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Production and Purification of Hydrolytic Enzymes and Enhanced Enzymatic Saccharification of Pine Needles – A Challenging Waste of Temperate Forests

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Abstract

In the present study, pine needles waste was used as the cost- effective carbon source for hydrolytic enzymes production by a potential fungal strain Trichoderma sp. R4 for the further purification process. The culture filtrate was subsequently partially purified by ammonium sulfate precipitation at 40% saturation level of laccase, 40% CMCase, 60% FPase, 50% β-glucosidase and 70% xylanase with purification fold of 3.06 (laccase), 2.20 (cellulase) and 1.59 (xylanase) with 82.94, 62.43 and 63.24 % recovery yield respectively. Gel exclusion chromatography was done for the purification of hydrolytic enzymes with 5.36, 5.51 and 6.33 purification fold and 45.77, 61.33 and 60.23 % recovery yield for laccase, cellulase and xylanase respectively. The molecular mass of purified laccase (40.0 KDa), cellulase – CMCase (45.0 KDa), FPase (31.0 KDa), β-glucosidase (29.0 KDa) and xylanase (65.0 KDa) was obtained by using SDS-PAGE. The maximum enzymatic degradation of pine needles was obtained in purified fractions of enzymes of Trichoderma sp. R4 with a release of 76.75 mg/g reducing sugars.

Keywords: *Pine needles, enzymes, laccase, cellulase, xylanase, purification, biodegradation, reducing sugars*

Introduction

The depletion of fossil fuel and its huge environmental problems are currently a concern for the scientific community in the area of energy. It has launched the research opportunities for searching alternate renewable energy sources especially biofuel production from lignocellulose biomass resources viz. agricultural and forestry biomass [1]. The structure is composed of complex polymers of lignin, cellulose and hemicellulose embedded in the plant cell wall [2]. These biological materials are identified as alternative energy sources for conventional fuel-receding fuel-petroleum [3]. Bioenergy also reduces adverse environmental impact (reduce the emission of greenhouse gases) caused by the use of fossil fuels, and at the same time, these are sustainable. Among woody biomass, pine needles are major recalcitrant lignocellulosic biomass rich in cellulose and hemicelluloses along with lignin that cannot serve as fodder. These do not even decay like any other biomass and piled-up pine needles are a major cause of wild forest fires and adversely affect biodiversity as well as soil fertility [4]. Top layer of soil left with pine needle litter prevents absorption of rain water by soil thus resulting in depletion of ground water and thus demolishing livestock of important food. Furthermore, dry pine needles fallen from the tree act as a barrier between the sunlight and the ground, thus slopping the growth of grasses. Pine needles are rich in complex carbohydrates- cellulose and hemicellulose upto 60- 80% apart from lignin. These complex carbohydrates can be broken down to simple monomeric sugars (glucose and xylose) with the help of hydrolytic enzymes- cellulase, xylanase and laccase produced by specialized microorganisms. This challenging unutilized waste can be employed for bioethanol production if a strategic approach is applied i.e. suitable pretreatment for simplifying its complex structure by enzymatic hydrolysis strong titers of hydrolytic enzymes for their efficient degradation into a good amount of fermentable

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reducing sugars. Biological pretreatments like grinding and microwave irradiation make the lignocellulosic woody biomass more accessible to enzymes by simplifying cellulose micro-fibrils. Among hydrolytic enzymes, cellulase is responsible for decomposing cellulose into its constituents, while xylanase decomposes hemicellulose. Xylanase further cleaves oligosaccharides to xylose [5]. Laccase catalyzes the oxidation of non-phenolic and phenolic compounds to their equivalent active free radicals in a reaction facilitated by four copper atoms positioned at the catalytic core. In the present study, the production and purification of hydrolytic enzymes by *Trichoderma* sp. R4 subsequently degradation of pine needles to release a significant amount of reducing sugars was investigated and the study revealed that it has significant potential for the biofuel industry.

Materials and Methods

Collection of Biomass

Pine needles were collected from the forests of adjoining Himalayas and brought to the laboratory. Dried biomass was chopped into small pieces and then ground into 2 mm sieve size.

