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Fungal Biodegradation and Enzymatic Activities on Sawdust.

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ABSTRACT

Lignin, cellulose and hemicellulose are components of the hard fibrous material as wood. Sawdust is a lignocellulosic waste form which pretreated before its use in compost pile and in bio-fuel production like ethanol. The most proper treatment of sawdust that implicates no side effects is utilizing microorganism. This work test the potentials of *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Fusarium* species, *Ganoderma* species, *Trichoderma* species to biodegrade softwood (*Fagussil vaticus*) providing the media and optimal conditions for testing fungal growth. These circumstances are temperature, pH, moisture and the carbon source applied is only the sawdust. The capability of fungi to biodegrade wood will be determined and proved through the testing soluble proteins and reducing sugars through the incubation time. *Sclerotinia sclerotiorum* had the major significant cellulase activity after 27th day incubation (1.09 U/ml) then *Ganoderma* sp. (0.69 U/ml). The protein content obtained by *Sclerotinia sclerotiorum* was 3.8 and *Ganoderma* sp. was 3.6 at the 27th day.The pectinase production was screened by using two states of fermentation the solid and submerged state. *Ganoderma* sp. using solid state give 0.95 IU/ml pectinase activity but in case of submerged was 0.53 IU/ml. *Ganoderma* sp. produced higher pectinase at 35^{o}C and pH 5.5.

Keywords: solid state fermentation, submerged fermentation, *Sclerotinia sclerotiorum*, *Ganoderma* sp., sawdust.

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INTRODUCTION

The biodegradation is natural process of breaking down organic matters to harmless compound or recycling pollutants to nutrients by microorganisms [1]. The degradation is achieved by massive assortment of organisms as bacteria, worms, insects and fungi that manage materials by recycling them into new shape [2]. Sawdust is cutting of wood into fine particles by a saw so hence its name [3]. Sawdust is generally considered as waste, as it the main product of wood processing in sawmills, unless it reprocessed into particle board, burnt in sawdust burner or applied as heat for other milling operations [4]. It produce energy in the USA also act as platforms in poultry houses, cow pens and horse stalls; moreover it mix with dirt and chicken dung for compositing [5]. Waste disposal is a subject of environmental worldwide concern especially when they non-biodegraded to useful goods and services [6].

The potency of lignocellulolytic fungi to produce lignocellulolytic enzymes has a great interest. These extracellular enzymes include peroxidases and oxidases, and hydrolyzing enzymes as cellulases, hemicellulases, pectinases, chitinases [7]. These enzymes have a spacious range of applications in environment and industry. They are economically valuable as they degrad lignocellulolytic compounds into simple sugars to obtain bioethanol [8]. Cellulases are used for amelioratment the nutritional values of animal feeding. The producing microorganisms were screened for producing cellulases which may be utilized for enhancement of livestock production [9].

Pectin contributes to the large quantities of plant fibers and builds up the cell wall of plants [10]. Pectin degradation leads to disassembly of the cellulose and hemicellulose network and plays remarkable role in wine industry and fruit juice and ripening [11]. Pectinases may be generated by two both methods solid-state and submerged fermentation state (SSF and SmF, respectively) [12]. SmF is liquid broth media requires continuous agitation with a lot of water volume to get high effluents [13, 14]. SSF is a solid media as microbial cultivated under aerobic conditions in relatively absence or without free water [15]. This process does not generally require aseptic condition [13]. Few reports are known in comparing of SmF and SSF to produce pectinases enzymes. This study used sawdust as a fermentation substrate to screen some fungal species for cellulolytic and pectinolytic activity also evaluate production of pectinase in SSF and SmF. The protein content produced by these fungi is tested.

MATERIALS AND METHODS

Source of microorganism and initial culturing

The fungal species used were *Sclerotinia sclerotiorum* (No: 4243), *Rhizoctonia solani* (No 4990), *Fusarium* sp. (No: 2807), *Trichoderma* sp. (No: 399) isolated and identified by **Mycological Centre at Assuit University (AUMC)** and *Ganoderma* sp. was isolated by **Arab Society for Fungal Conservation in Egypt, University of Suez Canal, Ismailia 41522, Egypt**. These fungi were cultured on PDA at 30^{o}C which maintained and stored at 4^{o}C.

Cellulolytic activity screening media

It consisted of 0.2% peptone, 0.6% yeast extract, 1.0 % sawdust. The pH was 6.0.

