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Production of enzymes by five *Pleurotus* spp. developed in solid and liquid state fermentation using three agricultural wastes

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Abstract. Oyster mushrooms (*Pleurotus* spp.) can bioconvert lignocellulosic residues due to the secretion of extracellular enzymes. The production of hydrolytic and oxidative enzymes by five *Pleurotus* spp. (*P. ostreatus, P. columbinus, P. pulmonarius, P. sajor-caju*, and *P. floridanus)*, developed in the solid and liquid state of fermentation using three argo-wastes (rice straw, sugarcane bagasse, and cotton waste), as substrate was evaluated in this work. The total nitrogen and potassium percentage were the highest in rice straw (0.96% and 0.60%). Also, the biological efficiency (BE), from these results, was the highest in *P. sajor-caju*, and *P. columbinus* recorded 64.4% on rice straw. It was observed that the submerged liquid fermentation (SmF) was suitable for the growth of all *Pleurotus* species. Also, the high value of enzymatic activity was determined through this study was higher in the submerged liquid fermentation SmF, than those produced during solid-state culture (SSF). Among proteolytic enzymes, protease is produced by the five *Pleurotus* spp. presenting the highest enzymatic activity (23.80 U/mL) on SmF and (22.56 U/mg) on SSF. The highest value (1.99 U/mL) of laccase activity of filtrate was estimated from *P. ostreatus* cultivated on sugarcane bagasse of SmF. Low enzyme level (0.39 U/mg) was manganese peroxidase, obtained from *P. floridanus* cultivated on the cotton waste of SSF. The enzymatic levels of α-amylase, β-amylase, cellulose, cellobiohydrolase, laccase, and lignin peroxidase were from 2.9-0.50 U/mL.

***Keywords**: Pleurotus spp., enzymes, solid and liquid state fermentation, agro wastes.*

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1. Introduction.

The genus *Pleurotus* is commonly known as oyster mushroom. However, *Pleurotus* species can be grown in a wide range of temperate and tropical areas as the wild mushroom. It belongs to the class *Basidiomycetes*, order *Agaricales*, family *Tricholomataceae*. This genus has 40 well-recognized species. Among species, *P. sajor caju* and *P. ostreatus* have been widely cultivated [1]. Oyster mushrooms (*Pleurotus* spp.) are wood-inhabiting white-rot *Basidiomycetes* with critical biotechnological and environmental applications [2,3,4]. They are highly adaptable to grow and fruit on a wide variety of forest and agro-industrial lignocellulosic substrates because of their ability to synthesize the relevant hydrolytic and oxidative enzymes that convert the individual component of the substrate (cellulose, hemicellulose, and lignin) into low molecular weight compounds, which can be assimilated for fungi nutrition [5]. Submerged liquid fermentation (SLF) gives rise to the possibility of high mycelial production in a compact space and shorter time with lesser chances of contamination.

Solid-state fermentation (SSF) will remain the chosen method for mushroom production [8]. Although, solid-state fermentation is a better system for the production of enzymes and metabolites than SmF [9]. While other studies reported that *Pleurotus ostreatus* grown in SmF produced higher laccases values than those grown in solid-state fermentation [10]. The lignocellulosic waste material is composed of three major structural polymers: cellulose (homopolymer of glucose),

hemicelluloses (heteropolymer) that include xylan, mannans, and lignin (complex phenolic polymer). Agricultural residues are the primary source of lignocellulosic biomass, which is renewable and inexpensive. These resources include corn fiber, sugarcane bagasse, rice husks, woody crops, and forest residues [11].

Hydrolytic enzymes used in industries can be produced in two ways solid-state fermentation (SSF) and submerged culture of the microorganisms [12]. Also, [13] recorded that the enzymatic degradation of cellulose is a complex process that requires at least 3 types of cellulolytic activity: Exo-β-I, 4-glucanase, endo- β-1, 4-glucanase, and β-glucosidase. Moreover, [14] reported that the hydrolysis of cellulose, exo-, and endoglucanase produce cellobiose and degraded glucose by β-glucosidase. The study of [15] reported that proteases represent one of the most prominent groups of world industrial enzymes, with perspective increasing around 7% until 2020 [16,17,18].

Lignocellulolytic enzymes such as laccase and Mn peroxidize [16] also produce xylanase [17]. On the other hand, Godínez et al. [6] reported that laccases) are copper-containing polyphenol oxidases implicated in lignin degradation by a one-electron oxidation mechanism [5]. Three ligninolytic enzyme families have been reported as the enzymatic complex from *Pleurotus* species; manganese peroxidase versatile peroxidase and laccase but lack lignin peroxidase.

It is needed for solving environmental pollution problems by using agricultural wastes as the substrate for the cultivation of mushrooms. Therefore, this work aims to cultivate five *Pleurotus* spp. in the solid and liquid state of fermentation SSF and SmF, using three argo-wastes and assessing the lignocellulolytic enzyme activity of these species cultivated on three different agro wastes.

2. Materials and methods

2.1. Agricultural wastes

2.1.1. Collection of samples

Rice straw, sugarcane bagasse, and cotton waste obtained from the private farm from the Assiut governorate were utilized as growing media for the different *Pleurotus* spp.

2.1.2. Determination of moisture content

Ten grams of sample was dried in the oven at 105 °C for about 24 h and then reweighted. The percentage of moisture content [19] was then calculated according to the following equation:

Moisture content (%) = [(A-B)/A] × 100, where A and B are the weight before and after drying, respectively.

2.1.3. The mineral content of agricultural wastes

The total nitrogen of dried agricultural wastes was determined using the Kjeldahl digestion method [20]. Total phosphorus was determined in agricultural wastes, spectrophotometrically (Specronic 200 Spectrophotometer) in the acid solution of the digested samples using ammonium molybdate and stannous chloride reagents [21]. The total potassium of agricultural wastes was determined in the acid solution of the digested samples using the flame photometric method.

2.2. Mushroom cultivation

The spawn packets and cultures of *Pleurotus ostreatus, Pleurotus columbinus, Pleurotus pulmonarius, Pleurotus sajor-caju*, and *Pleurotus floridanus* used in this research were obtained from the Agricultural Research Center, Food Technology, Egypt.