Isolation of multiple enzymes producing fungus-by *Trichoderma* sp. R4

To the 5g of rotten wood samples collected from different sites rich in pine forests, 2 % of cellulose powder was added and incubated at 28 ±2 °C for 4-5 days. One gram of enriched sample was serially diluted from 10^{-2} to 10^{-8} times using sterilized 9 ml dilution blanks. Each diluent (0.1 ml) was placed on the surface of Potato Dextrose Agar (PDA) medium and incubated at 28±2°C for 3 days. The pure cultures were obtained by bit method respectively. Extensive screening of fungal isolates capable of exhibiting appreciable levels of ligninolytic cellulolytic and xylanolytic, activity was done. The plates showing growth after 48 h of incubation were subjected to qualitative screening for visualizing corresponding zones of hydrolysis of different isolates for assessing their potential to produce multiple carbohydrates i.e. laccase, cellulase and xylanase.

Enzymes production

To each 5g of untreated pine needles biomass, 20 ml of moistening agent (Basal Salt medium) was added in 250 ml Erlenmeyer flask and autoclaved. After autoclaving one full plate of pure fungal culture (1×10^7 spores/ml) was added and flasks were incubated at 28±2°C for 7 days in static phase. After incubation, 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 a 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 for further purification steps and enzyme assays were performed [6].

Enzyme Assays

Laccase assay [7]

The reaction mixture contained 1ml of 2mM of guaicol in 3ml sodium acetate buffer (pH5.5) and 1ml of enzyme supernatant was added. The blank was also prepared with all components except the enzyme. The reaction mixture was incubated at 30°C for 15 min. The absorbance was read at 450 nm.

Cellulase assay [8]

The sub-enzymes of cellulase were measured by following standard assays. CMCase activity was determined by incubating 0.5 ml of culture supernatant with 0.5 ml of 1.1% CMC in citrate buffer (0.05M, pH 5.0) at 500C or 1 h. After incubation and 3 ml of 3, 5 –dinitrosalicylic acid (DNS) reagent was added. The tubes were immersed in a boiling water bath and removed after 15 min. The optical density was read at 540 nm. FPase activity was measured by Reese and Mandel method. The reaction containing 0.5 ml of culture supernatant, 50 mg strips of filter paper (Whatmann no. 1) and 0.5 ml of citrate buffer (0.05 M, pH 5.0) was incubated at 50^0C for 1 h. After incubation and 3 ml of DNS reagent were added. The tubes were boiled in a boiling water bath and removed after 15 min. The OD was read at 540 nm. For β-glucosidase activity the reaction mixture containing 1 ml of 1mM p-nitrophenol β-D-glucopyranoside in 0.05 M acetate buffer (pH 5.0) and 100 µl of enzyme solution was incubated at 45^0C for 10 min. After incubation, 2 ml of 1 M Na_2CO_3 was added and the mixture was heated in a boiling water bath for 15 min and OD was read at 400 nm [9].

Xylanase Assay [10]

To 0.5 ml of xylan, 0.3 ml of citrate buffer (pH 5) and 0.2 ml of the enzyme was added. The reaction

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mixture was incubated at 45°C for 10 min and then 3 ml of DNSA reagent was added, the mixture was then heated on a boiling water bath for 15 min, after cooling down at room temperature absorbance of the reaction mixture was read at 540 nm.

Protein assay [11]

To 0.1 ml of culture supernatant, 2.5 ml of Lowry's alkaline reagent was added and allowed to stand for 10 min. After incubation 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 color development. The absorbance of the reaction mixture was read at 670 nm.

