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ENZYMATIC AND BIOLOGICAL ACTIVITIES OF *Fomitopsis feei* IN BROTH MEDIA SUPPLEMENTED WITH AGRICULTURAL WASTES

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ABSTRACT

A basidiomycete*, Fomitopsis feei* (AY515327.1) was grown on Yeast extract Malt extract Broth medium (YEMB), Malt Extract Broth (MEB) and YEMB amended with extracts prepared from six agricultural wastes namely, whey, sugarcane bagasse, groundnut bran, rice bran, saw dust, dried stems of sweet sorghum individually and its exopolysaccharide production, antibacterial, antifungal, antioxidant and enzymatic activities were assayed with culture filtrates after 10 and 20 days of incubation. Exopolysaccharide production (6.6 g/lit.) was high in the medium supplemented with the extract of sweet sorghum after 20 days of incubation. Maximum growth inhibition zone (19mm) was observed on *Escherichia coli* with 10 days incubated culture medium supplemented with rice bran. Ten days incubated culture medium supplemented with saw dust showed maximum conidial germination inhibition (99.5%) of *Curvularia lunata*. Maximum antioxidant activity (93.5%) was observed with rice bran extract containing culture medium after 10 days of incubation. Lignin peroxidase (302 U/ml) and lipase activities (7.4 µm) were high in cultures amended with rice bran extract in their 10 days of incubation. Laccase (142 U/ml) and manganese peroxidase (40 U/ml) activities were high in the cultures amended with whey in their 10 and 20 days of incubations respectively. These results could be useful for evaluating liquid media to enhance *Fomitopsis feei* mycelial biomass and bio active substances.

KEY WORDS: *Fomitopsis feei*, antimicrobial, antioxidant, submerged culture, exopolysaccharide, agricultural wastes

INTRODUCTION

The search for bioactivity namely, antimicrobial, antifungal and antioxidant property has been escalating in recent years due to the rise of drug - resistant strains. Mushrooms have long been attracting a great deal of interest in many years of foods and biopharmaceuticals and are regarded as popular or effective medicines used to treat various human diseases [1,2]. Aiming at obtaining bioactive compounds from mushrooms, several researches have tried to cultivate medicinal mushrooms in solid artificial media for fruit body production rather than in submerged cultures for mycelial extract. However, submerged cultures have the potential for a higher mycelial production in a shorter period of time within a reduced space if compared to cultivation in solid media [3]. Cultivation of mushrooms on agricultural and industrial wastes is useful for obtaining food protein from such wastes and thus they can be marshalled to aid in solving many problems of global importance including protein shortages, resources recovery and environmental management. Among the 140 species studied for lignolytic activity and exopolysaccharide production in our previous studies [4,5]. *Fomitopsis feei* shown good results, hence it was selected for the present study. *Fomitopsis feei*, a basidiomycete fungus belonging to the family of Fomitopsidaceae, is common on dead hard wood trees and burned wood. This basidiomycete was also used earlier for decolorizing synthetic dyes [6].

The present work is carried out to establish suitable media and growth conditions of *Fomitopsis feei* in submerged culture using the extracts of six different agricultural wastes to determine the exopolysaccharide production, antibacterial, antifungal, antioxidant and enzymatic activities from its culture filtrate.

MATERIALS AND METHODS

Isolation and identification of fungus

The macrofungus was identified according to the spore print and other morphological characters in our laboratory from the wild fruit body of *Fomitopsis feei* collected from Pakhal forest, Warangal district, Andhra Pradesh, India. The tentatively identified fungus was phylogenetically confirmed by molecular analysis of D2 region of 28S rDNA as ***Fomitopsis feei* (AY 515327.1)** and was maintained on malt extract agar slants at 4°C and sub cultured for every two months.