2.3. Solid-State of Fermentation (SSF)

2.3.1. Preparation of substrate mixtures

The chopped to 2-4 cm pieces of each substrate from various agricultural wastes (rice straw, sugarcane bagasse, and cotton waste) were soaked in water for 24 h until the moisture content reached about 60-80%, sterilization of the substrates was carried out by autoclaving at 121 °C for 1 h. The sterilized substrate was left to cool down and drain excess water. 500 g of each substrate was thoroughly mixed with 10 g calcium carbonate, 10 g wheat bran, and moisture content was kept at 70% [22].

2.3.2. Spawning

After sterilization, the substrates were transferred to transparent polyethylene cultivation bags. After cooling under average temperature, each substrate (500 g) with 70% moisture was mixed with a 10% spawn (dry weight/wet weight basis) under the laminar flow hood. The spawn was thoroughly mixed with the substrate and then filled into plastic bags [23]. The mixtures of the spawn and organic substrates of the inoculated polythene bags were then tightly tied with string made from cotton cloth. Pinholes were made by sterilized needle through bags (1/100 cm^2).

2.3.3. Cultivation conditions and harvesting

Following spawning, the inoculated bags were kept at room temperature and sprinkled with water twice a day. The growth of mushrooms was observed daily for all the treatments. The bags were covered with full of mycelia, and kept inside the dark cropping room at 25 °C and relative humidity about 85%. The harvesting was done in 3 harvests. After the 2nd harvest, the substrate was turned upside down and regularly watered to harvest the 3rd harvests. The yields of mushrooms were recorded [24,25]. After 14 days' interval, three bags for each treatment were removed for enzyme assay. The contents of a set of three bags were mixed uniformly.

2.4. Dried mushroom samples

After harvest, the mushroom samples were cleaned by rubbing, scraping, and brushing to remove all foreign matters. After that, they were cut into small pieces of around 2 to 3 cm across using a sterile knife and then wrapped in newspaper and stored in a moisture-free open place. They were air-dried in the shade that took 15 days or more [22].

2.5. Submerged Fermentation (SMF)

2.5.1. Cultivation of oyster mushroom on liquid media

Preparation of rice straw, sugarcane bagasse, and cotton waste, the substrates were grinding, soaking for 24 h sterilization. Furthermore, put it in sterilized plugged conical flasks 500 ml. Ten gm of each ground agro-waste were added in each conical flask with 100 ml distilled water, 5 g $CaCo_3$, and 5 g of yeast extract. After autoclaving at 121 °C for 1 h and cooling the liquid media in plugged conical, then inoculated occurred with 5 g of spawn for each conical. The experiment was conducted in triplicates for each substrate. Incubation at room temperature for about 45 days avoids movement and light filtrate and then the weight of mycelium after filtration. After 14 days of cultivation, the cell suspension was agitated at 150 rpm for 1 h. The enzymatic extract (EE) obtained from the liquid medium was filtrate and stored at 4 °C for further use.

2.5.2. Determination of biological efficiency

The biological efficiency of mushrooms was calculated by dividing the weight of fresh mushroom yield (g) by weight of air-dried substrate (g) and multiplied by 100 as described by [26,27,34].

Biological efficiency (%) = [Yield of fresh mushroom (g)/ Total weight of used dry substrate (g)] × 100

2.6. Enzyme assays

2.6.1. Enzymatic extract (EE)

Samples of 20 g of each fermented substrate were homogenized and placed in a conical flask (250 mL) with 100 mL sodium citrate buffer and were agitated at 150 rpm for 1 h. The enzymatic extract (EE) was then filtrate. The EE was stored in a refrigerator until use. Enzymatic assays were done in triplicate, and a zero reaction was performed using the substrates before fungus inoculation [28].

A total of 9.5 g of fresh oyster mushroom were cut into small pieces of around 2 to 3 cm across using a sterile knife, and they were homogenized and soaked in 450 ml distilled water for 1 day. After filtration and centrifugation, the obtained supernatant was used for further studies.

2.6.2. α-Amylase

Amylase activity was calculated by the following formula [29,30], and 1 unit of alpha-amylase is the amount of protein that will hydrolyze 10 mL of starch/min under specific conditions. By addition 1 mL of starch solution, 1 mL of 0.1 M acetate buffer to

0.5 mL of the extract (EE), was incubated for 10 min, and then the reaction was stopped by the addition of 1 mL of iodine reagent and 3 mL of 0.05 N hydrochloric acids. Absorbance was recorded at 620 nm.

2.6.3. β- Amylase

The beta-amylase activity was assayed using a reaction mixture contained 0.5 mL of soluble starch in a phosphate buffer (0.5 mL, pH 7.5), 0.5 mL of enzyme extract, and the reaction was incubated for 15 min at 30 °C then 1 mL of dinitrosalicylic acid solution as added and the mixture was boiled for 15 min. After cooling, 1 mL of Rochelle salt (40% sodium potassium tartrate) was added, and the color was measured at 575 nm. One unit of amylase activity was defined as the amount of enzyme that releases one mg of reducing sugar as glucose per mL per min under the assay condition.

2.6.4. Cellulase

Filter paper assay (FPase) method [31,32]. A reaction mixture (1 mL) containing 50-mg Whatman no. 1 filter paper (1 by 6 cm) and 1 mL of 0.05 M citrate buffer (pH 4.8) was incubated with 0.1 mL of culture filtrate for 60 min at 50 °C and the glucose released was quantified by the glucose oxidase-peroxidase [33] reducing sugar was estimated by 3 mL of (DNS) 3,5-dinitrosalicylic acid [34]. Then the tubes were boiled for 15 min in a boiling water bath, and 1 mL of 40% sodium potassium tartrate was added while the tubes were still warm. After cooling to room temperature, absorbance was measured at 540 nm**)**. The activity, calculated as reducing sugar (mg of glucose) × 0.185, was expressed as filter paper units [31].

2.6.5. Cellobiohydrolase

Exoglucanase or cellobiohydrolase activity was measured [35]. Endoglucanase (CMCase) and exoglucanase or cellobiohydrolase (FPase) were assayed for the release of reducing sugar from carboxymethyl cellulose (CMC) and filter paper (Whatman No. 1), respectively. The reaction mixture for reducing sugar assay contained (total volume 2 mL) 0.5 mL enzyme solution, 1.5 mL buffer, 0.05 M citric acid (pH 4.8), and 0.05 g substrate. After incubation for 1 h at 50 °C, the reaction was stopped by adding 2 mL of 3,5-dinitrosalicylic acid reagent. The resulting mixture was boiled for 15 min, and the reduced content after cooling was measured by absorbance at 575 nm. CMCase and FPase activities were expressed as micromoles of glucose released per minute (international unit) per ml of culture extract [36,37].