Partial purification of laccase, cellulase and xylanase by *Trichoderma* sp. R4

Different concentrations of ammonium sulfate i.e. 0-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90% were evaluated to attain saturation point for each of laccase, cellulase subunits i.e. CMCase, FPase and β-glucosidase and xylanase from *Trichoderma* sp. R4. The preparations were kept at 4°C for overnight and then centrifuged that resulting in separation of pellets and supernatants. Laccase and CMCase were precipitated at 40%, FPase at 50%, β-glucosidase at 50% and xylanase at 70% level of saturation of ammonium sulfate, respectively. Precipitates of each fraction so obtained were dissolved in sodium citrate buffer (0.05 M, pH 5.0) and were refrigerated until further use. The enzyme solution was dialyzed against the same buffer overnight at 4^0C using 14 kDa cut-off dialysis membrane. The dialyzed α-amylase sample was then applied on to a Sephadex G-75 column, pre-equilibrated with 20mM Tris HCl (pH-8.0). The column was washed with 500 ml of equilibration buffer and the bound protein was eluted with the same buffer. Fractions (2.0 ml) were collected at a flow rate of 2ml/3.5min and assayed for enzyme activity. The active fractions which showed higher extracellular laccase, cellulase and xylanase activity were pooled and the purified enzyme was kept under refrigeration. The Concentrated supernatant containing enzyme dissolved in 20mM Tris HCl buffer (pH 8.0) was loaded on a DEAE-cellulose glass column. The column was eluted at a flow rate of 0.2 ml/min, with a linear NaCl gradient from of 0.1 M, 0.2 M…., 1.0 M in 20mM Tris HCl buffer (pH 8.0) and the protein content was measured at 280 nm. Active fractions were pooled and used for electrophoresis analysis.

Estimation of molecular weight of enzymes

SDS-PAGE was performed using 12% polyacrylamide gel under non-reducing conditions. The protein bands were visualized by staining coomassie brilliant blue. The molecular weight of the purified enzyme was determined by comparing it with Rf values of standard molecular weight markers, PAGE mark ™ Protein Marker (14.3-97.4 kDa).

Enzymatic degradation of pine needles by *Trichoderma* sp. R4

A comparative account of enzymatic degradation of pine needles was analyzed by crude enzyme, partially purified enzyme as well as purified enzyme of *Trichoderma* sp. R4 to establish the difference in their saccharification efficiency. To 1g of untreated pine needles biomass, 1ml of (0.05 M, pH 5.0) sodium citrate buffer was added to each Erlenmeyer flask and autoclaved. To each flask, enzymes preparations (Crude, partially purified and purified) @ 12.5 ml/g dose were added separately. All the flasks were incubated at 50°C for 72h in a static phase for degradation of pine needles. After incubation, contents were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected and reducing sugars were measured.

Reducing sugars [10]

To 1.0 ml of culture supernatant, 3 ml of DNSA reagent was added and mixture was then heated on a boiling water bath for 15 min, after cooling down at room temperature, absorbance of reaction mixture was read at 540 nm.

Results and Discussion

Production and purification of hydrolytic enzymes from *Trichoderma* sp. R4

Biological pretreatment is considered best over physicochemical pretreatment of lignocellulosic substrate because of its ecofriendly approach, economical along with higher degradation of biomass because of efficient synthesis of all three necessary enzymes i.e. laccase, cellulase and xylanase. The hydrolytic enzymes were produced by a potential fungal strain *Trichoderma* sp. R4 using native pine needles biomass under solid state fermentation and the maximum laccase, cellulase and xylanase titers

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Table1. Purification of laccase from Trichoderma sp.R4 using untreated pine needle biomass under solid state fermentation

Steps	Volume (ml)	Protein (mg/g)*	Laccase		Purification fold***	Recovery ****
			Laccase activity	Specific activity**		
Crude culture supernatant	100	45.96	6.90	0.15	1	100
Ammonium sulphate precipitation	90	38.12	14	0.46	3.06	82.94
Gel exclusion chromatography Sephadex G 100	5	17.45	49.8	2.47	5.36	45.77

* Protein concentration was determined by Lowry's method
**Specific activity is the activity unit/ protein
***Purification fold is increase in specific activity
****Recovery % is remaining protein concentration as % of the initial protein

Table 2. Purification of cellulase from Trichoderma sp.R4 using untreated pine needle biomass under solid state fermentation

