Research Article

Enhanced Biodegradation of Pine Needles by Optimizing Temperature for Different Degrading Fungi under Solid State Fermentation

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Abstract

The present study was focused on the biodegradation of pine needles by the production of hydrolytic enzymes viz. cellulase, xylanase, laccase as well as reducing sugars production w.r.t optimization of temperature for the newly isolated fungi *Phanerochaete* sp. S7, *Armillaria* sp. K3 and *Phanerochaete chrysosporium* [NCIM 1197] were optimized under solid state fermentation and maximum of cellulase (20.54 U/g) with specific activity of 0.769 was observed in *P. chrysosporium* using untreated pine needles as substrate at 35°C temperature, while maximum of xylanase and laccase were expressed by fungi *Armillaria* sp K3 at 30°C temperature i.e. 178.87 U/g and 6.86 U/g using pretreated and untreated pine needles respectively. In case of reducing sugars maximum of 22.26 mg/g was released at 35°C temperature using untreated pine needles which is followed by *P. chrysosporium* in untreated pine needles. In our study the newly isolated hydrolytic fungi *Phanerochaete* sp. S7 and *Armillaria* sp. K3 were found to be potential strains for the hyper production of lignin degrading enzyme i.e. laccase as compared to the standard strain *Phanerochaete chrysosporium* [NCIM 1197].

Keywords: Cellulase, Xylanase, Laccase, Solid state fermentation, Pine needles

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Introduction

Today's world is presently facing an unprecedented energy crisis as the conventional energy resources are consistently deteriorating. The continued production of waste is creating management problems. The use of traditional waste management methods, such as incineration and landfill, releases gases that may cause global warming. There are two main problems which we have need to be addressed by our society i.e. shortage of fossil fuel and environmental pollution [1]. Rapid increase in industrialization, modernization of agricultural, house hold practices as well as transportation have tremendous impact on our environment and also increased the demand for energy. Production of second generation bioethanol from lignocellulosic biomass, holds a potential to meet the current energy demand as well as sort out the greenhouse gas emissions problem for a sustainable clean environment [2]. The lignocellulosic biomass mainly composed of cellulose, hemicellulose and lignin which have the great potential for the production of industrially important enzymes cellulase and xylanases under solid sate fermentation [3]. Among various lignocellulosic biomass, pine needles have a great potential to be used as the substrate for second generation biofuel production [1]. *Pinus roxburghii* is one of the six pines of India and the most widely occurring, also known as long leaved Indian pine. The pine needles catch fire immediately and become highly combustible can lead to a forest fire, thereby destroying most of the forest. So it is very interesting to use this forest waste for bioethanol production. To the complete degradation of pine needles there is a need of potential hydrolytic enzymes/ microorganisms which make it more accessible for hydrolysis into simpler sugars. Enzymes are industrially important and are routinely being used for various processes [4]. Microorganisms serve as a potential producer of enzymes that have been successfully used on industrial level [5]. Enzymes from microbes have applications in biofuel, textile, detergents, paper and pulp, food as well as leather industries [6]. In recent years, fungi are the potentials source of industrially important enzymes [7]. Fungi are used as a source of multiple carbohydrases due to their easy cultivation and high production of enzymes. Most of the fungi i.e. *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp. are important producers of xylanase due to high yields and extracellular release of the enzymes [8]. Xylanases (E.C.3.2.1.8) are the key enzymes, which play an important role in the breakdown of xylan and most commonly used for biopulping and biobleaching processes in industries [9]. *Trichoderma harzianum* is also reported as the best producer of hydrolytic enzymes [10].

Therefore, development of a viable technology that can utilize pine needles as a substrate for production of bioethanol is of great interest. The ability of certain microorganisms to produce multiple enzymes such as cellulase,

hemicellulases and laccase can be used to generate resources for a cleaner process that can produce simple sugars from a readily available and cheap biomass source i.e. pine needles. Solid state fermentation is an attractive method to produce enzymes from fungi due to its high productivity and low cost. Therefore it's important to select a desirable substrate for solid state fermentation. The hyphae of the fungi support to the filamentous to penetrate into the solid substrates and break down the structure of substrate. Penetration increases the accessibility of all available nutrients within particles. The present study describes the production of multiple hydrolytic enzymes and further optimization of temperature for enhancing enzymes production under solid state fermentation.

