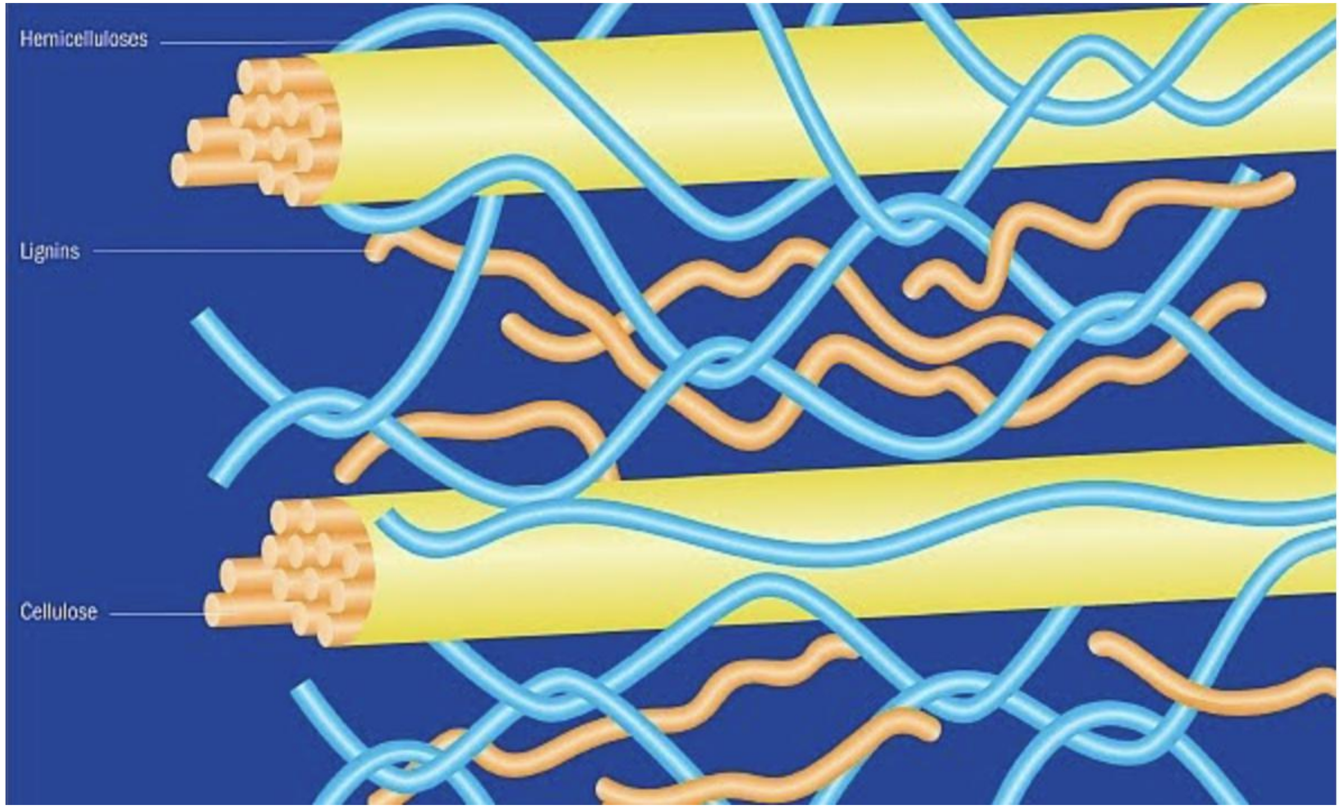
CURRENT SCENARIO (burnt openly in fields)

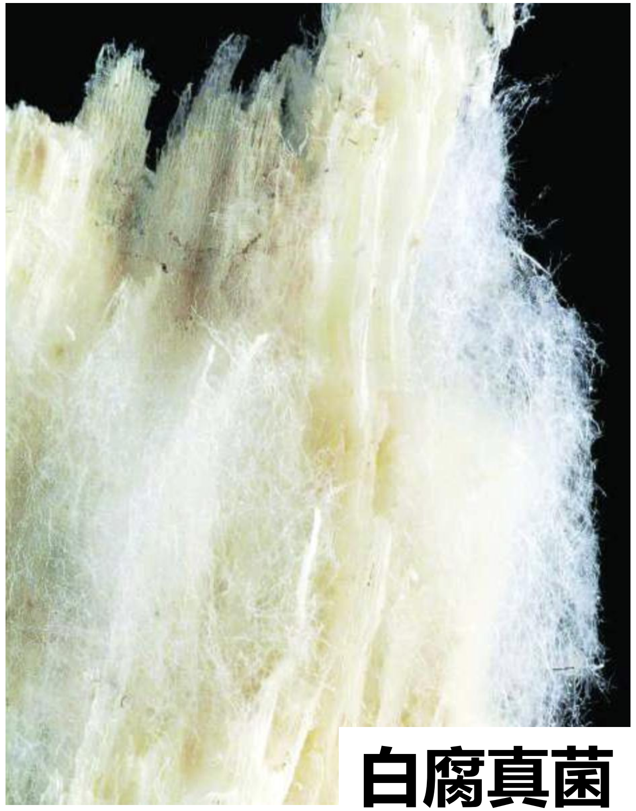
低消化率、高硅含量以及低营养价值，导致这种废料不能用作饲料

纤维素（44%）和半纤维素（20.1%）与木质素（19%）和不易消化的二氧化硅（9.8%）

⇒ 造纸和生物燃料工业生产中最不优选的底物

Introduction





白腐真菌

白腐真菌是一类使木材呈白色腐朽的真菌,能够分泌胞外氧化酶降解木质素,且降解木质素的能力优于降解纤维素的能力,这些酶可以促使木质腐烂成为淡色的海绵状团块——白腐,故称为白腐真菌。

它既不专指某一种真菌也不是泛指某一些真菌，而是限定为一类腐生木质上对木质有相同进攻能力并造成木质发生相同结构与外观变化—白腐的丝状真菌的总称

Introduction

Laccase
(漆酶)

氧化还原酶; EC 1.10.3.2

Ligno-hemicellulases

Xylanase

鉴于上述要求，本研究的目的是分离真菌菌株，该菌株能在木质纤维素残留物上共同产生漆酶，木聚糖酶和甘露聚糖酶的混合物，并能同时使稻秆脱木质素以获得有效的酶促作用水解。