2.6.6. Laccase

Laccase assay was carried out at room temperature for 10 min [12] and [15] using 2,2'-azino Bis-3-ethylbenzthiazoline-6-sulfonate (ABTS) as substrate. For the reaction using 20 µM of sodium citrate buffer (0.1 M, pH 4.0), 100 µM of ABTS (1 mM) dissolved in distilled water 100 mL to 0.5 mL of crude enzyme extract. The reaction was incubated at 37 ºC for 15 min. The oxidation of ABTS was measured by increased absorbance with 420 nm at 37 ° C for 1 min. Enzyme activity was expressed in U/ml as described previously [38].

2.6.7. Lignin peroxidase

Lignin peroxidase activity was evaluated as the same procedure for laccase [39], by using 0.5 mL of sodium tartrate buffer (pH 5), 0.5 mL of 100 µM guaiacol, 1 mL of distilled water, 0.1 mL of culture filtrate, and 0.5 mL of hydrogen peroxide (30% w/v) containing a reaction mixture and by reading its optical density at 465 nm. For these 3 enzymes, 1 activity unit was defined as the amount of enzyme necessary to oxidize 1 µmol substrate/min.

2.6.8. Manganese peroxidase

Manganese peroxidase activity was assayed spectrophotometrically [40,41] by using 0.5 mL of sodium tartrate buffer (pH 5), 0.5 mL of 100 µM guaiacol, 1 mL of distilled water, 0.1 mL of culture filtrate, and 0.5 mL of hydrogen peroxide (30% w/v) containing a reaction mixture and by reading its optical density at 465 nm. For these 3 enzymes, 1 activity unit was defined as the amount of enzyme necessary to oxidize 1 µmol substrate/min.

2.6.9. Protease

The activity of the protease enzyme was assayed by the spectrophotometric method [42,43]. Incubating the enzyme extract with casein by using 110 mM trichloroacetic acid. Then, the filtrate was obtained after filtration through filter paper Whatman No. 1. Then sodium carbonate was added to the Folin-Ciocalteu reagent and incubated at 37 °C for 30 min. A spectrophotometer measures the absorbance at 660 nm. To generate the standard curve, the absorbance value attributable to the amount of tyrosine in the standard solutions. After this simple calculation, create the standard curve.

2.7. Soluble protein

The soluble protein concentrations were evaluated using a colorimetric method based on the standard curve of bovine serum albumin at 595 nm [44].

2.8. Statistical analysis

The data were statistically analyzed following the Randomized Complete Block Design (RCBD) with the arrangement of three replications, and means were compared following Duncan's Multiple Range Test (DMRT) test at a 5% level of probability for interpretation of results**.**

3. Results and discussion

3.1. Mineral content

The total nitrogen and potassium percentage were the highest in rice straw (0.96% and 0.60%), followed by sugarcane bagasse and cotton waste (0.61% and 0.57%), respectively. While in the case of cotton waste were lowered by 0.21% and 0.24%) respectively (Table 1). During this study, the total nitrogen and potassium percentage were the highest in rice straw (0.96% and 0.60%), followed by sugarcane bagasse and cotton wastes (0.61% and 0.57%), respectively. While in the case of cotton, wastes were lowered (0.21% and 0.24%), respectively. Silveira et al. [45] reported that the average value for the nitrogen content of the straw was 5.8%, representing a 63% reduction compared to the supplemented and sterilized substrate. This value is higher than the results of this study and also the results reported [46,47].

Table 1. Moisture content and mineral content (nitrogen, phosphorus, and cotton wastes) for the different agricultural wastes (rice straw, sugarcane bagasse, and cotton waste).

	Moisture content	**N%**	**P%**	**K%**
Rice straw	8 ± 0.78c[z]	0.96 ± 0.04a	0.22 ± 0.03b	0.60 ± 0.05a
Sugarcane bagasse	29 ± 1.13a	0.61 ± 0.02b	0.48 ± 0.02a	0.57 ± 0.02a
Cotton wastes	14 ± 0.98b	0.21 ± 0.01c	0.13 ± 0.02c	0.24 ± 0.01b

[z]Each value represented the mean value of three replicates ± standard error (SE). Data were analyzed by Duncan's multiple range tests (DMRT) at $p \leq 0.05$ level.

3.2. Biological efficiency

The biological efficiency (BE) was the highest in *P. sajor-caju*, and *P. columbinus* recorded 64.4% on rice straw also, and the total yield was 225.6 and 225.4 g in the case of *P. sajor-caju* and *P. columbinus,* respectively (Table 2). The biological efficiency (BE), from these results, was the highest in the case of *P. sajor-caju*, and *P. columbinus* recorded 64.4% on rice straw. Nevertheless, Zinabu et al. [48] pointed to biological efficiencies from 85% to 71% on some locally available substrates; the reason may be the harvesting and nutritional composition of substrates. This study's result was lower than the results reported by [49], which recorded that the highest biological efficiencies 191.8 were produced from the different substrates composed from wheat straw, waste paper supplemented with cottonseed waste.

3.3. Enzymes activity

3.3.1. α-Amylase activity

The results in Table 3 reveal that the highest value (2.8 U/mg) of α-amylase activity was obtained from *P. pulmonarius* cultivated on rice straw and cotton waste of solid state of fermentation (SSF). While the highest value (2.9 U/mL) of α-Amylase activity was estimated from *P. pulmonarius* and *P. sajor-caju* cultivated on rice straw of liquid fermentation (SmF). On the other

hand, the lowest value (2.1 U/mg and 2.2 U/mL) of α-Amylase activity was obtained from *P. columbinus* cultivated on rice straw of both SSF and SmF. On the other hand, the lowest value (2.1 U/mg and 2.2 U/mL) of α-Amylase activity was obtained from *P. columbinus* cultivated on rice straw of both SSF and SmF. All types of enzymes detected for their activity during this study were α-Amylase, β-amylase, cellulase, cellobiohydrolase, laccase, lignin peroxidase, manganese peroxidase, and protease. These were equilibrium for each of both fermentation conditions SSF and SmF. When *Pleurotus* spp. are cultivated under SSF in lignocellulosic substrates, they can produce cellulases and xylanases [31].