Materials and methods

Collection of Biomass

Pine needles were collected from the forests of adjoining Himalayas and brought to the laboratory. Washed and dried biomass was chopped into small pieces and then grinded into 2 mm sieve size and stored for the further experiments.

Pretreatment

Pine needles were used as the substrate and were pretreated with microwave irradiation dose at 600 W for 3 minutes to make it more accessible for hydrolysis.

Biodegradation of pine needles by different white rots under solid state fermentation.

White rots used:

- *Armillaria* sp. K3: The strain was isolated form degraded pine wood samples collected from Kumarhatti forest, district Solan- Himachal Pradesh.
- *Phanerochaete* sp. S7: The strain was isolated form degraded pine wood samples collected from Sultanpur forest, district Solan- Himachal Pradesh.

These two hyper laccase producers were isolated in our Microbiology research laboratory and identified as *Armillaria* sp. K3 and *Phanerochaete* sp. S7 on the basis of their morphological and phenotypic characteristics.

- *Phanerochaete chrysosporium* [1197]: A standard strain procured from NCIM-National chemical laboratory-Pune, India.

Biodegradation of pine needles

To 5 g of each untreated and microwave pretreated pine needles, moistening agent *viz.* Basal salt medium in 250 ml Erlenmeyer flask and autoclaved. After autoclaving, the flasks were inoculated with 1×10^7 spores/ml of white rot *Phanerochaete* sp. S5, *Armillaria* sp. K3 and *Phanerochaete chrysosporium* and incubated at different temperatures i.e. 25°C, 30°C, 35°C, 40°C, and 45°C for 7 days under static phase. Hydrolytic enzymes i.e. cellulase, xylanase and laccase produced during degradation as well as reducing sugars and proteins in tern were estimated as given below.

Extraction of cellulase xylanase enzyme by Repeated Extraction method [11]

To 5 g of each untreated and microwave pretreated pine needles, 50 ml of sodium citrate buffer (0.1M, pH 6.9) was added and the contents were kept in the shaker for 1 h at 120 rpm and then filtered through muslin cloth. The process was repeated twice with 50 ml of sodium citrate buffer. After filtration, contents were centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected and enzyme assays were performed.

Enzyme assays

Cellulase assays

Carboxy methyl cellulase, Filter Paperase and β-glucosidase assays was determined as described by [12] and [13] using 1% CMC, 50 mg filter paper strips and 1mM ρ-nitrophenyl ß-D-glucopyranoside as substrate respectively. The released reducing sugars were quantified using glucose and ρ-nitrophenol standard curves as a reference. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of glucose/ρ-nitrophenyl ß-D-glucopyranoside released per min under assay conditions.

Xylanase assay

Xylanase activity was determined using 1% (w/v) oat spelt xylan in 0.055 mM sodium acetate buffer pH 4.0 as substrate [14]. The released reducing sugars were quantified using a xylose standard curve as a reference. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of xylose per min under assay conditions.

Laccase assay
Laccase was determined as described by [15] using 2mM guaiacol and 10mM sodium acetate buffer. To the 1.0 ml of enzyme supernatant, 2.0 ml of 10mM sodium acetate buffer and 1.0 ml of 2mM guaicol was added and incubated at 35◦C temperature for 15 minutes. Absorbance of reaction mixture was read at 450 nm against a reagent blank [15].

Protein assays
To 0.1 ml of culture supernatant, 2.5 ml of Lowry's alkaline reagent was added, mixed and allowed to stand for 10 min. Diluted (1N) Folin Ciocalteau's reagent (0.25 ml) was added. The contents were shaken quickly and allowed to stand for 30 min for maximum colour development. Absorbance of reaction mixture was read at 670nm against a reagent blank. The contents of protein in culture supernatant were estimated from standard curve which was prepared by using Bovine Serum Albumin (BSA) in concentration of 10-100 µg/ml [16].

Reducing sugars produced

To the 1.0 ml of culture supernatant, 3 ml of Dinitrosalicylic acid (DNSA) reagent was added and the mixture was boiled in boiling water bath for 10 minutes. Absorbance of the reaction mixture was read at 540 nm against a reagent blank [14].