Steps	Volume (ml)	Protein (mg/g)*	CMCase activity (U/g)	FPase activity (U/g)	β-glucosidase activity (U/g)	Total cellulase		Purification fold***	Recovery (%) ****
						Total cellulase (U/g)	Specific** activity (U/mg)		
Crude culture supernatant	100	46.72	18.95	16.53	2.4	37.88	0.81	1	100
Ammonium sulphate precipitation	90	29.17	22.4	24	7.8	54.2	1.79	2.20	62.43
Gel exclusion chromatography Sephadex- G 100	5	17.89	108.6	78	12.4	199	9.88	5.51	61.33

Table 3. Purification of xylanase from Trichoderma sp. R4 using untreated pine needle biomass under solid state fermentation

Steps	Volume (ml)	Protein (mg/g)*	Xylanase		Purification fold***	Recovery (%) ****
			Xylanase activity (U/g)	Specific activity (U/mg)**		
Crude culture supernatant	100	47.7	398	8.34	1	100
Ammonium sulphate precipitation	90	30.17	402.6	13.34	1.59	63.24
Gel exclusion chromatography Sephadex-G 100	5	18.20	1016	50.44	6.33	60.32

i.e. 6.90, 37.86 and 398U/g which were obtained in crude supernatant further subjected to multistep purification. The cell free extract of *Trichoderma sp.* R4 was subjected to sequential ammonium sulfate saturation and maximum laccase activity i.e. 14.00 U/g with purification fold i.e. 3.06 and 82.94% yield was observed between 30-40% saturation, (Table 1-3). Similarly, partial purification of cellulase was attained at 40% (22.4 U/g) for CMCase, 60% for FPase (24.00 U/g) and 50% for β-glucosidase (7.80 U/g) with 2.20 purification fold and 62.45 % yield. Partial purification of xylanase was performed and the precipitation was obtained at 60-70% saturation level i.e. 402.6 U/g with 1.59 purification fold and 63.24 % yield. Hernandez *et al.*, reported the maximum laccase, cellulase and xylanase activity at 40-60% saturation levels i.e. 12.16, 65 and 161U/g with purification fold i.e. 2.7, 1.6 and 2.8 respectively by *Fomes* sp. EUM1 [12]. Senthivelan *et al.*, cited that the maximum laccase activity after ammonium sulphate precipitation was obtained at 70% saturation with a specific activity of 1.26 U/mg with 1.57 fold purification for *Penicillum chrysogenous* [13].

Dialysis

Enzyme solution obtained after ammonium sulfate precipitation at suitable saturation ratio was kept in dialysis membrane of cut-off 14kDa at 4 °C and dialyzed against distilled water with three increments

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Table 4. Enzymatic degradation of pine needles

Sr. No	Steps	Reducing Sugars (mg/g)	Percent increase (%)
1	Crude culture supernatant	39.50	-
2	Partially purified enzyme with ammonium sulphate precipitation	53.00	34.17
3	Purified enzyme by gel exclusion chromatography	76.75	44.81

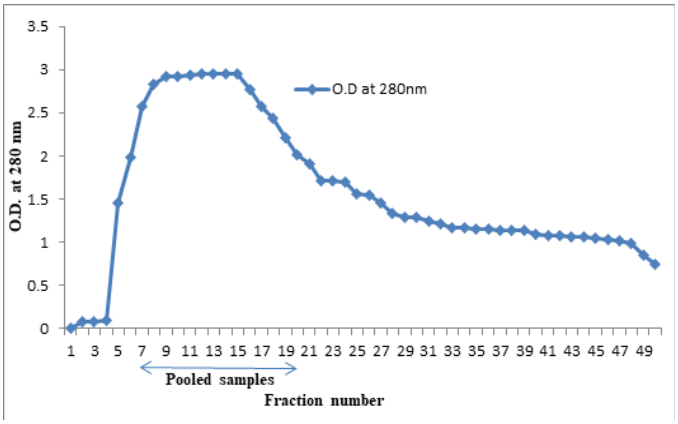


Fig 1. Elution profile of purified enzymes i.e. laccase, cellulase and xylanase of *Trichoderma* sp. R4 on Sehpadex G-100 column