CYM constituent: Dextrose 20 g, peptone 2 g, $MgSO_4$. $7H_2O$ 0.5 g, yeast extracts 2.0 g, KH_2PO_4 0.46 g, K_2HPO_4. $3H_2O$ 1.0 g, agar 20 g, dist. H_2O 1.0 L.

Reagents

3,5 –dinitro salicylic acid reagent (1% DNS)

10 g 3,5-dinitrosalicylic acid are shaked well with 500 ml dist. H_2O. Then 300 g of potassium sodium tartrate (Rochelle salt) added until it dissolved completely. Finally added 200 ml of 2M NaOH and the solution completed to 1.0 L dist. H_2O [16].

Coomassie blue dye reagent

Dissolve Coomassie brilliant blue (100 mg) in 50 ml ethanol (95%). 100 ml phosphoric acid (85%) was added and then mixture diluted to 1.0 L dist. H_2O.

Total cellulolytic activity

Fungi were grown in media containing 50 ml peptone (0.2%), yeast extract (0.6%) with cellulose powder (1%), the pH (5.5- 6.0) [17]. The Erlenmayer flasks (100 ml) inoculated with agar discs (1.0 cm) of every fungus cut from 5th day old culture growing in Complete Yeast Extract medium (CYM). Flasks were incubated at 25°C for 27 days. The mycelia then filtered and the filtrate stored at 0°C prior to enzyme assay.

Assay of cellulases

Activities of cellulases determined colorimetrically by the method of [18].Culture filtrate (1.0 ml) was added to 1.0 ml of citrate buffer (0.05 M, pH 4.8) and saw dust (10 mg). The mixture incubated for 10 min at 40°C, and the reaction ended by adding 3 ml of (1 g/ 220 ml) DNS reagent. The liberating sugar determined by DNS method [16].The tubes placed in boiling water bath for 5 min then cooled to room temperature. The absorbance was read at 575 nm.

Degradation (%) = {glucose (mg/0.5 ml) / substrate (mg/0.5 ml)} x 100

Since sawdust consists of 50% cellulose, substrate concentration in 0.5 ml can be derived as 25 mg (cellulose). The degradation was calculated by applying the glucose concentration retrieved from the standard glucose curve.

Screening of fungal isolates for pectinolytic enzyme activity

Plate method was modified using Czapek-Dox agar with commercial citrus pectin (sole carbon source) inoculated with fungi [19]. The clearance zone obtained after five days around the colonies determined using potassium iodide - Iodine solution [dist. water (330 ml) with potassium iodide (5.0 g) and iodine (1.0 g)].

Solid-state fermentation in contrast with submerged fermentation

The medium of SmF contained per liter of dist. water: sawdust (10.0 g), $(NH_4)_2SO_4$ (6.0 g), K_2HPO_4 (6.0 g), KH_2PO_4 (6.0 g), $MgSO_4$. $7H_2O$ (0.1 g) and pH value was 5.0. This medium inoculated with the suspension of fungus spore (2.0 ml). The media for SSF contained 5.0 g sawdust and 10 ml of the mineral salt solution: 6.0 g $(NH_4)_2SO_4$, 6.0 g K_2HPO_4, 6.0 g KH_2PO_4 and 0.1 g $MgSO_4$. $7H_2O$. Cultures incubated at 30°C with shaking (100 Osci min^{-1}) using Griffins shaker. The culture harvested by centrifugation after five days at 4000 rpm for 10 min. The flasks of SSF culture harvested after the addition of 0.05 M sodium acetate buffer (100 ml) with pH 5.5 [20]. The supernatant was preserved as the enzyme source.

Pectinase assay

The reaction of pectinase: equal amounts of crude enzyme and citrus pectin (1%) in 0.05 M of sodium acetate buffer (pH 5.5) was prepared. The mixture incubated for 30 min with water bath 50 °C. The reaction stop with 1.0 ml of DNS solution [16] and boiling for 10 min then cooled. The resulting color read at 540 nm using spectrophotometer. The reducing sugar released was quantified by standard solution of galactouronic acid (20-120 µg, Y = 0.0004 X; R2 = 1).

The enzyme activity (IU mg^{-1} protein) is enumerated by the enzyme amount that required for releasing 1.0 µmol equivalent of galactouronic acid per 1.0 minute per 1.0 mg protein under condition assay.

Protein assay

The total protein of crude enzyme content was evaluated according to the method of [21] using coomassie brilliant blue dye reagent. The absorbance recorded at 595 nm and the protein content measured

from calibration curve of bovine serum albumen in the range (20-100 µg, Y = 0.008 X; R2 = 0.991) at interval days from 9th to 27th day.