Table 2. Total yield and biological efficiency of *Pleurotus* sp on rice straw, sugarcane bagasse, and cotton wastes.

Oyster mushroom species	Type of waste	First flush (28 days) (%)	Second flush (35 days) (%)	Third flush (45 days) (%)
P. ostreatus	Rice straw	84.0 ± 3.1b[z]	66.2 ± 0.68c	45.2 ± 0.86g
	Sugarcane bagasse	77.4 ± 4.25d	61.2 ± 0.97e	57.2 ± 0.71c
	Cotton wastes	82.4 ± 3.25c	70.4 ± 2.10b	51.4 ± 0.66f
P. columbinus	Rice straw	83.0 ± 4.18bc	78.8 ± 1.78a	63.6 ± 0.63a
	Sugarcane bagasse	81.4 ± 2.16c	74.4 ± 0.74bc	60.0 ± 0.57bc
	Cotton wastes	83.8 ± 3.17b	67.8 ± 0.86c	45.8 ± 0.59g
P. pulmonarius	Rice straw	84.0 ± 4.19b	72.8 ± 2.17bc	57.4 ± 0.63c
	Sugarcane bagasse	69.6 ± 1.14e	62.2 ± 0.98e	46.8 ± 0.46g
	Cotton wastes	85.4 ± 2.16ab	76.8 ± 2.15a	41.0 ± 0.78h
P. sajor-caju	Rice straw	87.2 ± 3.24a	76.4 ± 1.63b	62.0 ± 0.67b
	Sugarcane bagasse	83.4 ± 2.89b	64.2 ± 0.97d	52.6 ± 0.77f
	Cotton wastes	81.6 ± 3.15c	67.0 ± 1.99c	55.0 ± 0.64e
P. floridanus	Rice straw	84.4 ± 4.12ab	64.0 ± 3.12d	55.8 ± 0.61e
	Sugarcane bagasse	79.6 ± 2.25c	65.8 ± 2.15d	62.4 ± 0.54a
	Cotton wastes	84.6 ± 2.17ab	72.0 ± 1.85bc	58.4 ± 0.36c

[z]Each value represented the mean value of three replicates ± SE. Data were analyzed by Duncan's multiple range tests (DMRT) at $p \leq 0.05$ level.

Table 3. Assay of α-Amylase activity from the filtrate of five *Pleurotus* spp. (*P. ostreatus, P. columbinus, P. pulmonarius, P. sajor-caju,* and *P. floridanus*) cultivated on three agro-wastes (rice straw, sugarcane bagasse, and cotton waste of solid state of fermentation (SSF) and liquid fermentation (SmF).

Type of mushroom / Type of waste		Filtrate from (SSF) (U/mg)	Filtrate from (SmF) (U/mL)
P. ostreatus	Rice straw	2.2 ± 0.03d[z]	2.5C ± 0.04c
	Sugarcane bagasse	2.3 ± 0.02d	2.6 ± 0.05c
	Cotton wastes	2.5 ± 0.04c	2.5 ± 0.04c
P. columbinus	Rice straw	2.1 ± 0.01d	2.2 ± 0.03e
	Sugarcane bagasse	2.4 ± 0.04c	2.8 ± 0.02a
	Cotton wastes	2.6 ± 0.03b	2.3 ± 0.04d
P. pulmonarius	Rice straw	2.8 ± 0.02a	2.9 ± 0.03a
	Sugarcane bagasse	2.4 ± 0.03c	2.8 ± 0.05a
	Cotton wastes	2.8 ± 0.01a	2.6 ± 0.02c
P. sajor-caju	Rice straw	2.7 ± 0.04a	2.9 ± 0.03a
	Sugarcane bagasse	2.6 ± 0.02b	2.8 ± 0.04a
	Cotton wastes	2.6 ± 0.03b	2.6 ± 0.02c
P. floridanus	Rice straw	2.6 ± 0.02b	2.7 ± 0.03b
	Sugarcane bagasse	2.6 ± 0.01b	2.3 ± 0.02d
	Cotton wastes	2.7 ± 0.02a	2.4 ± 0.04d

[z]Each value represented the mean value of three replicates ± SE. Data were analyzed by Duncan's multiple range tests (DMRT) at $p \leq 0.05$ level.

3.3.2. β-Amylase activity

Data in Table 4 showed that the highest value (1.76 U/mg) of β-amylase activity was obtained from *P. sajor-caju* cultivated on rice straw of SSF. However, the highest value (1.95 U/mL) of β-amylase activity was estimated from *P. floridanus* cultivated on

sugarcane bagasse of SmF. On the other hand, the lowest value of β-amylase activity (1.51 U/mg) was obtained from *P. floridanus* on rice straw of SSF. In contrast, the lowest value of β-amylase activity (1.37 U/mL) was obtained from *P. columbinus* on the cotton waste of SmF. On the other hand, the lowest value of β-amylase activity (1.51 U/mg) was obtained from *P. floridanus* on rice straw of SSF. In comparison, the lowest value of β-amylase activity (1.37 U/mL) was obtained from *P. columbinus* on the cotton waste of SmF. It was observed during this study that α-Amylase, more active than β-amylase. The highest value (2.90 U/mL) of α-Amylase activity was estimated from *P. pulmonarius,* and *P. sajor-caju* cultivated on rice straw of liquid fermentation (SmF).

Table 4. Assay of β-amylase activity from the filtrate of five *Pleurotus* spp. (*P. ostreatus, P. columbinus, P. pulmonarius, P. sajor-caju,* and *P. floridanus*) cultivated on three agro-wastes (rice straw, sugarcane bagasse, and cotton waste of solid state of fermentation (SSF) and liquid fermentation (SmF).