Organisms used

Bacteria causing infectious diseases to both animals and humans and food spoilage causing fungal strains were tested for antimicrobial activity. *Bacillus subtilis* (ATCC-6633), *Staphylococcus aureus* (ATCC-29737), *Micrococcus luteus* (ATCC-10054), *Escherichia coli* (ATCC-2343), *Proteus*

mirabilis (NCIM-2241), *Enterobacter aerogens* (NCIM-5139), *Curvularia lunata* (KUCCC-C-12), *Aspergillus terreus* (KUCCC-A-25), and *Aspergillus fumigatus* (KUCCC-A-9) were obtained from the IMTECH, Chandigarh, NCIM, Pune and Kakatiya University Culture Collection Centre (KUCCC) respectively and were maintained on nutrient agar and potato dextrose agar slants respectively.

Preparation of extracts from agricultural wastes

Ten grams of each shade dried agricultural wastes namely, whey, sugarcane bagasse, groundnut bran, rice bran, saw dust, dried stems of sweet sorghum were mixed in 150 ml of distilled water and extracted in autoclave at 121^{o}C for 15 min. After filtration, the supernatant obtained was used for the preparation of media containing extracts of agricultural wastes.

Preparation of media having extracts of agricultural wastes

Ten milliliters of the filtrate of each agricultural waste extract obtained was mixed with 40 ml of YEMB individually.

Culture media

The following media were used (g/lit.): Malt Extract Broth (MEB) medium (Malt extract 15, Dipotassium hydrogen phosphate 1, Ammonium chloride 1, 1N Citric acid 15 ml); YEMB medium (Yeast extract 2, Calcium sulphate 1, Malt extract 10) along with the YEMB media having extracts of agricultural wastes.

Inoculation and incubation

50 ml aliquots of each type of broths distributed in 100 ml Erlenmeyer flasks were sterilized and inoculated with single 8 mm disk of mycelia from the edge of *Fomitopsis feei* mat, grown on malt extract agar for 7 days at 28°C and triplicates were maintained. After incubation under still condition at 28^{o}C for 10 days and 20 days all these broths were filtered and the filtrates were used to determine exopolysaccharide, antibacterial, antifungal, antioxidant and enzymatic studies.

Mycelial dry weight and Exopolysaccharide (EPS) determination

The mycelial dry weight was measured after repeated washing (with distilled water) of the mycelial pellet, obtained after filtration and then drying at 70°C for 15 min. To measure EPS, filtrates collected were mixed with isopropanol (1:4 ratio), stirred vigorously, then left overnight at 4^{o}C. The precipitated EPS were centrifuged at 10,000 rpm for 15 min. and the supernatants were discarded and pellet were dried and weighed for the exopolysaccharide determination [7].

Antibacterial activity

Antibacterial activity was done by agar well method. Six bacterial cultures were inoculated in nutrient broth tubes and incubated overnight. Inocula of these cultures were standardized using 0.5 Mc Farland solution [8]. Nutrient agar plates were spread with 100 µl of each inoculum by using cotton swab and 100 µl of 10 and 20 days incubated culture filtrates were added to 6 mm wells, which were made by using sterile borer. All plates were incubated at 37^{o}C for 18 hrs.

Antifungal activity

Spore germination inhibition method [9] was followed for antifungal activity. One millilitre of conidial suspension of each type of pathogenic fungal culture was placed on a slide and mixed thoroughly with 1 ml each type of both 10 and 20 days incubated culture filtrate separately. Slides were placed in a moist chamber at 25^{o}C and were observed under microscope after 24 hrs. incubation and number of germinating spores were recorded. Control was prepared using distilled water instead of culture filtrate. Percentage of germination inhibition was calculated using the following formula:

$$\text{Percentage of germination inhibition} = 100 - \frac{\text{No. of germination in treated}}{\text{No. of germination in control}} \times 100$$