挑战！

产量低、培养基配方昂贵
主要研究集中在纯化和表征上，
而不是在更便宜的木质纤维素底物上过量生产所有三种酶

章节

Part 02

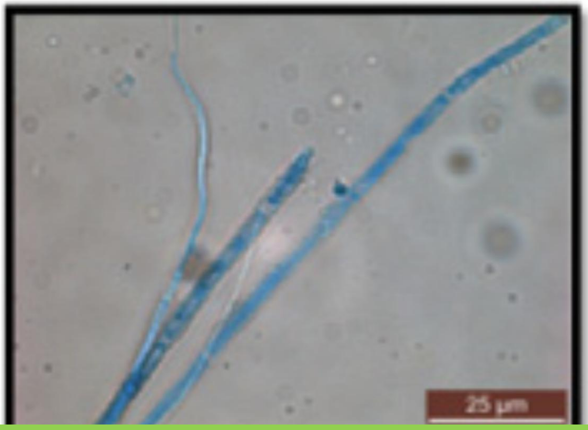
Material and methods

Material and methods

Microorganism

02

腐烂的木质样品



印度昌迪加尔市

选择菌株是基于它能够在麦麸固态发酵（SSF）4天后共同产生木质 - 半纤维素分解混合物的所有三种酶，并具有相当的滴度。

愈创木酚发红区域

漆酶

刚果红透明圈

木聚糖酶

刺槐豆胶板透明圈

甘露聚糖酶

Material and methods

Solid state fermentation of wheat bran

5g麦麸+7.5ml蒸馏水

250ml锥形瓶中

灭菌15 psi for 30 min

从PDA平板上生长良好的菌落周围切下5个菌丝盘（7mm）接种，然后在28°C下在静态条件下温育4天，用手动每天摇晃一次。

200ml蒸馏水
金属筛过滤
4°C以10,000rpm离心10分钟
无菌丝上清液作为粗酶制剂

Material and methods

Enzyme assays

漆酶

酶活性以国际单位(IU)表示
每分钟氧化1 μmoles 愈创木酚所需的酶

10分钟内在50°C氧化时测量愈创木酚在470nm的吸光度变化来测定漆酶

木聚糖酶

使用spelt xylan
作为底物测定木聚糖酶活性

50°C下每分钟释放的木糖的摩尔数，使用二硝基水杨酸 酸试剂

甘露聚糖酶

使用瓜尔胶
作为底物测定甘露聚糖酶活性

50°C下每分钟释放的甘露糖的摩尔数，使用二硝基水杨酸 酸试剂

Material and methods

Identification of the strain

18S rDNA测序，
基于测序，BLAST和Multialn结果，
使用MEGA 4软件构建了系统发育树

Material and methods

Assessment of different lignocellulosic residues for supporting the growth and co-production of laccase, xylanase and mannanase by solid state cultures of P. phaeocomes S-1

1	1米糠	Rice Bran
2	2脱油米糠	De-oiled Rice bran
3	3小麦麸	Wheat Bran
4	4甘蔗渣	Sugarcane baggase
5	5二手茶叶	Used Tea leaves
6	6小麦秸秆	Wheat Straw
7	7稻草	Rice Straw
8	8稻壳	Rice husk
9	9干树叶	Dried Tree Leaves
10	10土豆皮	Potato Peels
11	11干草	Dried Grasses
12	12玉米秸秆	Corn Stover
13	13地螺母壳	Ground Nut Shells
14	14菠萝提取物	Pineapple Extracts
15	15 Mausami	Mausami Peels
16	16木屑	Saw Dust
17	17香蕉茎	Banana Stalks
18	18厨房垃圾	Kitchen Waste
19	19Sarkanda	Sarkanda
20	20纸浆	Pulp (Wheat straw alkali pretreated)

salt solution containing (g/L):

NH_4NO_3,	4.0;
KH_2PO_4,	0.8;
Na_2HPO_4 $7H_2O$,	0.75;
$MgSO_4$ $7H_2O$,	0.5;
yeast extract,	2.0;
ZnSO4 $7H_2O$,	0.002;
FeSO4 $7H_2O$,	0.005;
$CaCl_2$ $2H_2O$,	0.06;
$MnSO_4$ $4H_2O$,	0.05
$CuSO_4$ $7H_2O$,	0.5

5g不同木质纤维素残留物

Material and methods

稻秆固态发酵期间生产木质素 - 半纤维素分解酶混合物的时间过程

250ml锥形瓶

时间：固态发酵10天

操作：以24小时的间隔取出锥形瓶

目的：研究了在稻草上生产漆酶，木聚糖酶和甘露聚糖酶的时间过程
研究三种酶的生产曲线

Material and methods

Characterization of crude ligno-hemicelluloytic enzymes cocktail of P. *phaeocomes S-1*

温度和热稳定性

测定不同温度范围为25至70°C的酶活性，在50°C下培养酶制剂96小时来研究热稳定性曲线，并且通过以相同的时间间隔取出样品来测定残留活性

pH

乙酸盐缓冲液pH 3.0-6.0，磷酸盐缓冲液pH 7.0-8.0和Tris-HCl缓冲液pH 8.0-10.0，100mM浓度

金属离子

$BaCl_2 2H_2O$, $CuSO_4 5H_2O$, $HgSO_4$, NaCl, $MgSO_4 7H_2O$, $CaCl_2 2H_2O$, $MnSO_4$, $FeSO_4 7H_2O$, $ZnSO_4 7H_2O$, KCl, $CoCl_2$ and CH_3COONa

有机溶剂

EDTA，十二烷基硫酸钠（SDS），叠氮化钠（NaN3），丙酮，甲醇，乙腈和福尔马林

Material and methods

***P. phaeocomes* S-1在稻草生物脱木素中的应用**

15g稻草+蒸馏水

若干500ml锥形瓶中

灭菌121℃下30 min

从PDA平板上生长良好的菌落周围切下15个菌丝盘（7mm）接种，然后在28℃下在静态条件下培养60天，用手动每天摇晃一次。

4,8,10,15,20,30,40和60天后，一式两份取出烧瓶

每个烧瓶中的内容物与600ml蒸馏水混合，在实验室混合器中混合，然后通过机械筛。用蒸馏水将固体残渣洗涤2-3次，然后在80℃下干燥。

上清液进行漆酶，木聚糖酶和甘露聚糖酶活性检测，以及总量还原糖检测

Material and methods

稻草样品的成分分析

根据NREL实验室分析程序（Sluiter等，2008），根据纤维素，半纤维素和木质素含量测定未处理和预处理的稻草样品的组成。 预处理期间各种成分的损失计算如下：

$$\% \text{ Loss of the cellulose/hemicelluloses/lignin} = 1 - \frac{(\text{Residue recovery} \times \text{cellulose/hemicellulose/lignin content in the residue})}{\text{Native cellulose/hemicellulose/lignin content}} \times 100$$

$$\%\text{纤维素/半纤维素/木质素的损失}=1-\frac{\text{残留物回收}\times\text{残余物中的纤维素/半纤维素/木质素含量}}{\text{天然纤维素/半纤维素/木质素含量}}\text{x100}$$