Effect of incubation time, temperature and pH on pectinase activity by *Ganoderma* sp.

Ganoderma was selected to determine the favorable conditions for pectinase so it subjected to different culture conditions [incubation at regular intervals days (4th day, 5th day, 6th day, and 7th day), temperature (15^{O}C, 25 ^{O}C, 30 ^{O}C, 35 ^{O}C) and pH (4.5, 5.0, 5.5, and 6.0)].

Statistical Analysis

The results were expressed as mean values and the standard error was statistically analyzed in completely randomized design by Statistical Analysis Software (SPSS v.11). Statistically significant difference (at 0.05 level) among means of experiment results was evaluated by the analysis of variance and means compared by one way ANOVA test and paired T-test.

RESULTS AND DISCUSSION

Fungi have the degradation abilities to decompose organic substances by the help of some enzymes [22]. Cellulose is the most dominant renewable resources in the biosphere as form of lignocellulosic wastes. Fungi degraded cellulose to produce valuable products.

The selected fungi in this study had different ability to decay cellulose as *Sclerotinia sclerotiorum* possess the highest activity about 1.09 U/ml followed by *Ganoderma* sp. 0.69 U/ml (**Table 1**) then *Trichoderma* sp. The results are similar to reports of [23] for *A. niger* KJ365316.1 (0.92 ± 0.02 U/ml), *Colletotrichum kahawae* NR144787.1 (0.80 ± 0.06 U/ml) and *Chaetomium cf. subglobosum* NR144826.1 (1.17 ± 0.08 U/ml). The cellulolytic activity of *Trichoderma* sp. was smaller than *Trichoderma* cf. *longibrachiatum* activity (1.39 ± 0.03) that reported by [23]. Both *Rhizoctonia solani* and *Fusarium* sp. had the lowest enzyme activity. This difference may be due to that fungal growth is influenced by the environmental conditions, specific host species, and the habitat nature [24]. The degradation mechanism of lignocelluloses by enzymes is still not understood, but great advances is made to gain knowledge about lignocellulolytic genes of microorganisms and various enzymes involved in the process.

Pectinases are abundantly produced by saprophytic fungi. The decaying plant tissue represents the most prevalent substrate for pectinase-producing microorganisms [25]. Pectinase production occupies about 10% of the manufacturing of enzyme preparations [26]. The mixtures of cell wall degrading enzymes with pectinases are applied for improving more than 100 % of juice extraction yields [27]. The selected fungal isolates were screened for pectinolytic activity and the diameter of hydrolysis zones was measured. *Sclerotinia sclerotiorum* and *Ganoderma* sp. cultures had a zone of clearance 3 mm and 3.5 mm respectively followed by *Trichoderma* sp. and *Rhizoctonia solani*. The lowest zone of clearance was obtained by *Fusarium* sp. as observed in **Table 2**. *Ganoderma* as a famous genus is a traditional stimulant in Asian medicines due to its characteristic and biological activities [28]. As *Ganoderma* species grow on wood and classified as wood-decaying fungi that degraded lignin, cellulose and hemicellulose (the wood cell wall substrate) by secreting cellulase, hemicellulase, and ligninase, respectively [29], this confirmed our results. The protein content was measured for all tested fungi, as seen in **Figure 1.** *Sclerotinia sclerotiorum* had the highest protein content followed by *Ganoderma* sp. *Rhizoctonia solani* and *Trichoderma* sp. On the other hand, *Fusarium* sp. had the lowest protein content.

SSF is more advantageous than SmF for microbial enzyme production as it had a higher yield of products [30], generation of less effluent and requiring simple equipment [31]. As represented in **Table (3)**, SSF showed more enzyme production than SmF. In SSF *Ganoderma* sp. showed the highest pectin lyase activity than other fungi followed by *Trichoderma* sp and *Sclerotinia sclerotiorum*. No significant difference was observed between SSF and SmF in both *Trichoderma* sp. and *Rhizoctonia solani*. These results are supported our results as SSF showed more enzyme production when compared to SmF. *G. neo-japonicum, G. lucidum* GBGL-01 and *G. lucidum* ASI 7039 show weak pectinase and cellulase activity as reported by [32]. These disagree with our results as *Ganoderma* sp. showed a moderate activity for both enzymes. Reports of [20] for *A. flavus* showed the maximum pectinase production (0.79±0.070 IU/ml) at 7th day on Pine apple. These results

were slightly similar to our results on sawdust by *Ganoderma* sp. (0.50±0.01 IU/ml). This difference is due to the different constitution and structure of agrowastes so the degradation differs from microorganism to other.