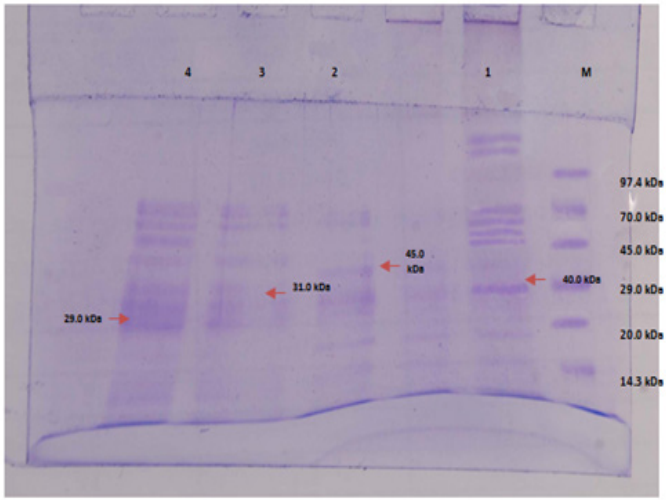
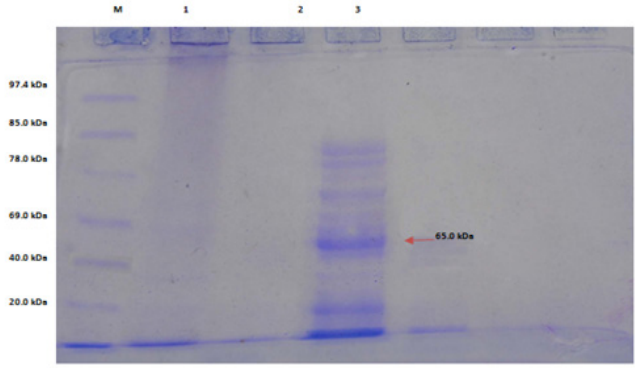


Fig 2. (a) SDS PAGE to determine molecular weight of purified cellulase subunits-Lane 1- Purified Laccase ; Lane 2- Purified CMCase; Lane 3- Purified FPase; Lane 4- Purified β-glucosidase



Lane1- Marker and Lane3-Purified xylanase

Fig2 (b) SDS PAGE of purified xylanase enzyme Trichoderma sp. R4

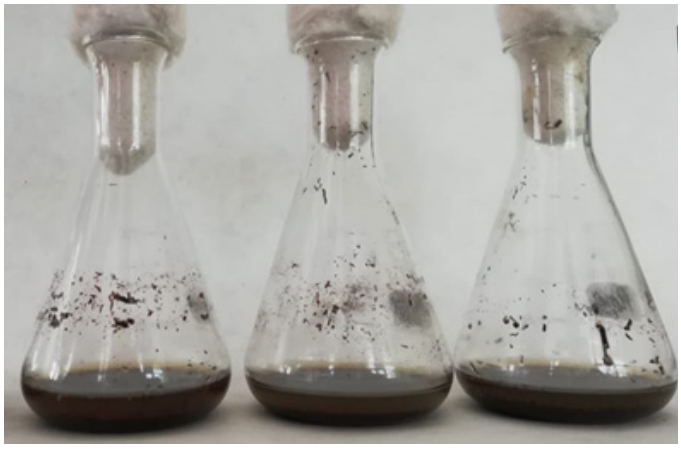


Fig3. Biodegradation of pine needles biomass by using enzymes (a) crude supernatant (b) partially purified (c) purified

of substitutions every six hours. Literature survey revealed the significance of dialysis in the purification of proteins. Several workers have dialysed their samples to concentrate before loading onto the column for gel filtration chromatography [14].