Type of mushroom / Type of waste		Filtrate from (SSF) (U/mg)	Filtrate from (SmF) (U/mL)
P. ostreatus	Rice straw	1.60 ± 0.02c[z]	1.654 ± 0.13c
	Sugarcane bagasse	1.76 ± 0.03a	1.820 ± 0.16b
	Cotton wastes	1.73 ± 0.01a	1.76 ± 0.18b
P. columbinus	Rice straw	1.61 ± 0.03c	1.55 ± 0.09d
	Sugarcane bagasse	1.68 ± 0.02b	1.49 ± 0.15d
	Cotton wastes	1.65 ± 0.01b	1.37 ± 0.19e
P. pulmonarius	Rice straw	1.58 ± 0.03c	1.61 ± 0.09c
	Sugarcane bagasse	1.59 ± 0.02c	1.50 ± 0.08d
	Cotton wastes	1.56 ± 0.01d	1.59 ± 0.11c
P. sajor-caju	Rice straw	1.76 ± 0.03a	1.39 ± 0.12f
	Sugarcane bagasse	1.59 ± 0.02c	1.42 ± 0.13e
	Cotton wastes	1.67 ± 0.03b	1.75 ± 0.14b
P. floridanus	Rice straw	1.51 ± 0.02e	1.87 ± 0.12a
	Sugarcane bagasse	1.56 ± 0.02d	1.95 ± 0.13a
	Cotton wastes	1.62 ± 0.03c	1.45 ± 0.12e

[z]Each value represented the mean value of three replicates ± SE. Data were analyzed by Duncan's multiple range tests (DMRT) at $p \leq 0.05$ level.

Table 5. Assay of cellulase activity from the filtrate of *Pleurotus* spp. (*P. ostreatus, P. columbinus, P. pulmonarius, P. sajor-caju,* and *P. floridanus*) cultivated on three agro-wastes (rice straw, sugarcane bagasse, and cotton waste) of solid-state of fermentation (SSF) and liquid fermentation (SmF).

Type of mushroom / Type of waste		Filtrate from (SSF) (U/mg)	Filtrate from (SmF) (U/mL)
P. ostreatus	Rice straw	0.89 ± 0.03a[z]	1.11 ± 0.06b
	Sugarcane bagasse	0.86 ± 0.04c	1.10 ± 0.07b
	Cotton wastes	0.87 ± 0.02c	1.11 ± 0.05b
P. columbinus	Rice straw	1.00 ± 0.05a	1.17 ± 0.04a
	Sugarcane bagasse	0.92 ± 0.04b	1.00 ± 0.02c
	Cotton wastes	0.88 ± 0.02b	1.01 ± 0.05c
P. pulmonarius	Rice straw	0.86 ± 0.03c	1.14 ± 0.06a
	Sugarcane bagasse	0.83 ± 0.04d	1.14 ± 0.07a
	Cotton wastes	0.84 ± 0.01d	1.16 ± 0.06a
P. sajor-caju	Rice straw	0.72 ± 0.03f	1.00 ± 0.03c
	Sugarcane bagasse	0.70 ± 0.04g	0.92 ± 0.02d
	Cotton wastes	0.75 ± 0.03e	0.99 ± 0.04c
P. floridanus	Rice straw	0.73 ± 0.02e	0.89 ± 0.01d
	Sugarcane bagasse	0.71 ± 0.04f	0.90 ± 0.03d
	Cotton wastes	0.75 ± 0.03e	0.89 ± 0.04d

[z]Each value represented the mean value of three replicates ± SE. Data were analyzed by Duncan's multiple range tests (DMRT) at $p \leq 0.05$ level.

However, the highest value (1.95 U/mL) of β-amylase activity was estimated from *P. floridanus* cultivated on sugarcane bagasse of SmF. Keay and Wildi [42] observed that the highest activity CMCase and amylase were 0.6 ± 0.05 U/mL and 1.1 ± 0.06 mL of amylase at very low titers. However, minimum activities of this enzyme were observed by *P. ostreatus*, and *P. sajor-caju* cultivated in rice straw: 0.090 U/mL and 0.087 U/ mL. Similar results were found by when cultivated *P. ostreatus* in rice straw. While, McComb, and Yushok, [33] reported that the highest activity amylase activities in SSF were found as 6 U/L by *P. ostreatus* on the 7thday. The maximum amylase activity was observed in dry PPW, pre-treated with KOH on 15 days of incubation.

3.3.3. Cellulase activity

Table 5 showed that the highest values (1.00 U/mg) and (1.17 U/mL) of cellulase activity from the filtrate were obtained from *P. columbinus* cultivated on rice straw of SSF and SmF, respectively. While the lowest value (0.70 U/mL) of cellulase activity from the filtrate was obtained from *P. sajor-caju* cultivated on sugarcane bagasse of SSF, and (0.89 U/mL) was obtained from *P. floridanus* on rice straw of SmF. Moreover, (0.89 U/mL) was obtained from *P. floridanus* on rice straw of SmF. It was found that during these results, the highest values (1.00 U/mg) and (1.17 U/mL) of cellulase activity from filtrate at 14 days were obtained from *P. columbines* cultivated on rice straw of SSF and SmF, respectively.

3.3.4. Cellobiohydrolase activity

The results in Table 6 reveal that the highest value (0.82 U/mg) of cellobiohydrolase activity of filtrate was obtained from *P. columbinus* cultivated on rice straw of SSF. At the same time, the highest value (1.66 U/mL) of the cellobiohydrolase activity of filtrate was obtained from *P. ostreatus*, and *P. columbinus* cultivated on the cotton waste of SmF. On the other hand, the lowest value (0.50 U/mg) of cellobiohydrolase activity of filtrate was estimated from *P. floridanus on* sugarcane bagasse of SSF. Also, the lowest value (1.08 U/mL) of cellobiohydrolase activity of filtrate was obtained from *P. sajor-caju* on the cotton waste of SmF. On the other hand, the lowest value (0.50 U/mg) of cellobiohydrolase activity of filtrate was estimated from *P. floridanus on* sugarcane bagasse of SSF. Also, the lowest value (1.08 U/mL) of the cellobiohydrolase activity of filtrate was obtained from *P. sajor-caju* on the cotton waste of SmF. Moreover, the highest value (1.66 U/mL) of the cellobiohydrolase activity of filtrate was obtained from *P. ostreatus* and *P. columbinus* cultivated on the cotton waste of SmF. However, in other results, total cellulase activity was observed to a high value at 10 days of fermentation for both strains. For *P. ostreatus* it was observed as 3.51 units, while for *P. sajor-caju* it was 0.82 units.

Table 6. Assay of cellobiohydrolase (CBH) activity from the filtrate of five *Pleurotus* spp. (*P. ostreatus, P. columbinus, P. pulmonarius, P. sajor-caju,* and *P. floridanus*) cultivated on three agro- wastes (rice straw, sugarcane bagasse, and cotton waste) of solid-state of fermentation (SSF) and liquid fermentation (SmF).