Results and Discussion

Pine needles are a difficult and challenging unutilized forest waste. They cannot serve as fodder. They do not even decay like the other biomass and piled up pine needles are a major cause of wild forest fires. They are however, a good source of biomass fuel. Lignocellulosic biomass is a potential source of carbohydrate polymers for fermentation and saccharification of its structural polysaccharides into simple sugars is highly challenging due to its intrinsic recalcitrant nature. Biodegradation of pine needles increases with increase in extracellular cellulase and xylanase production from hydrolytic microorganisms. One effective approach to reduce the cost of enzyme production is to replace conventional carbon source i.e. pure cellulose and xylan by relatively cheaper substrates such as lignocellulosic forest waste i.e. pine needles. Thus keeping in view the above facts, cost effective production of cellulase, xylanase and laccase by optimizing temperature under solid state fermentation for the selected fungi *Armillaria* sp. K3, *Phanerochaete* sp. S7 and *P. chrysosporium* using untreated/ pretreated pine needles was done in the present study. white rot *Armillaria* sp. K3 and *Phanerochaete* sp. S7 were isolated from decaying pine wood samples collected from forests of Kumarhatti and Sultanpur sites of district Solan, Himachal Pradesh –India and were identified as *Armillaria* sp. K3 and *Phanerochaete* sp. S7 on the basis of their morphology and phenotypic characterization.

Incubation temperature is the most important physical factor which affects enzyme production dramatically and their stability. Microorganisms grow slowly at a temperature below or above the normal temperature because of reduced rate of cellular production. Temperature is a cardinal factor affecting the amount and rate of growth of an organism and increasing temperature has the general effect of increasing enzyme activity but the enzyme begins to suffer thermal inactivation at higher temperature. The reduction in enzyme activity at higher temperature could be due to denaturation of enzymes. Maximum enzyme activity at optimum temperature may be due to the faster metabolic activity and increase in protein content and extracellular enzyme production in culture supernatant. At very low temperatures, membranes solidify and high temperatures damage microorganisms by denaturing enzymes, transport carriers and other proteins thus lowering enzyme activity. As indicated in Tables 1-3, the enzymes production was optimized by using different temperatures i.e. 25°C, 30°C, 35°C, 40°C, and 45°C. Different fungus gave their hyper enzyme production at their own optimum temperature. As data revealed in **Table 1** hydrolytic enzymes produced by fungus *Armillaria* sp. K3 using untreated and pretreated pine needles and maximum of cellulase (9.97 U/g) with specific activity of 0.46, laccase of 6.86 U/g (specific activity 0.32) as well as reducing sugars 12.88 mg/g were observed by using untreated pine needles, while maximum xylanase (178.87 U/g) was achieved using pretreated pine needles at 30°C. On the other hand minimum of cellulase (3.77 U/g) was observed at 45°C. So the optimum temperature for the *Armillaria* sp. K3 was 30°C which gave its maximum of enzyme productions. **Figure 1** shows the biodegradation of pine needles by *Armillaria* sp K3 under solid state fermentation at different temperatures.

Table1 Biodegradation of untreated and pretreated pine needles at different temperatures by fungus *Armillaria* sp. K3 under SSF