Material and methods

用室内生产的纤维素酶进行酶水解

分别将未处理（U），生物预处理（B），NaOH提取（N）以及生物处理的+ NaOH提取的（B + N）稻秆残余物各2g分别分散在30ml的0.1M乙酸钠缓冲液（pH 4.5）在250ml锥形瓶中。将它们在15psi下蒸3小时，冷却至室温，随后加入10ml来自黑曲霉NS-2（Bansal等，2014）的粗制纤维素制剂以提供5FPU / g 干基质和0.1%甲醛以防止微生物污染。在水浴振荡器（150rpm）中于50℃进行水解144小时。定期24小时取出样品，以10,000rpm离心10分钟，并通过DNS分析还原糖（Miller，1959）。得到的信息用于计算糖化效率如下：

$$\text{Saccharification } \% = \frac{\text{Total reducing sugars released} \times 0.9 \times 100}{\text{Carbohydrate content in pretreated rice straw}}$$

$$\text{糖化}\% = \frac{\text{总还原糖释放 X 0.9 X 100}}{\text{预处理稻草中碳水化合物的含量}}$$

章节

Part 03

Results and discussion

Results and discussion

S-1

它可以在非常短的时间内以相当高的滴度共产生三种重要的木质纤维素分解酶，使其成为酶商业化的潜在候选物。但是，通过研究酶生产的时间进程，选择合适的底物和补充营养素，可能会进一步提高酶的生产力。

S-1

培养4天后

漆酶 1618 IU gds^{-1}

木聚糖酶90.37 IU gds^{-1}

甘露聚糖酶20.83 IU gds^{-1}

Pleurotus ostreatus

是第一个被报道的，在小麦秸秆培养

after 28 days到达最高值

Trametes versicolor

培养21天后

漆酶 72.9 ± 1.4 U gds^{-1}

木聚糖酶98.9 ± 6.4 U gds^{-1}

甘露聚糖酶35.5 ± 3.9 U gds^{-1}

Results and discussion

Table 1
Enzyme activities of the selected fungal strain on different lignocellulosic residues moistened with distilled water.

Sr. No.		Lignocellulosic residues	Enzyme activities (IU gds^{-1})		
			Laccase[a]	Xylanase[b]	Mannanase[c]
1	1米糠	Rice Bran	301.65 ± 15.08	32.21 ± 0.32	11.57 ± 0.57
2	2脱油米糠	De-oiled Rice bran	9.09 ± 0.45	5.05 ± 0.05	51.04 ± 2.55
3	3小麦麸	Wheat Bran	631.40 ± 31.57	56.38 ± 0.07	4.16 ± 0.20
4	4甘蔗渣	Sugarcane baggase	5.78 ± 0.028	27.33 ± 0.03	3.67 ± 0.18
5	5二手茶叶	Used Tea leaves	10.74 ± 0.53	25.90 ± 0.03	4.44 ± 0.22
6	6小麦秸秆	Wheat Straw	19.83 ± 0.99	40.64 ± 0.03	1.93 ± 0.09
7	7稻草	Rice Straw	102.47 ± 5.12	31.84 ± 0.06	1.97 ± 0.09
8	8稻壳	Rice husk	0	26.73 ± 0.08	1.26 ± 0.06
9	9干树叶	Dried Tree Leaves	604.13 ± 30.20	316.93 ± 0.42	1.00 ± 0.05
10	10土豆皮	Potato Peels	241.32 ± 12.06	173.38 ± 0.42	0
11	11干草	Dried Grasses	563.63 ± 28.18	51.33 ± 0.16	6.38 ± 0.31
12	12玉米秸秆	Corn Stover	238.01 ± 11.90	28.99 ± 0.06	3.72 ± 0.18
13	13地螺母壳	Ground Nut Shells	387.60 ± 19.38	29.88 ± 0.05	2.53 ± 0.12
14	14菠萝提取物	Pineapple Extracts	42.14 ± 2.10	310.04 ± 0.89	3.59 ± 0.17
15	15 Mausami	Mausami Peels	106.61 ± 5.33	6.06 ± 0.08	44.23 ± 2.21
16	16木屑	Saw Dust	47.10 ± 2.35	0	6.37 ± 0.31
17	17香蕉茎	Banana Stalks	5.78 ± 0.28	272.72 ± 0.78	3.78 ± 0.18
18	18厨房垃圾	Kitchen Waste	316.52 ± 15.82	5.52 ± 0.05	37.36 ± 1.86
19	19Sarkanda	Sarkanda	1336.36 ± 66.81	303.14 ± 0.67	40.94 ± 2.04
20	20纸浆	Pulp (Wheat straw alkali pretreated)	26.44 ± 1.32	0	0.89 ± 0.04

ANOVA results – [a,b,c]p-value P = <0.001; F_L-value 1516.438; F_X-value 9112.506; F_M-value 30839.759.
Overall significance level = 0.05.

Results and discussion

Table 2
Enzyme activities of the selected fungal strain on different lignocellulosic residues moistened with stajic medium.