Type of mushroom / Type of waste		Filtrate from (SSF (U/mg)	Filtrate from (SmF) (U/mL)
P. ostreatus	Rice straw	0.60 ± 0.03d[z]	1.65 ± 0.12a
	Sugarcane bagasse	0.72 ± 0.02b	1.64 ± 0.13a
	Cotton wastes	0.75 ± 0.04b	1.66 ± 0.15a
P. columbines	Rice straw	0.82 ± 0.05a	1.55 ± 0.09b
	Sugarcane bagasse	0.77 ± 0.03b	1.45 ± 0.16c
	Cotton wastes	0.79 ± 0.04a	1.66 ± 0.11a
P. pulmonarius	Rice straw	0.60 ± 0.03d	1.41 ± 0.21c
	Sugarcane bagasse	0.62 ± 0.02c	1.37 ± 0.22d
	Cotton wastes	0.64 ± 0.01c	1.29 ± 0.10e
P. sajor-caju	Rice straw	0.62 ± 0.03c	1.10 ± 0.09g
	Sugarcane bagasse	0.61 ± 0.02c	1.11 ± 0.17f
	Cotton wastes	0.61 ± 0.04c	1.08 ± 0.08g
P. floridanus	Rice straw	0.62 ± 0.03c	1.34 ± 0.16d
	Sugarcane bagasse	0.50 ± 0.02f	1.25 ± 0.11e
	Cotton wastes	0.51 ± 0.01e	1.15 ± 0.09f

[z]Each value represented the mean value of three replicates ± SE. Data were analyzed by Duncan's multiple range tests (DMRT) at $p \leq 0.05$ level.

On the other hand, the low value of three components of cellulase complex, namely cellobiohydrolase, CMCase, and β-glucosidase with specific activity levels of 10.00, 71.40, and 21.60 U/mg protein that the cellulase and xylanase activities increased during the after 15 days were found in *P. sajor-caju* when grown on cotton-waste [45]. Matsumoto found that development of the fruiting bodies, with highest levels during mushroom maturation [46]. While, Low value of three components of cellulase complex, namely cellobiohydrolase, CMCase, and β-glucosidase with the specific activity level of 10.00, 71.40, and 21.60 U/mg protein after 15 days were observed in *P. sajor-caju* when cultivated on cotton-waste [25].

Table 7. Assay of laccase activity from the filtrate of five *Pleurotus* sp. (*P. ostreatus, P. columbinus, P. pulmonarius, P. sajor-caju,* and *P. floridanus*) cultivated on three agro- wastes (rice straw sugarcane bagasse and cotton waste) of solid-state of fermentation (SSF) and liquid fermentation (SmF).

Type of mushroom / Type of waste		Filtrate from (SSF) (U/mg)	Filtrate from (SmF) (U/mL)
P. ostreatus	Rice straw	1.35 ± 0.02d[z]	1.85 ± 0.06b
	Sugarcane bagasse	1.32 ± 0.03d	1.99 ± 0.05a
	Cotton wastes	1.34 ± 0.01d	1.86 ± 0.04b
P. columbinus	Rice straw	1.36 ± 0.04c	1.87 ± 0.05b
	Sugarcane bagasse	1.33 ± 0.02d	1.89 ± 0.06b
	Cotton wastes	1.36 ± 0.03c	1.84 ± 0.04c
P. pulmonarius	Rice straw	1.41 ± 0.04b	1.86 ± 0.03b
	Sugarcane bagasse	1.41 ± 0.05a	1.87 ± 0.02b
	Cotton wastes	1.42 ± 0.01a	1.89 ± 0.01b
P. sajor-caju	Rice straw	1.38 ± 0.03c	1.77 ± 0.03d
	Sugarcane bagasse	1.36 ± 0.02c	1.71 ± 0.04d
	Cotton wastes	1.39 ± 0.04b	1.72 ± 0.02d
P. floridanus	Rice straw	1.41 ± 0.02a	1.81 ± 0.05c
	Sugarcane bagasse	1.42 ± 0.01a	1.81 ± 0.03c
	Cotton wastes	1.44 ± 0.03a	1.78 ± 0.02e

[z]Each value represented the mean value of three replicates ± SE. Data were analyzed by Duncan's multiple range tests (DMRT) at $p \leq 0.05$ level.

Table 8. Assay of lignin peroxidase activity from the filtrate of five *Pleurotus* spp. (*P. ostreatus, P. columbinus, P. pulmonarius, P. sajor-caju,* and *P. floridanus*) cultivated on three agro- wastes (rice straw sugarcane bagasse, and cotton waste) of solid-state of fermentation (SSF) and liquid fermentation (SmF).

Type of mushroom / Type of waste		Filtrate from(SSF) (U/mg)	Filtrate from (SmF) (U/mL)
P. ostreatus	Rice straw	0.94 ± 0.05b[z]	1.64 ± 0.17e
	Sugarcane bagasse	0.92 ± 0.06b	1.73 ± 0.16c
	Cotton wastes	0.93 ± 0.07b	1.71 ± 0.22d
P. columbinus	Rice straw	0.88 ± 0.03c	1.86 ± 0.23a
	Sugarcane bagasse	0.90 ± 0.05c	1.84 ± 0.16b
	Cotton wastes	0.88 ± 0.04c	1.76 ± 0.11c
P. pulmonarius	Rice straw	0.78 ± 0.06d	1.54 ± 0.21g
	Sugarcane bagasse	0.92 ± 0.05b	1.60 ± 0.12f
	Cotton wastes	0.96 ± 0.04a	1.58 ± 0.10f
P. sajor-caju	Rice straw	0.86 ± 0.03c	1.45 ± 0.13h
	Sugarcane bagasse	0.87 ± 0.05c	1.47 ± 0.12h
	Cotton wastes	0.92 ± 0.06b	1.49 ± 0.14h
P. floridanus	Rice straw	0.93 ± 0.04b	1.75 ± 0.17c
	Sugarcane bagasse	0.91 ± 0.05b	1.73 ± 0.19c
	Cotton wastes	0.94 ± 0.03b	1.71 ± 0.18d

[z]Each value represented the mean value of three replicates ± SE. Data were analyzed by Duncan's multiple range tests (DMRT) at $p \leq 0.05$ level.