Gel filtration chromatography

Gel filtration chromatography also known as size exclusion chromatography separates proteins in their native state according to their relative size. The probability of a protein diffusing into these cavities increases with decreasing protein size in such a way so that smaller proteins are retained longest. Therefore, a mixture of proteins will elute from the column in order of decreasing size. This is the most frequently used technique for the purification of biological macromolecules by facilitating their separation from larger and smaller molecules in the supernatant [6]. The dialyzed fractions of partially purified enzymes of *Trichoderma* sp. R4 was applied onto Sephadex G-100 (1.5 x 40 cm) column for gel exclusion chromatography pre-equilibrated with 0.05M Sodium citrate buffer (pH-5.0). In total, 40 fractions of 3 ml volume each were collected with a flow rate of 3 ml/3min. Most of the low-expressed background proteins were removed but the major contributing proteins responsible for enzyme

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hydrolysis were collected in fractions. Fraction number 7-20 were pooled for laccase, cellulase and xylanase activity (Fig 1). Pooled fractions showed 49.8 U/g enzyme with 2.47 U/mg specific activity for laccase, 199 U/g with 9.88 U/mg specific activity for cellulase (108.6 U/g CMCase, 78 U/g FPase and 12.4 U/g β-glucosidase) and 1016 U/g enzyme activity with 50.44 U/mg specific activity for xylanase. Gel chromatographic separation resulted in purification fold for laccase, cellulase and xylanase i.e. 5.36, 5.51 and 6.33 with 45.77, 61.33 and 60.32% recovery yield respectively (Table 1, 2, 3). Walia *et al*., reported the purification of xylanase enzyme by using gel exclusion chromatography [15]. The purified xylanase exhibited a specific activity of 48.46 U/mg of protein, an overall recovery of 21.13% and 5.46 fold purification of *C. cellulans* CKMX1. More *et al*., observed 72.2 purification fold with 22.4% yield of purified laccase from *Pleurotus* sp [16]. The purification process was done using DEAE cellulose column chromatography and specific activity was found to be 25.30 and 3.42 fold purification for *Penicillum chrysogenous* [13]. Laccase of *Trichoderma giganteum* was purified using ammonium sulfate precipitation and column chromatographic technique. Purification was done by Sephadex G-100 gel chromatography and four active fractions having laccase activity were pooled. The specific activity of 1.07×10^5 U/mg and 10.49 % yield were achieved with 3.33 fold purification [17].

Molecular mass determination

The purity and molecular mass of the purified laccase, cellulase and xylanase of. *Trichoderma* sp. R4 was determined on SDS-PAGE. The molecular mass of purified enzyme was calculated according to the RF values of the standards used in the protein molecular weight marker. The molecular mass of laccase (40.0 kDa), different units of cellulase i.e. CMCase (45.0 kDa), FPase (31.0 kDa) and β-glucosidase (29.0 kDa) and xylanase (65.0 kDa) were observed by SDS-PAGE (Fig 2). Vivekanandan *et al.*, **s**tudied laccase enzyme produced by *Aspergillus nidulans* [18]. The molecular weight of laccase enzyme was found to be 66kDa. Another study revealed that the purified laccase enzyme had a single protein band on SDS-PAGE with a molecular mass of 40± 1kDa by *Pleurotus* sp. [16]. Cellulase produced by *Trichoderma viride* was purified by column chromatography and the molecular weight was estimated i.e. 87 kDa by SDS-PAGE [19]. Hernandez *et al*., purified the laccase enzyme by *Fomes* sp. with-molecular weight i.e. 70 kDa [12].