Sr. No.		Lignocellulosic residues	Enzyme activities (IU gds^{-1})		
			Laccase[a]	Xylanase[b]	Mannanase[c]
1	1米糠	Rice Bran	330.57 ± 23.37	13.39 ± 3.87	11.57 ± 0.57
2	2脱油米糠	De-oiled Rice bran	4008.26 ± 58.43	212.27 ± 0.57	38.60 ± 1.56
3	3小麦麸	Wheat Bran	14652.89 ± 58.43	83.61 ± 0.57	20.93 ± 0.17
4	4甘蔗渣	Sugarcane baggase	3123.96 ± 350.63	22.42 ± 0.43	1.17 ± 0.15
5	5二手茶叶	Used Tea leaves	74.38 ± 58.43	24.25 ± 0.71	10.67 ± 0.21
6	6小麦秸秆	Wheat Straw	25413.23 ± 35.06	38.76 ± 0.28	8.66 ± 0.07
7	7稻草	Rice Straw	10859.51 ± 46.74	22.01 ± 1.00	10.36 ± 0.25
8	8稻壳	Rice husk	669.42 ± 35.06	16.13 ± 0.71	2.48 ± 0.01
9	9干树叶	Dried Tree Leaves	1396.69 ± 35.06	38.96 ± 10.04	42.16 ± 1.33
10	10土豆皮	Potato Peels	1669.42 ± 46.75	39.87 ± 1.00	25.94 ± 4.72
11	11干草	Dried Grasses	16669.42 ± 58.43	45.66 ± 0.57	4.54 ± 0.08
12	12玉米秸秆	Corn Stover	22305.79 ± 128.55	41.80 ± 1.14	11.42 ± 0.04
13	13地螺母壳	Ground Nut Shells	578.51 ± 46.75	22.01 ± 1.00	2.42 ± 0.014
14	14菠萝提取物	Pineapple Extracts	429.75 ± 70.12	32.77 ± 1.00	49.57 ± 0.71
15	15 Mausami	Mausami Peels	396.69 ± 70.12	35.81 ± 0.71	6.37 ± 0.46
16	16木屑	Saw Dust	90.90 ± 81.81	26.48 ± 0.43	4.98 ± 1.03
17	17香蕉茎	Banana Stalks	1123.96 ± 93.5	31.76 ± 1.29	46.70 ± 0.04
18	18厨房垃圾	Kitchen Waste	479.33 ± 116.87	33.07 ± 4.30	57.31 ± 1.38
19	19Sarkanda	Sarkanda	264.46 ± 70.12	19.58 ± 1.00	0.89 ± 0.04
20	20纸浆	Pulp (Wheat straw alkali pretreated)	595.04 ± 70.12	24.35 ± 0.57	4.22 ± 0.19