The pectinase production by *Ganoderma* sp. was optimized using different periods range from 4- 7 days. In solid state fermentation the maximum pectinase production was noticed at the 6th day (0.52 ±0.02 IU/ml). As showed in **Figure (2**) the lower growth was at the 4th day. The incubation temperature has a promising effect on the enzyme yield and the time of enzyme synthesis phase [33]. The pectinase production was optimized for sawdust using different temperature range from 15^{O}C - 35^{O}C. In SSF the maximum pectinase production of *Ganoderma* sp. (0.62±0.071 IU/ml) was noticed at 35^{O}C (**Figure 3**). At lower temperature (15^{O}C) the production of enzyme was decreased. No significant difference in enzyme activity was observed at 25^{O}C and 30^{O}C. Most pectinase production of fungi showed optimum growth at 15^{o}C to 35^{o}C [34] which agrees with our results. At higher temperature, the yield was less this might be due to the enzyme denaturation. In other work, the effective leaching of the -amylase from fermented bran was reported at 25^{o}C. The pH regulates the synthesis of extracellular enzyme and the growth of microorganisms particularly fungal strains [35]. As observed in **Figure (4)** *Ganoderma* sp. had the ability to grow well at pH 5.5 with the highest pectinase activity obtained. With increasing the pH the pectinase activity will decrease. At pH 4.5 lower activity of pectinase was observed. These results were comparable with [36] for the production of pectinase by *A. oryzae*. The optimal pectinase of *A. niger* was at pH 6.0 when citrus peel and sugarcane bagasse used in SSF [37]. Results of [20] on *A. flavus* are agreed with our work. The fermentation period depends up on the nature of medium, fermenting organisms, nutrients concentration and the physiological conditions process [20]. The biological, chemical, pharmacological studies and clinical applications have been extensively reported for *Ganoderma*. There is limited information on *Ganoderma* industrial implementation such as the use of extracellular enzyme activity, so these data will be useful to apply high-quality enzymes obtained from *Ganoderma* sp. to industries which include food processing, brewery, biofuels, and bioremediation.

Table 1: Total cellulolytic activity (U/ml) of the tested fungi at 27th day.

Total cellulolytic activity (U/ml) at 27th day						
Fungi	Control	*Sclerotinia sclerotiorum*	*Ganoderma* sp.	*Rhizoctonia solani*	*Fusarium* sp.	*Trichoderma* sp.
Absorbance	0.00±0.00^{a}	1.09 ±0.09^{b}	0.69±0.01^{bc}	0.31±0.00^{cd}	0.25±0.01^{d}	0.49±0.01^{cd}

All values are mean ±of triplicates. Values bearing different letters in the same row are significant at 0.05 level.

Table 2: Screening of fungal isolates for pectinase production

Zone of clearance (mm) after 5 days				
Sclerotinia sclerotiorum	*Ganoderma* sp.	*Rhizoctonia solani*	*Fusarium* sp.	*Trichoderma* sp.
3 mm	3.5 mm	1.5 mm	1.0 mm	2.0 mm

Table 3: Comparison of SSF and SmF under optimum conditions for all fungi.

Pectin lyase activity (IU/ml)					
Fungi	*Sclerotinia sclerotiorum*	*Ganoderma* sp.	*Rhizoctonia solani*	*Fusarium* sp.	*Trichoderma* sp.
SmF	0.32±0.020	0.53±0.020	0.30±0.020	0.24±0.020	0.43±0.020
SSF	0.40±0.004	0.95±0.023	0.36±0.051	0.39±0.020	0.47±0.070

All values are mean ±of 3 replicates.

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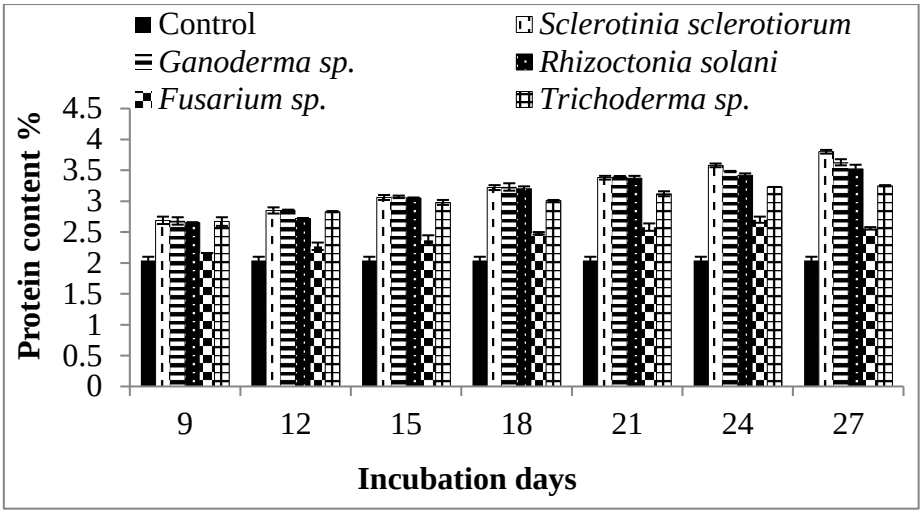