Sr No.	Temperature	Cellulase activities						Protein (mg/g)	Xylanase activities		Laccase activities		Reducing sugars (mg/g)
		Biomass	CMCase activity (U/g)	FPase activity (U/g)	β-glucosidase activity (U/g)	Total cellulase (U/g)	Specific activity		Xylanase activity (U/g)	Specific activity	Laccase activity (U/g)	Specific activity	
1.	25◦C	Untreated	4.33	4.86	0.176	9.36	0.44	20.83	172.46	8.27	6.63	0.31	11.85
		Pretreated	3.94	4.08	0.173	8.19	0.36	22.20	166.50	7.50	6.11	0.27	11.08
2.	30◦C	Untreated	4.83	4.98	0.169	9.97	0.46	21.30	178.50	8.38	6.86	0.32	12.88
		Pretreated	4.06	4.16	0.158	8.37	0.38	21.57	178.87	8.29	6.66	0.30	11.63
3.	35◦C	Untreated	4.12	4.15	0.124	8.39	0.45	18.54	173.93	9.38	5.60	0.30	11.85
		Pretreated	3.69	3.51	0.119	7.31	0.35	20.52	169.51	8.26	5.28	0.25	10.70
4.	40◦C	Untreated	2.68	2.05	0.073	4.80	0.28	16.94	157.84	9.31	2.31	0.13	7.20
		Pretreated	2.01	2.48	0.069	4.55	0.25	18.13	156.75	8.64	1.38	0.07	5.84
5.	45◦C	Untreated	1.69	2.02	0.065	3.77	0.32	11.52	108.52	9.42	1.88	0.16	5.53
		Pretreated	1.66	2.18	0.050	3.89	0.34	11.18	126.50	11.31	0.96	0.08	4.18
C.D0.05			0.40	0.64	0.02	0.09	0.98	0.04	0.02	0.01	0.06	0.07	0.08
S.E. (m)			0.10	0.15	0.17	0.13	0.20	0.12	0.11	0.09	0.10	0.13	0.11

(i) Untreated (ii) Pretreated

Figure 1 Solid state fermentation by *Armillaria* sp. K3 using untreated and pretreated pine needles at different temperatures (a) 25˚C (b) 30˚C (c) 35˚C (d) 40˚C (e) 45˚C

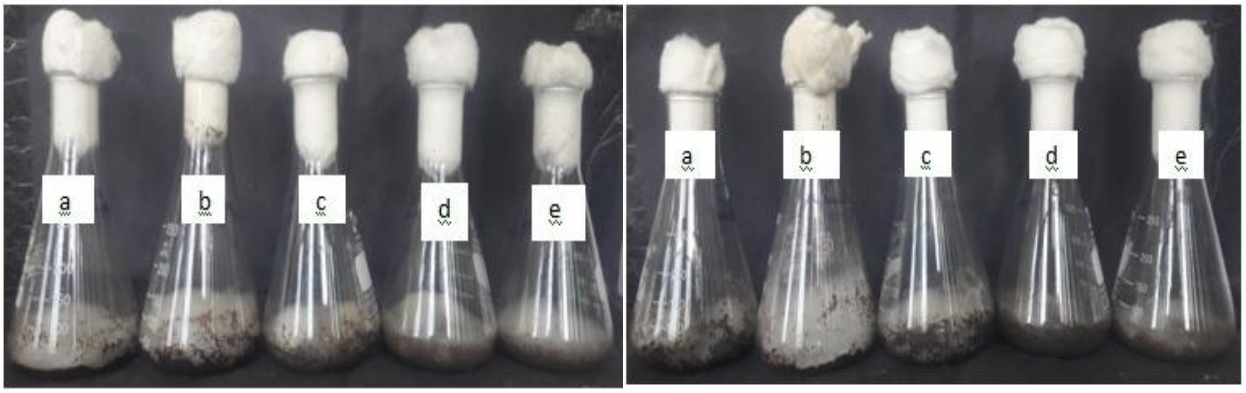
Same as in case of white rot fungi *Phanerochaete* sp. S7 maximum of cellulase as well as reducing sugars were achieved at 30˚C i.e. 12.46 U/g within specific activity of 0.64 and 14.46 mg/g in untreated biomass respectively (**Table 2**). While the hyper xylanase of 138.70 U/g **(**7.22 specific activity) and laccase of 6.19 U/g (0.31 specific activity) were observed at 30˚C in untreated pine needles biomass. Where as minimum of cellulase (2.26 U/g), xylanase (105.36 U/g), and laccase (1.49 U/g) were produced at 45˚C in pretreated biomass. **Figure 2** shows the biodegradation of pine needles as well as growth of white rot *Phanerochaete* sp. S7 at different temperature. As the **Table 3** depicts the results of hydrolytic enzymes produced by *P. chrysosporium* using untreated and pretreated biomass in which the maximum cellulase (20.54 U/g within specific activity 0.769), reducing sugars (22.26 mg/g) as well as xylanase (147.43 U/g with in specific activity 6.46), while the highest value of laccase (3.90 U/g with specific activity 5.50) was observed at 35◦C temperature. *P. chrysosporium* released maximum cellulase and xylanase by using pretreated pine needles, while maximum of laccase and reducing sugars by using untreated pine needles. Minimum of cellulase i.e. 8.06 U/g, xylanase of 105.15 U/g, laccase of 1.67 U/g and reducing sugars i.e. 11.04 mg/g were recorded at 45˚C, 40˚C and 40˚C respectively.