ANOVA results – [a,b,c]p-value P = <0.001; F_L-value 12,464.922; F_X-value 531.059; F_M-value 437.167.
Overall significance level = 0.05.

漆酶：小麦秸秆、玉米秸秆、干草、稻草
木聚糖酶：脱油米糠、麦麸、干草、玉米秸秆
甘露聚糖酶：厨房垃圾、菠萝提取物、香蕉茎、干树叶

不同底物上的各种真菌的固态培养物　　比较木质纤维素酶的产率

Table 3
Comparison of lingo-hemicellulytic enzyme yields by solid state cultures of various fungi on different substrates.

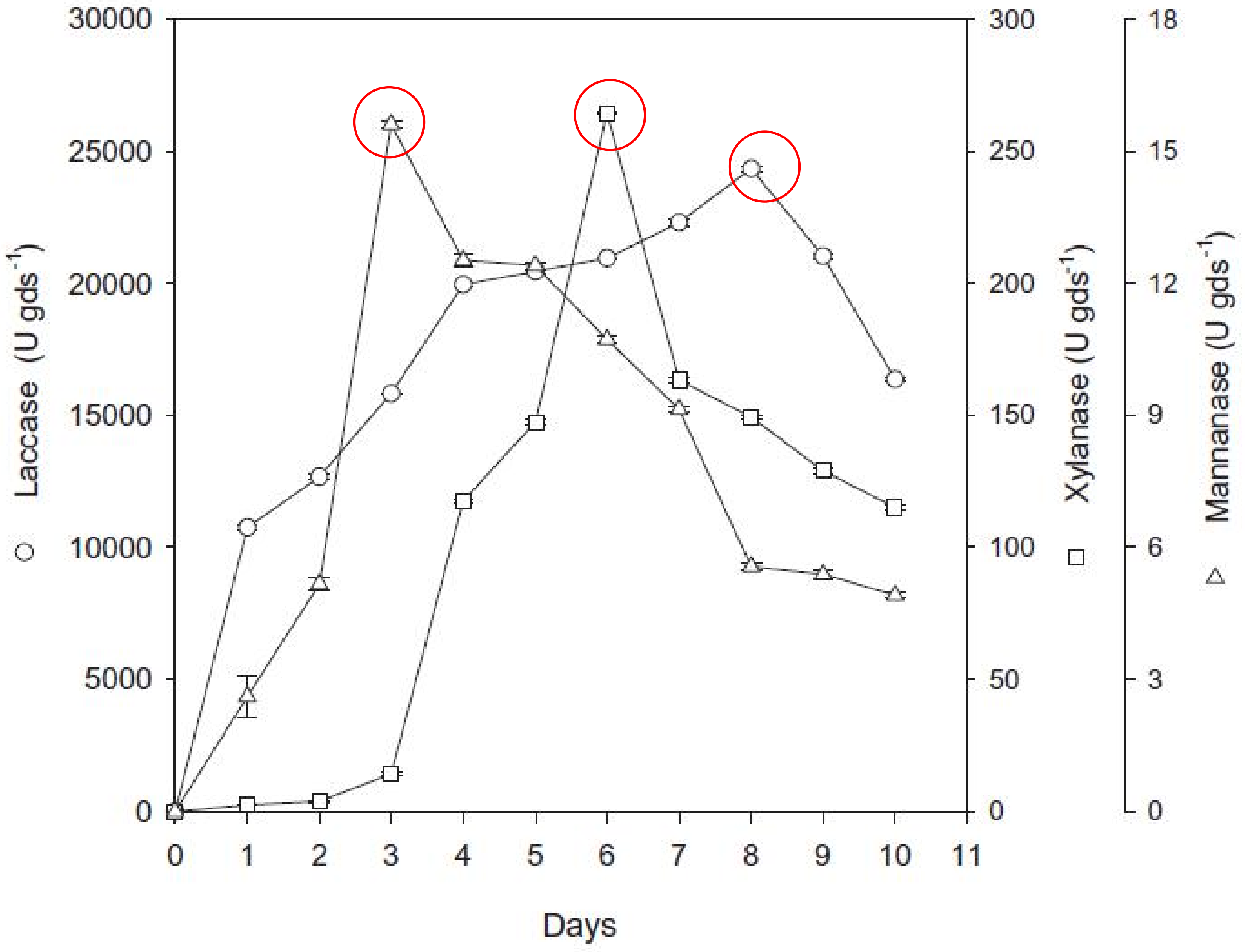
Fungi	Enzyme yields (U gds^{-1})			Time (d)	Substrate used	References
	Laccase	Xylanase	Mannanase			
P.phaeocomes S-1	10859.51 ± 46.74	22.01 ± 1.00	10.45 ± 0.128	8	Rice straw	Present study
Panus tigrinus	1090	–	–	10	Rice straw	Ruqayyah et al. (2013)
Aspergillus heteromorphus	8.2	160.8	–	12/6	Microwave-alkaline pretreated rice straw	Singh et al. (2011)
Phlebia floridensis	1.46	–	–	20	Rice straw	Sharma and Arora 2011
T.versicolor	72.9 ± 1.4	98.9 ± 6.4	35.5 ± 3.9	21	Wheat straw	Valaskova and Baldrian (2006)
Trametes taogii	900	413.15		14	Saw dust	Levin et al. (2008)
Pleurotus ostreatus	1360.5 ± 22.2	–	33.9 ± 0.8	21	Wheat straw	-do-
P. citrinopileatus	3.73 ± 0.55	0.12 ± 0.04	–	30	Wheat straw	Carabajal et al. (2012)
P. ostreatus	8.22 ± 0.79	0.14 ± 0.05	–	30	-do-	-do-
L. edodes	57 ± 4.7	200 ± 14	–	7	Tree leaves	Elisashvili et al. (2008)
P. ostreatus	15	9	–	3/2	Tomato pomace	Iandolo et al. (2011)
Trametes versicolar	35	50	–	16/13	-do-	-do-
P. ostreatus	161.3	–	–	5	Sugar cane baggase	Karp et al. (2015)