Enzymatic degradation of pine needles

Pine needles biomass is a potential source of carbohydrate polymers for fermentation into simple sugars and subsequently to biofuel. Saccharification of structural polysaccharides into simple sugars is highly challenging due to intrinsic recalcitrant nature of the lignocellulosic pine needles. It has been established that with an increase in extracellular cellulase and xylanase production from hydrolytic microorganisms, the biodegradation of pine needles can increase [20]. Biodegradation of pine needles by *Trichoderma* sp. R4 was accomplished by using hydrolytic enzymes laccase, cellulase and xylanase in crude, partially purified and purified form at 50°C temperature for 72h (Table 4). Crude enzyme, partially purified enzyme and purified have resulted in the release of reducing sugars i.e. 39.50 mg/g, 53.00 mg/g and 76.75 mg/g respectively-after the degradation of pine needles (Fig 3). The results clearly showed that maximum hydrolysis of the pine needles biomass had occurred with purified enzyme. Due to the presence of highest titers in the purified enzyme, maximum amount of sugars had been released and thus significantly give 44.81 percent increase over the partially purified enhanced the rate of degradation. Kumar *et al*., studied the production of crude cellulase enzyme from *Aspergillus niger* BK01 using pretreated rice straw under solid state fermentation [21]. The enzymatic degradation of rice straw was obtained after 2.5h. The maximum reducing sugars 23.78 % of rice straw were obtained. Bhavsar *et al*., studied the saccharification of rice straw and banana stem by crude enzyme and partially purified enzyme from the isolate 2b [22]. The maximum reducing sugars of rice straw and banana stem was obtained i.e. 1.38 and 1.34 mg/g respectively with enzymatic hydrolysis using pretreated substrates. Enzymatic degradation of untreated substrates produced less reducing sugars as compared to pretreated substrates. Pine needles are a challenging forest waste worldwide with the least utilization scope. In this study, two-way utilization of pine needles has been establishing successfully i.e. cost-effective production of a good yield of hydrolytic enzymes viz. laccase, cellulase and xylanase using pine needles as a substrate by multiple enzyme producing fungal isolate and secondly use of hydrolytic enzymes for saccharification of pine needles to solubilize to fermentable sugars for final conversion to bioethanol.

Extracellular synthesis of laccase along with cellulase

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and xylanase by these potential fungal strains prove that ligninolytic fungal isolates are successfully capable of breaking down the recalcitrant lignin shield around holocellulose present in pine needles and thus facilitating the hydrolytic action of cellulase and xylanase to release more amount of reducing sugar from the complex carbohydrates (cellulose and hemicellulose) however the use of hydrolytic enzymes in their concentrated form is finally recommended to maximize the yield of reducing sugar. This study indicates the importance of using robust hydrolytic enzymes for the degradation of lignocellulosic biomass from a commercial angel.

Conclusion

The present study strongly proves pine needles as a substrate for microbial degradation with a cost-effective approach of enzyme production due to inexpensive native and abundant pine needle waste. Multiple enzymes i.e. laccase, cellulase and xylanase introduced from *Trichoderma sp. R4* had been partially purified by ammonium sulfate precipitation. The precipitation of *Trichoderma* sp. R4 was attained at 40% for laccase with the activity of 14.00 U/g and purification fold of 3.06 with 82.94% yield. Partial purification of cellulase by *Trichoderma sp.* R4 was achieved at 40% salt saturation for CMCase (22.4 U/g), 60% for FPase (24.00) and 50% for β-glucosidase activities (7.80) with 2.20 purification fold and 62.43% yield. Partial purification of xylanase by *Trichoderma* sp. R4 was obtained at 70% of salt saturation level i.e. 402.6 U/g with 1.59 purification fold and 63.24 % yield. Gel exclusion chromatography was further performed to purify the laccase, cellulase and xylanase enzymes. An increase in enzyme activity of laccase, cellulase and xylanase was observed i.e. 49.8, 199 and 1016 U/g for *Trichoderma sp.* R4 and 5.36, 5.51 and 6.33 and 45.77, 61.33 and 60.32% of purification fold and recovery yield respectively. The molecular mass of purified laccase, cellulase and xylanase of *Trichoderma sp. R4* i.e. 40.0 kDa (laccase), 45.0 kDa (CMCase), 31.0 kDa (FPase) and 29.0 kDa (β-glucosidase) and 65.0 kDa (xylanase) were recorded. The enzymatic degradation of pine needles was compared using all the three fractions of enzymes i.e. crude, partially purified and purified enzymes. The maximum degradation of pine needles was observed in a purified fraction of enzymes (76.35 mg/g) with a combination of laccase, cellulase and xylanase at 50°C temperature, 5.5 pH after 72 h of incubation with the achievement of 76.75 mg/g reducing sugars with 44.81 % increase over the partially purified enzymes. The reducing sugars so obtained can be used for further fermentation processes i.e. conversion into bioethanol which will be an innovative technology for biorefineries. The technique will be proven highly cost efficient with the utilization of challenging waste of Himalayan forests i.e. pine needles.

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