The overall study showed that, the maximum of cellulase (20.54 U/g) with specific activity of 0.679 was observed in *P. chrysosporium* using untreated pine needles as substrate at 35˚C temperature (**Figure 3**), while maximum of xylanase (178.87 U/g) (**Figure 4**) and laccase (6.86 U/g) (**Figure 5**) were expressed by white rot *Armillaria* sp. K3 at 30˚C temperature using untreated pine needles. In case of reducing sugars maximum of 22.26 mg/g (**Figure 6**) was released at 35˚C temperature using untreated pine needles which is followed by the value of 20.08 mg/g by *P. chrysosporium* in pretreated pine needles. **Figure 7** shows the degradation of pine needles by *P. chrysosporium* at different temperatures under solid state fermentation. Pine needles are basically rich in silica and phenolic compounds such as tannins, it is highly resistant to degradation in nature. However, pine needles biomass can be converted to useful renewable fuels such as biogas, biooil, bioethanol, biodiesel etc. by the use of microorganisms having the

potential to degrade it. In a study, the production of xylanase and laccase hydrolytic enzymes was reported from a lignolytic polypore fungi i.e. *Trametes versicolor* and *Aspergillus niger* [17, 18]. The ability of white-rot fungi to withstand and degrade tannin is well known [19]. Crystallinity of biologically pretreated lignocellulosic biomass of *Pinus densiflora* decreased by using enzymes from brown-rot fungi [20]. By the analysis of foregoing results and parameters governing the biodegradation process of pine needles by releasing reducing sugars, it can be concluded that pine needles are feasible and has enough potential for bioethanol yield as a green fuel.

Table 2 Biodegradation of untreated and pretreated pine needles at different temperatures by fungus *Phanerochaete* sp. S7 under SSF

Sr No.	Temperature	Cellulase activities						Protein (mg/g)	Xylanase activities		Laccase activities		Reducing sugars (mg/g)
		Bio mass	CMCase activity (U/g)	FPase activity (U/g)	β-glucosidase activity (U/g)	Total cellulase (U/g)	Specific activity		Xylanase activity (U/g)	Specific activity	Laccase activity (U/g)	Specific activity	
1.	25◦C	Untreated	6.19	4.11	0.174	10.47	0.54	19.17	135.23	7.05	6.19	0.32	12.67
		Pretreated	3.94	4.00	0.173	8.11	0.42	19.12	134.60	7.03	6.02	0.31	11.15
2.	30◦C	Untreated	7.57	4.71	0.183	12.46	0.64	19.21	138.70	7.22	5.91	0.30	14.46
		Pretreated	4.33	3.59	0.174	8.09	0.45	17.93	136.49	7.62	5.94	0.33	12.81
3.	35◦C	Untreated	5.33	4.39	0.173	9.89	0.47	20.83	138.49	6.64	5.97	0.28	12.58
		Pretreated	3.80	3.58	0.113	7.49	0.35	21.01	134.18	6.38	5.57	0.26	11.15
4.	40◦C	Untreated	4.10	3.89	0.108	8.09	0.39	20.38	131.86	6.43	2.97	0.14	11.04
		Pretreated	2.47	1.31	0.065	3.84	0.32	11.70	106.52	9.10	1.57	0.13	10.91
5.	45◦C	Untreated	3.78	1.63	0.108	5.51	0.29	18.42	125.97	6.83	1.74	0.09	6.80
		Pretreated	1.54	0.66	0.069	2.26	0.17	13.14	105.36	8.01	1.49	0.11	2.22
C.D0.05			0.20	0.34	0.05	0.09	0.06	0.04	0.02	0.03	0.08	0.05	0.08
S.E. (m)			0.09	0.17	0.10	0.14	0.10	0.11	0.10	0.08	0.07	0.14	0.14