在稻秆固态发酵过程中由*P. phaeocomes* S-1生产木质素－半纤维素混合物的各种酶的时间过程

漆酶：192h
24,347.13±1
16.91 IU gds^{-1}

木聚糖酶：
144h
264.43±0.43
IU gds^{-1}

甘露聚糖：
72h
15.61±0.08
IU gds^{-1}



温度(a)和pH(b)对*P. phaeocomes S-1*生产的木质素－半纤维素混合物的各种酶的活性影响

在25℃的温度下

漆酶 44.8％

木聚糖酶 59％

甘露聚糖酶 57％

在pH=3下

漆酶 27％

木聚糖酶 56％

甘露聚糖酶 55％

在pH=9下

漆酶 25％

木聚糖酶 57％

甘露聚糖酶 48％

最高酶活性

漆酶 50℃

木聚糖酶 55℃

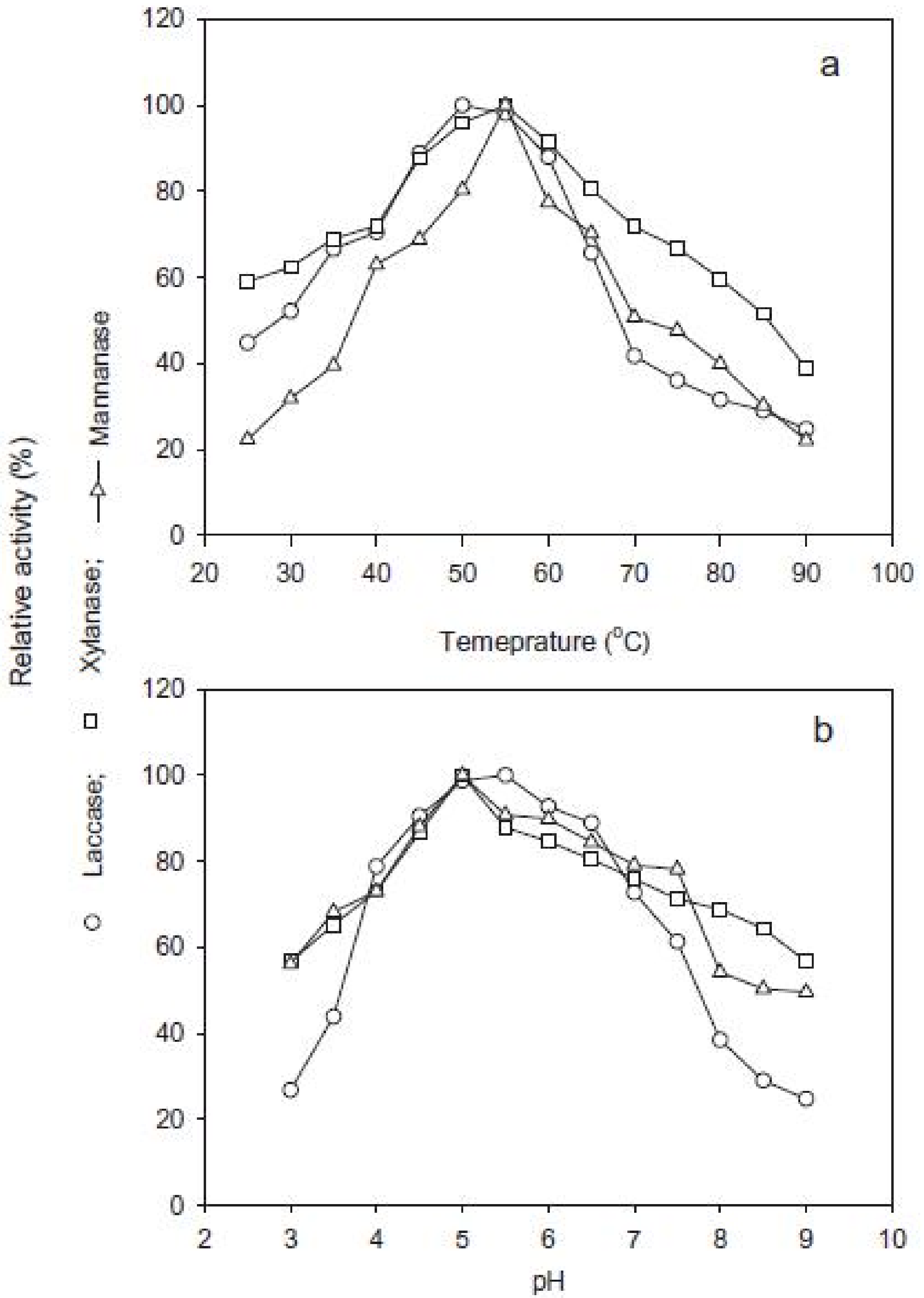
甘露聚糖酶 55℃

最适pH

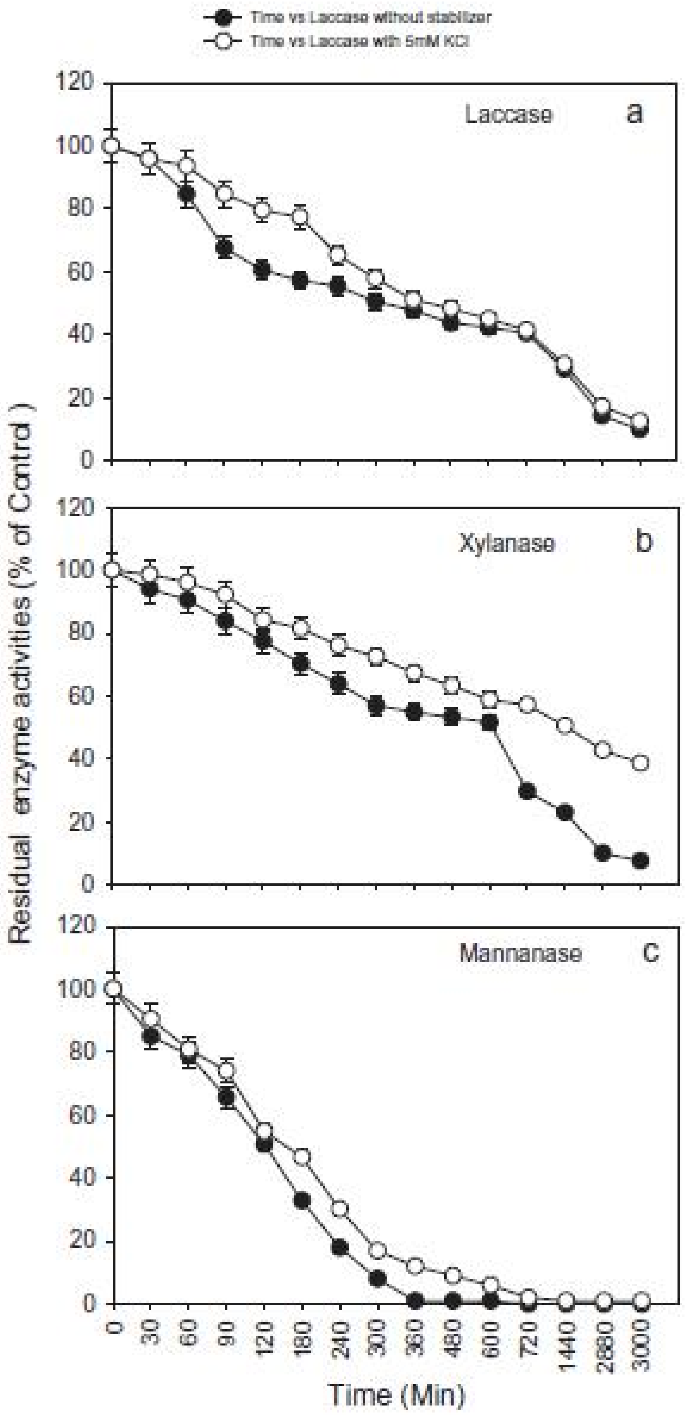
漆酶 5.5

木聚糖酶 5

甘露聚糖酶 5



热稳定性曲线
在50℃下
5mM KCl
漆酶(a)
木聚糖酶(b)
甘露聚糖酶(c)



半衰期

漆酶 5h→8h
木聚糖酶 10h→24h
甘露聚糖酶 2h→3h

Fig. 3. Thermostability profiles of laccase (a), xylanase (b) and mannanase (c) from *P. phaeocomes* S-1 at 50 °C without and with 5 mM KCl. Open symbols represent enzymes with 5 mM KCl and the closed symbols represent the enzymes without KCl.