3.3.5. Laccase activity

The results in Table 7 reveal that the highest value (1.44 U/mg) of laccase activity of filtrate was obtained from *P. floridanus* cultivated on the cotton waste of SSF. While the highest value (1.99 U/mL) of laccase activity of filtrate was estimated from *P. ostreatus* cultivated on sugarcane bagasse of SmF. On the other hand, the lowest value (1.32 U/mg) was obtained from *P. ostreatus* cultivated on sugarcane bagasse of SSF and (1.71 U/mL) from *P. sajor-caju* on sugarcane bagasse of SmF.

3.3.6. Lignin peroxidase

Table 8 showed that the highest value (0.96 U/mg) of lignin peroxidase activity was obtained from the filtrate of *P. pulmonarius* cultivated on the cotton waste of SSF. On the other hand, the highest value (1.86 U/mL) of the lignin peroxidase activity was estimated from *P. columbinus* cultivated on rice straw of SmF. The lowest value (0.78 U/mg) lignin peroxidase activity was observed from *P. pulmonarius* on rice straw of SSF. In contrast, the lowest value (1.45 U/mL) was obtained from *P. sajor-caju* on rice straw of SmF. In comparison, the lowest value (1.45 U/mL) was obtained from *P. sajor-caju*on rice straw of SmF. It was observed that the highest value (1.86 U/mL) of the lignin peroxidase activity through these results was estimated from *P. columbinus* cultivated on rice straw of SmF. However, [12] observed high laccase and manganese peroxidase activity during the colonization stage and declined activity during the first primordial formation in *P. ostreatus*. Page et al. [21] reported that *P. ostreatus* growth stimulated higher hydrolysis of lignin (80.36%), followed by hemicellulose (78.64%) and cellulose (60.37%). Significant differences between one and two harvests were also found for the production parameters (biological efficiency and yield) for both types of liquid and solid.

3.3.7. Manganese peroxidase

The results in Table 9 reveal that the highest value (0.58 U/mg) of manganese peroxidase activity was obtained from the filtrate of *P. ostreatus* cultivated on rice straw of SSF. While, the highest value (2.00 U/mL) of manganese peroxidase activity was estimated from the filtrate of *P. columbinus* cultivated on the rising straw of SmF. The lowest value (0.39 U/mg) was estimated from *P. floridanus* on the cotton waste of SSF, and (1.09 U/mL) was obtained from *P. ostreatus* on the cotton waste of SmF. The lowest value (0.39 U/mg) was estimated from *P. floridanus* on the cotton waste of SSF, and (1.09 U/mL) was obtained from *P. ostreatus* on cotton wastes of SmF. On the other hand, the lowest value (0.39 U/mg) of enzymatic activity during this study was obtained from the assay of manganese peroxidase activity from *P. floridanus* cultivated on the cotton waste of SSF Table 9.

Table 9. Assay of manganese peroxidase activity from the filtrate of five *Pleurotus* spp. (*P. ostreatus, P. columbinus, P. pulmonarius, P. sajor-caju,* and *P. floridanus*) cultivated on three agro-wastes (rice straw, sugarcane bagasse, and cotton waste) of solid-state of fermentation (SSF) and liquid fermentation (SmF).

Type of mushroom / Type of waste		Filtrate from (SSF) (U/mg)	Filtrate from (SmF) (U/mL)
P. ostreatus	Rice straw	0.58 ± 0.03a[z]	1.15 ± 0.12f
	Sugarcane bagasse	0.56 ± 0.02b	1.12 ± 0.13f
	Cotton wastes	0.52 ± 0.04e	1.09 ± 0.21f
P. columbinus	Rice straw	0.49 ± 0.01f	2.00 ± 0.17a
	Sugarcane bagasse	0.49 ± 0.03f	1.98 ± 0.18a
	Cotton wastes	0.45 ± 0.05g	1.97 ± 0.22a
P. pulmonarius	Rice straw	0.52 ± 0.02e	1.89 ± 0.13b
	Sugarcane bagasse	0.49 ± 0.01f	1.87 ± 0.14b
	Cotton wastes	0.50 ± 0.03f	1.89 ± 0.10b
P. sajor-caju	Rice straw	0.53 ± 0.04d	1.82 ± 0.16c
	Sugarcane bagasse	0.55 ± 0.02c	1.83 ± 0.20c
	Cotton wastes	0.56 ± 0.03b	1.84 ± 0.19c
P. floridanus	Rice straw	0.50 ± 0.04f	1.76 ± 0.23d
	Sugarcane bagasse	0.41 ± 0.02h	1.72 ± 0.21e
	Cotton wastes	0.39 ± 0.05h	1.78 ± 0.17d

[z]Each value represented the mean value of three replicates ± SE. Data were analyzed by Duncan's multiple range tests (DMRT) at $p \leq 0.05$ level.

3.3.8. Protease activity

Data in Table 10 showed that the highest value (22.56 U/mg) of the assay of protease activity from the filtrate was obtained from *P. ostreatus* cultivated on cotton wastes of a solid medium. While the highest value (23.80 U/mL) of the assay of protease activity from the filtrate was estimated from *P. sajor-caju* cultivated on rice straw of SmF. The lowest value was observed from (22.32 U/mg) and (23.62 U/mL) from *P. floridanus* on the cotton waste of both SSF and SmF. It was observed that the highest value of enzymatic activity was obtained in this study from protease (23.80 U/mL) on SmF and (22.56 U/mg) on SSF Table 10. While, Bradford [44] recorded the maximum protein values were 89.20 ± 1.10 and 82.90 ± 1.10 ppm in SSF by dry PPW pretreated with distilled water and KOH on the 7thday of cultivation, while their values of fresh PPW were found as 36.70 ± 0.7 and 37.40 ± 0.7 ppm. Submerged media with complex sources provide higher protease yields compared to simple media, such as casein or gelatin [50]. The maximum proteolytic activity was observed from *P. ostreatoroseus* (142.22 U/mL), while the minimum proteolytic activity (24.88 U/mL) was observed. The results of Paranthaman et al. [43] study with *P. ostreatoroseus* were higher than the ones [40], who found a significant proteolytic activity (7.89 U/mL) from *P. ostreatoroseus* using cupuaçuexocarp with 20% rice bran as substrate.

In general, it was observed that rice straw was a suitable substrate for enzymatic activity and gave high enzymatic production by all *Pleurotus* spp. Also, it was observed that *P. sajor-caju*and, *P. columbinus* were associated with rice straw. At the same time, *P. ostreatus* and *P. floridanus* were associated with sugarcane bagasse. On the other hand, *P. ostreatus* and *P. columbinus* were associated with cotton waste.