(i) Untreated (ii) Pretreated

Figure 2 Solid state fermentation by fungus *Phanerochaete* sp. S7 using untreated and pretreated pine needles at at different temperatures (a) 25°C (b) 30°C (c) 35°C (d) 40°C (e) 45°C

Table3 Biodegradation of untreated and pretreated pine needles at different temperatures by *P. chrysosporium* under SSF

Sr No.	Temperature	Cellulase activities						Protein (mg/g)	Xylanase activities		Laccase activities		Reducing sugars (mg/g)
		Bio mass	CMCase activity (U/g)	FPase activity (U/g)	β-glucosidase activity (U/g)	Total cellulase (U/g)	Specific activity		Xylanase activity (U/g)	Specific activity	Laccase activity (U/g)	Specific activity	
1.	25◦C	Untreated	8.77	8.77	0.173	17.71	0.777	22.77	128.71	5.65	2.50	0.109	19.38
		Pretreated	7.73	8.23	0.168	16.12	0.647	24.90	127.45	5.11	2.52	0.101	15.64
2.	30◦C	Untreated	9.48	9.66	0.182	19.32	0.715	27.02	142.59	5.27	3.39	0.125	21.33
		Pretreated	8.80	8.77	0.174	17.74	0.703	25.20	141.96	5.63	2.85	0.113	16.64
3.	35◦C	Untreated	10.43	9.94	0.174	20.54	0.769	26.70	147.01	5.50	3.90	0.146	22.26
		Pretreated	9.04	9.06	0.170	18.27	0.801	22.79	147.43	6.46	3.75	0.164	20.08
4.	40◦C	Untreated	6.15	5.56	0.121	11.83	0.583	20.29	126.50	6.23	3.06	0.150	17.57
		Pretreated	5.15	8.02	0.104	13.27	0.655	20.25	105.15	5.19	1.67	0.082	11.04
5.	45◦C	Untreated	3.80	4.16	0.108	8.06	0.404	19.95	126.82	6.35	3.03	0.151	14.41
		Pretreated	4.15	3.82	0.105	8.07	0.473	17.03	115.99	6.81	1.83	0.107	11.05
C.D0.05			0.30	0.34	0.06	0.04	0.06	0.06	0.04	0.01	0.06	0.03	0.09
S.E. (m)			0.11	0.26	0.19	0.13	0.20	0.11	0.19	0.08	0.10	0.17	0.16

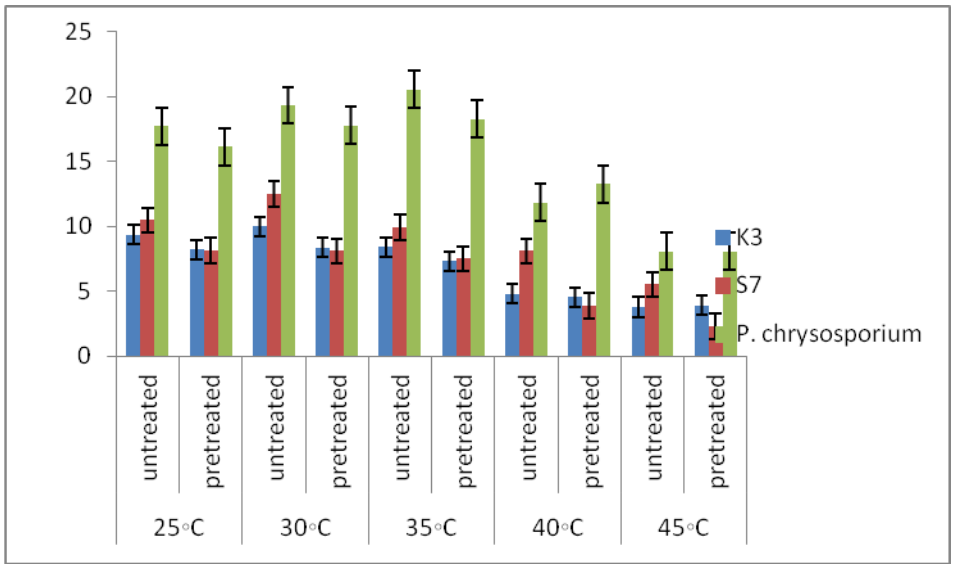


Figure 4 Comparison of Cellulase production by *Armillaria* sp. K3, *Phanerochaete* sp. S7 and *P. chrysosporium* under SSF