1mM（a）和5mM（b）的各种金属盐对由*P.Phaeocomes S-1*生产的木质素 - 半纤维素混合物的各种酶的活性的影响。

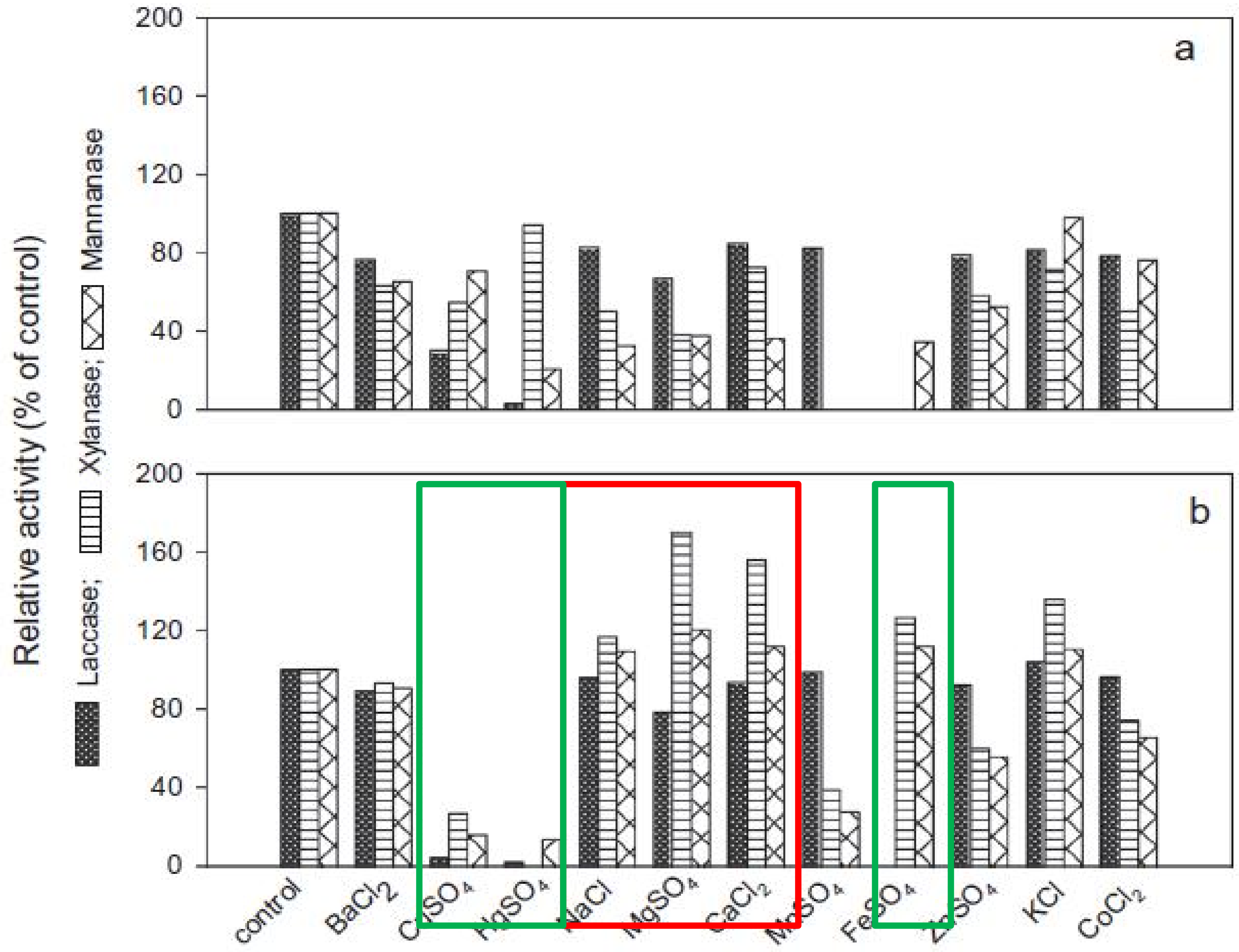


Fig. 4. Effect of various metal salts at 1 mM (a) and 5 mM (b) on the activities of various enzymes of the ligno-hemicellulytic cocktail produced by *P. phaeocomes* S-1.

EDTA，抑制剂，表面活性剂和有机溶剂对由*P. phaeocomes S-1*生产的木质素 - 半纤维素混合物的各种酶的活性的影响。

NaN3被证明是漆酶的抑制剂，即使最低浓度为0.375mM也被抑制，可能是由于活性位点中的2型和3型铜阻止内部电子转移而形成的复合物

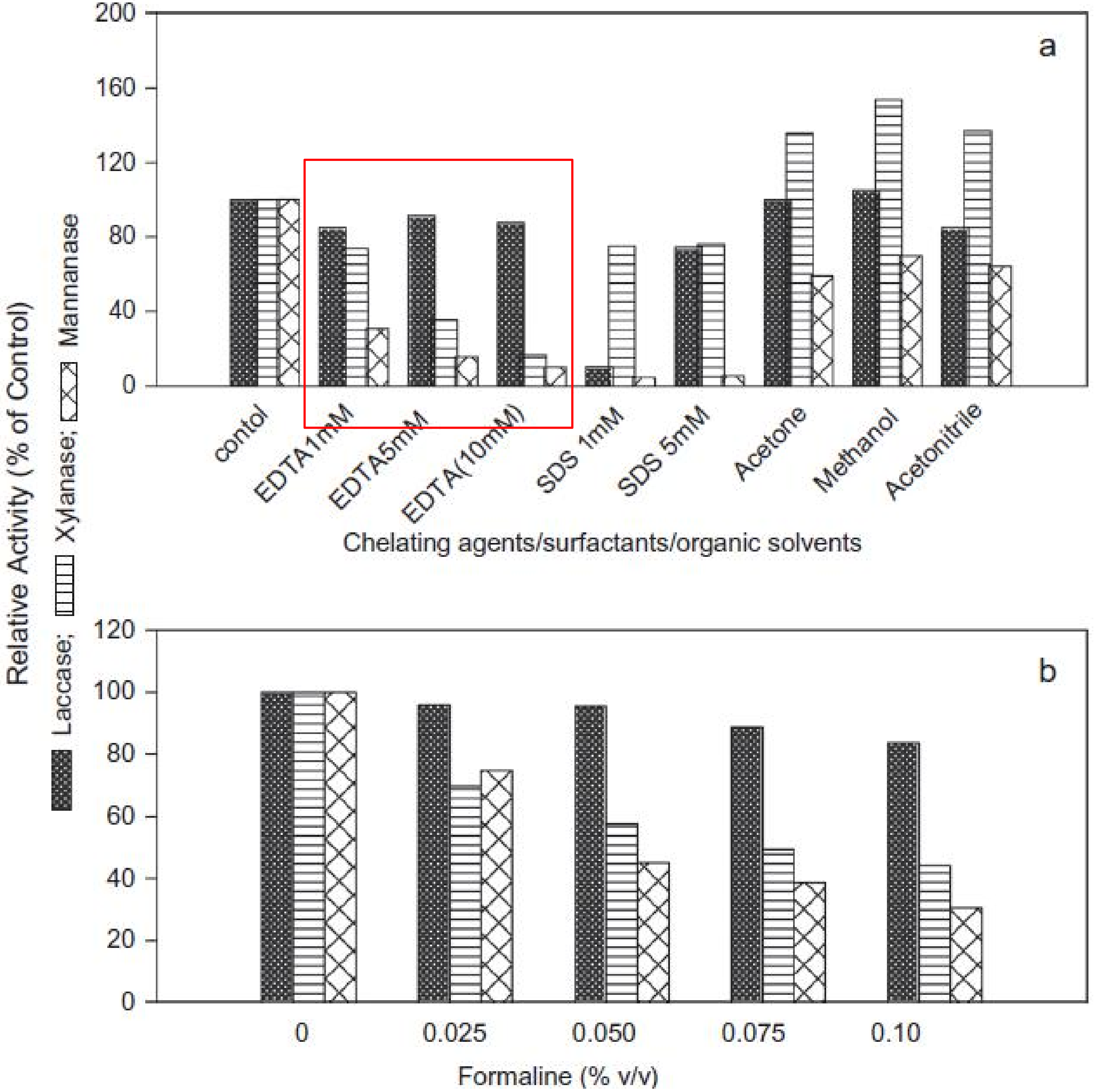


Table 4
Effect of biological pretreatment of rice straw by the growth of *Pyrenophora phaeocomes* S-1 on the disintegration of lignin and hemicellulose due to the production of laccase, xylanase and mannanase.