Figure 1: Protein content of the selected fungi at interval days from 9th to 27th day.

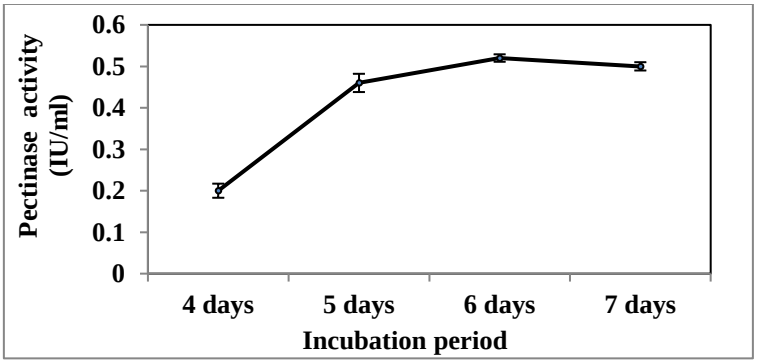

Figure 2: Effect of incubation time on pectinase activity by *Ganoderma* sp. All values are means ± SEM of 3 replicates.

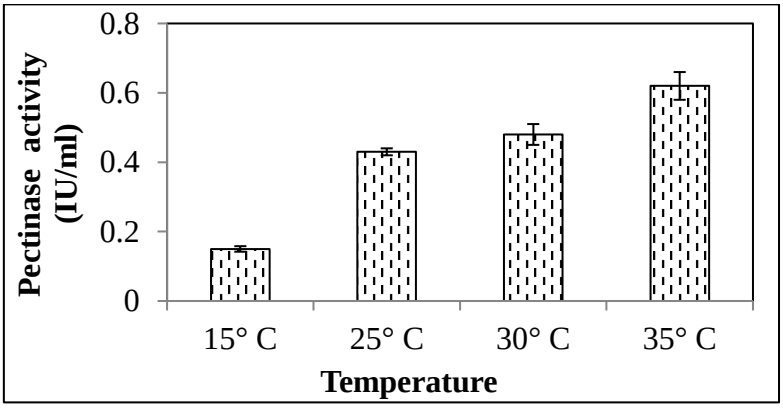

Figure 3: Effect of temperature on Pectinase activity by *Ganoderma* sp. All values are means ± SEM of 3 replicates.

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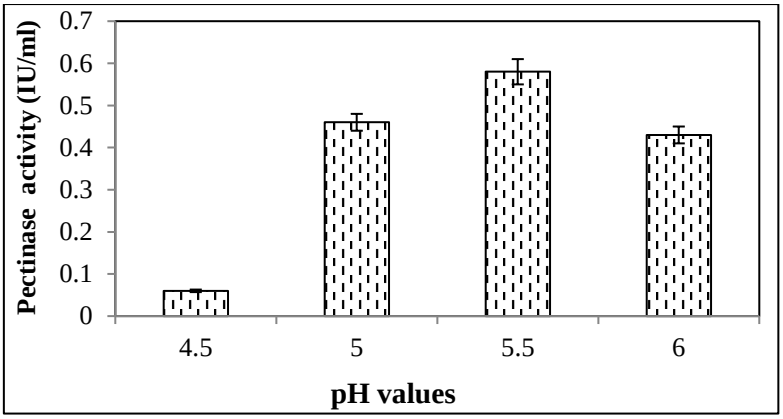

Figure 4: Effect of pH on pectinase activity by *Ganoderma* sp. All values are means ± SEM of three replicates.

CONCLUSIONS

The seeking for promising strains of pectinase producers is a continuous process. The isolates which show pectinase activity were selected. On the basis of data obtained in the present work it can be concluded that *Ganoderma* sp. can be employed in the production of pectinase.

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