Table 10. Assay of protease activity from the filtrate of five *Pleurotus* spp. (*P. ostreatus, P. columbinus, P. pulmonarius, P. sajor-caju*, and *P. floridanus*) cultivated on three agro-wastes (rice straw, sugarcane bagasse, and cotton waste) of solid-state of fermentation(SSF) and liquid fermentation (SmF).

Type of mushroom / Type of waste		Filtrate from (SSF) (U/mg)	Filtrate from (SmF) (U/mL)
P. ostreatus	Rice straw	22.51 ± 0.04a[z]	23.75 ± 0.05a
	Sugarcane bagasse	22.52 ± 0.03a	23.78 ± 0.04a
	Cotton wastes	22.56 ± 0.01a	23.76 ± 0.02a
P. columbinus	Rice straw	22.49 ± 0.05a	23.81 ± 0.03a
	Sugarcane bagasse	22.48 ± 0.02a	23.73 ± 0.01a
	Cotton wastes	22.48 ± 0.04a	23.75 ± 0.03a
P. pulmonarius	Rice straw	22.41 ± 0.05b	23.69 ± 0.02b
	Sugarcane bagasse	22.46 ± 0.06a	23.65 ± 0.04b
	Cotton wastes	22.43 ± 0.02b	23.69 ± 0.05b
P. sajor-caju	Rice straw	22.48 ± 0.04a	23.81 ± 0.02a
	Sugarcane bagasse	22.47 ± 0.03a	23.75 ± 0.03a
	Cotton wastes	22.42 ± 0.05b	23.77 ± 0.04a
P. floridanus	Rice straw	22.55 ± 0.02a	23.65 ± 0.01b
	Sugarcane bagasse	22.36 ± 0.01b	23.69 ± 0.03b
	Cotton wastes	22.32 ± 0.03b	23.62 ± 0.05b

[z]Each value represented the mean value of three replicates ± SE. Data were analyzed by Duncan's multiple range tests (DMRT) at $p \leq 0.05$ level.

3.3.9. Soluble protein

The results in Table 11 reveal that the highest count of soluble protein determined from supernatant on the liquid medium was (40.10 mg/g) from *P. ostreatus* cultivated on sugarcane bagasse. While the highest count of soluble protein obtained from dried mycelium cultivated in liquid medium was (50.60 mg/g) from *P. columbinus* cultivated on rice straw and cotton wastes and from *P. pulmonarius* cultivated on cotton wastes. However, in dried mushrooms, the highest value (66.00 mg/g) of soluble protein was obtained from *P. floridanus* cultivated on sugarcane bagasse.

It has been reported that in this study, the highest count of soluble protein determined from supernatant on the liquid medium was (40.10 mg/g) from *P. ostreatus* cultivated on sugarcane bagasse**.** While the highest count of soluble protein obtained from dried mycelium cultivated in liquid medium was (50.60 mg/g) from *P. columbinus* cultivated on rice straw and cotton wastes and from *P. pulmonarius* cultivated on cotton wastes. Nevertheless, in dried mushrooms, the highest value **(**66.00

mg/g) of soluble protein was obtained from *P. floridanus* cultivated on sugarcane bagasse. In contrast**,** Khalil et al. [11] reported that *P. sajor-caju* was more efficient in insoluble protein production (40.20 mg/g) as compared to *P. ostreatus* (3.10 mg/g).

Table 11. Determination of soluble protein from supernatant on liquid medium, dried mycelium cultivated on liquid medium, and dried mushroom.

Type of mushroom / Type of waste		Supernatant from liquid media (mg/g)	Dried mycelium (mg/g)	Dried mushroom (mg/g)
P. ostreatus	Rice straw	37.40 ± 0.03b[z]	46.70 ± 0.06d	56.80 ± 0.01c
	Sugarcane bagasse	40.10 ± 0.02a	46.90 ± 0.04c	56.90 ± 0.02c
	Cotton wastes	38.70 ± 0.01a	46.20 ± 0.03c	55.00 ± 0.03d
P. columbinus	Rice straw	34.40 ± 0.04d	50.60 ± 0.05a	59.90 ± 0.04a
	Sugarcane bagasse	34.50 ± 0.02c	49.70 ± 0.05ab	60.00 ± 0.03a
	Cotton wastes	34.60 ± 0.03c	50.60 ± 0.03b	59.00 ± 0.02b
P. pulmonarius	Rice straw	34.70 ± 0.02b	49.70 ± 0.02ab	59.20 ± 0.01b
	Sugarcane bagasse	34.60 ± 0.01c	49.30 ± 0.01a	59.70 ± 0.02a
	Cotton wastes	35.20 ± 0.04c	50.60 ± 0.03a	60.30 ± 0.04a
P. sajor-caju	Rice straw	34.30 ± 0.03d	50.20 ± 0.05a	60.30 ± 0.04a
	Sugarcane bagasse	32.60 ± 0.02e	50.20 ± 0.04a	58.60 ± 0.03c
	Cotton wastes	38.30 ± 0.04a	47.10 ± 0.02c	60.80 ± 0.02a
P. floridanus	Rice straw	38.30 ± 0.03a	50.20 ± 0.01a	62.50 ± 0.03a
	Sugarcane bagasse	36.50 ± 0.05b	50.00 ± 0.03ab	66.00 ± 0.04a
	Cotton wastes	39.20 ± 0.06a	50.20 ± 0.04a	63.40 ± 0.02a

[z]Each value represented the mean value of three replicates ± SE. Data were analyzed by Duncan's multiple range tests (DMRT) at $p \leq 0.05$ level.

Conclusion

Our results support the use of rice straw, sugarcane bagasse, and cotton waste as substrates for the production of necessary industrial enzymes from *Pleurotus* spp. were able to produce hydrolytic and oxidative enzymes. It was observed that the submerged liquid fermentation (SmF) was suitable for the growth of all *Pleurotus* species. Also, the high value of enzymatic activity was determined through this study was higher in the submerged liquid fermentation SmF, than those produced during solid-state culture (SSF). Among proteolytic enzymes, protease is produced by the five *Pleurotus* spp., presenting the highest enzymatic activity on SmF and SSF.

Conflicts of interest. There is no conflict of interest.

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