Incubation time (d)	Weightloss (%)	Cellulose (%)	Hemi-cellulose (%)	Lignin (%)	APPL (mg g^{-1})	Soluble lignin (mg ml^{-1})	Reducing sugars (mg g^{-1})	Ligno-cellulytic enzymes (IU gds^{-1})		
								Laccase[a]	Xylanase[b]	Mannanase[c]
0	5.50	32.4	28.1	20	88.00	0.12	17.26	0	0	0
4	15.00	32.820	25.48	19.56	140.80	0.21	12.78	2018.18	11.04	11.66
8	17.80	33.08	24.89	18.89	157.60	0.29	23.37	2451.24	13.91	5.47
10	19.80	36.17	23.25	17.89	159.20	0.31	27.78	1652.83	10.78	4.90
15	22.20	38.35	21.07	13.98	164.00	0.33	36.60	1542.19	3.97	1.42
20	23.80	39.84	20.98	12.34	172.53	0.34	41.01	1290.99	2.50	2.82
30	25.13	41.56	19.78	11.67	176.80	0.36	43.21	796.62	2.43	2.48
40	26.26	42.00	18.80	10	181.60	1.28	49.83	727.27	2.32	2.00
60	27.53	41.89	19.00	9.85	163.20	1.05	47.18	320.62	1.25	0.85

ANOVA results – [a,b,c]p-value < 0.001; F_L-value 178.815; F_X-value 345.00; F_M-value 338.026.

32.4％纤维素，
28.1％半纤维素
20.0％木质素

单独生物处理后的稻草组成百分比变化（40 d），用0.1N NaOH（30分钟，室温）单独提取，生物预处理，然后用0.1N NaOH萃取。

B
纤维素5.2％
半纤维素50.9％
木质素63.3％

B+N
纤维素8％
半纤维素60％
木质素78％

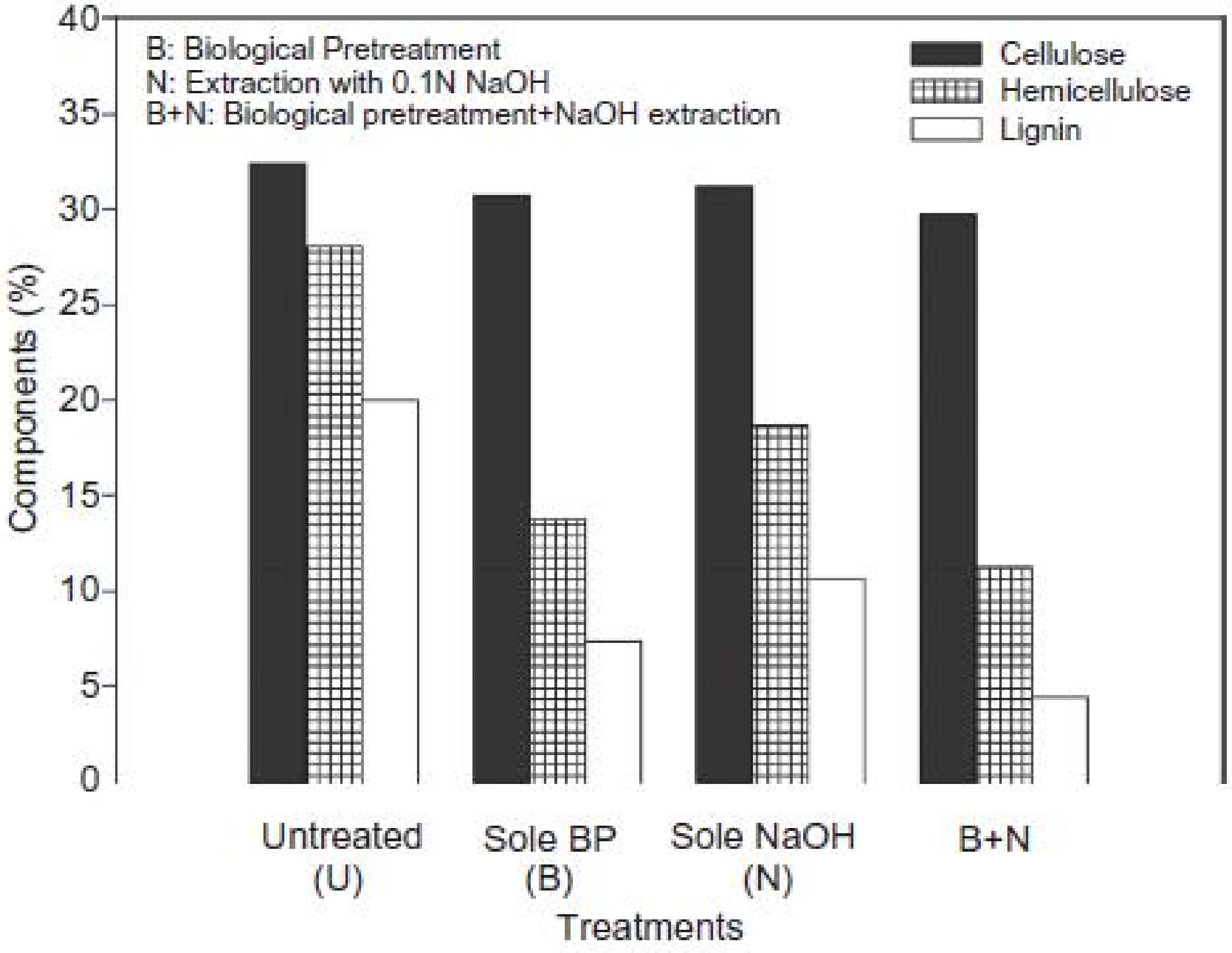


Fig. 6. Changes in percentage composition of rice straw after sole biological treatment (40 d), sole extraction with 0.1 N NaOH (30 min; room temperature) and the Biological pretreatment followed by extraction with 0.1 N NaOH.

Table 5
Pattern of reducing sugars and glucose formation after enzymatic hydrolysis of rice straw after various pretreatments.

Treatment of rice straw	Time (h)						
	0	24	48	72	96	120	144
Total reducing sugars (mg/ml)							
Untreated	1.12	2.34	2.85	3.75	4.96	6.16	6.76
Biologically treated	1.27	1.38	5.56	6.46	8.87	9.92	10.37
0.1 N NaOH	1.21	5.71	10.37	11.87	12.78	14.13	14.88
Biological + 0.1 N NaOH	1.45	13.48	21.33	28.16	29.16	29.83	31.00
Total reducing sugars (mg g^{-1})							
Untreated	4.43	24.39	41.01	50.99	65.41	78.71	95.34
Biologically treated	8.86	23.28	90.90	143.01	157.42	185.14	189.57
0.1 NaOH	9.97	27.71	103.10	172.94	220.62	256.09	264.96
Biological + 0.1 NaOH	12.82	150.99	223.64	368.94	425.92	445.86	470.08

（B+N）÷ U=4.90倍
（B+N）÷ N=1.77倍

2018

THANK YOU

感谢聆听，批评指导