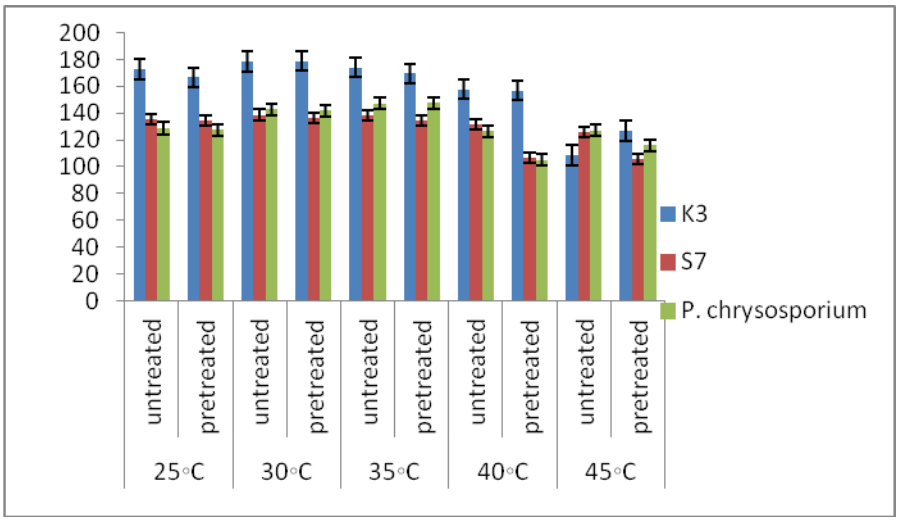


Figure 4 Comparison of xylanase production by *Armillaria* sp. K3, *Phanerochaete* sp. S7 and *P. chrysosporium* under SSF

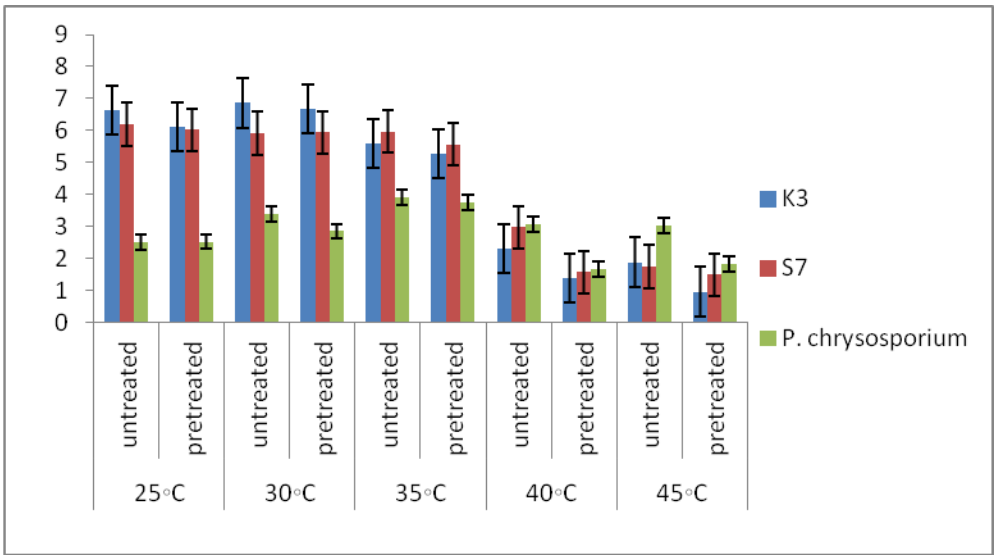


Figure 5 Comparison of laccase production by *Armillaria* sp. K3, *Phanerochaete* sp. S7 and *P. chrysosporium* under SSF

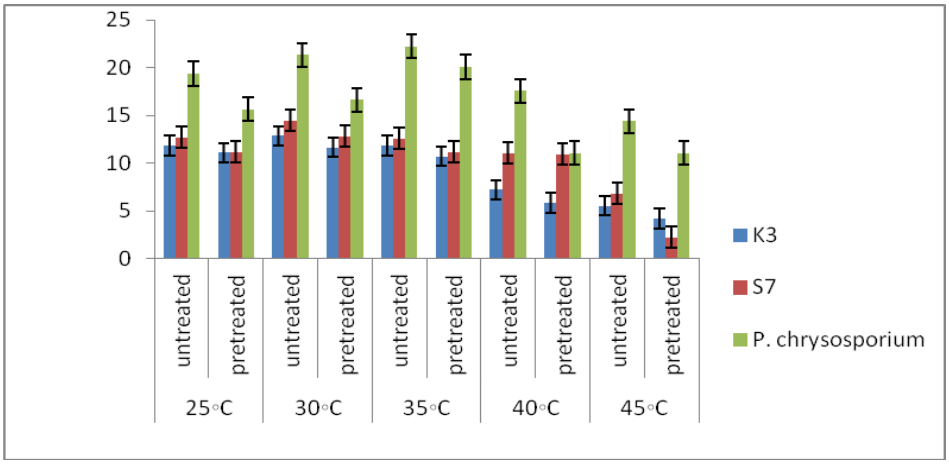
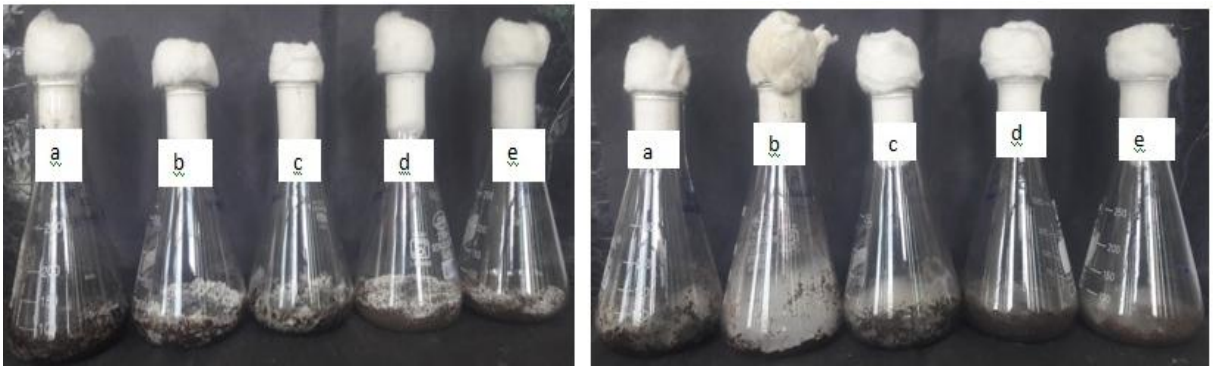


Figure 6 Comparison of reducing sugar production by *Armillaria* sp. K3, *Phanerochaete* sp. S7 and *P. chrysosporium* under SSF



(i) Untreated (ii) Pretreated

Figure 7 Solid state fermentation by *P. chrysosporium* using untreated and pretreated pine needles at different temperature (a) (a) 25°C (b) 30°C (c) 35°C (d) 40°C (e) 45°C

Conclusion

In the present study, the white rot fungi i.e. *Armillaria* sp. K3, *Phanerochaete* sp. S7 and *P. chrysosporium* had the potential to utilize lignocellulosic waste such as pine needles as a carbon source to produce valuable enzymes, thus reducing enzyme production cost. These strains also play their role in biological pretreatment as shown in the study, because there is no such observation for maximum enzyme as well as reducing sugar production in microwave pretreated pine needles as compared to untreated pine needles and our newly isolated strains i.e. *Armillaria* sp. K3 and *Phanerochaete* sp. S7 were found to be hyper laccase producers (which degrade lignin) as compared to reference strain *P. chrysosporium*. Optimization of temperature is a pre-requisite to enhance the yield under solid state fermentation process which is very helpful in large-scale production. Therefore, being rich in cellulose and hemicelluloses, pine needles can serve as a substrate for biodegradation and for the production of useful byproducts like biofuels besides its safe disposal helping to solve the burning issue of global warming and serve as an alternative source for better utilization of environmental waste.

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