Antioxidant activitiy

Antioxidant activity was performed [10] by mixing 1 ml of 0.5 mM 2,2 – diphenyl – 1 – picrylhydrazyl (DPPH) radical solution in methanol with 3 ml of each type of both 10 and 20 days incubated culture filtrates. After incubation for 30 min. in the dark, absorbance was read at 517 nm. The control contained 1 ml of DPPH solution mixed with 3 ml methanol. DPPH scavenging effect was calculated using the following equation [11]:

$$\text{DPPH scavenging effect (\%)} = \frac{A0 - A1}{A0} \times 100$$

where,

A0 = The absorbance of control
A1 = The absorbance of sample

Enzymatic Studies

Extracellular laccase (EC 1.10.3.2) activity was measured [12] by mixing 0.5 ml of distilled water, 1 ml of sodium acetate buffer (pH 4.5), 0.5 ml of substrate solution (46mM guaiacol) to 0.5 ml of culture filtrate and optical density of this mixture was read at 440 nm up to 90 sec with 30 sec of time interval. Lignin peroxidase activity (EC 1.11.1.14) was done [13] by same procedure of laccase but 0.5 ml of hydrogen peroxide (30% w/v) was added in addition to that mixture. Managanese peroxidase activity was assayed [14] using 0.5 ml of sodium tartarate (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 containing reaction mixture by reading optical density at 465 nm. For the above three enzymes under evaluation, one activity unit was defined as

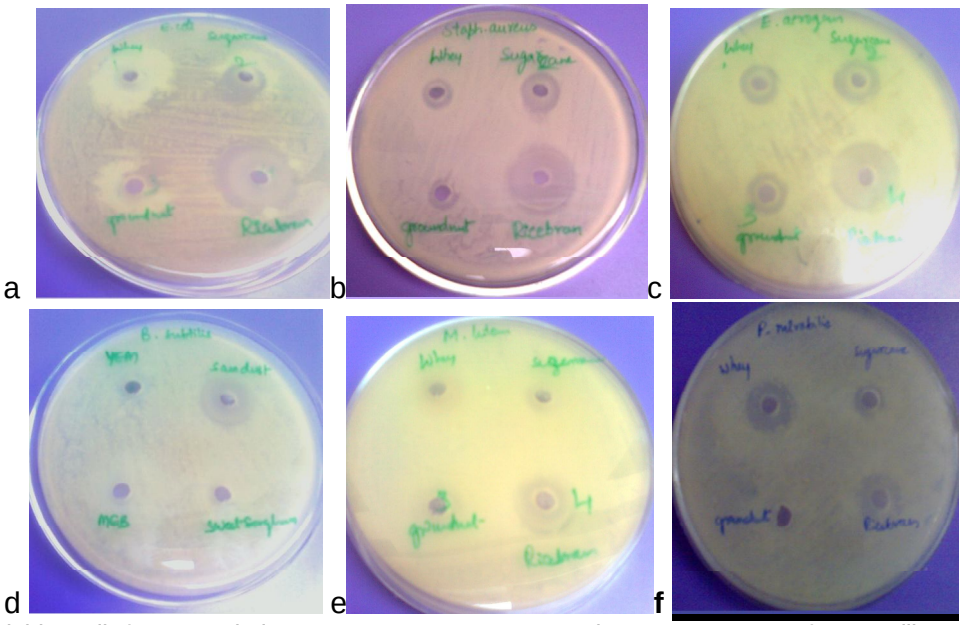
the amount of enzyme necessary to oxidize 1 µmol of substrate per minute. All these activities determined were expressed in U/ml.

Lipase (EC 3.1.1.3) activity was measured by universal titrimetric method [15, 16]. The oil – water emulsion and enzyme extract (0.1 ml: 9.9 ml: 1 ml) was titrated against 0.1N sodium hydroxide using phenolphthalein indicator. A blank (9.9 ml water: 0.1ml Tween 20: 1 ml sterilized broth) was previously run to find the standard deduction in titre value. The activity was measured as amount of enzyme required liberating one micromole equivalent fatty acid per ml/min.

RESULT AND DISCUSSION

The results of antibacterial (Table I), antioxidant and antifungal (Table II), lignolytic enzyme activities (Table III), lipase activity (Table IV), dry weight, exopolysaccharide production and pH (Table V) of *Fomitopsis feei* grown on different agricultural waste extracts amended media were presented with statistical representation i.e. mean, standard deviation (SD), standard error (SE), t-value and p-value.

Figure 1:Antimicrobial activity of filtrates of *Fomitopsis feei* grown in different agricultural waste extracts amended media.



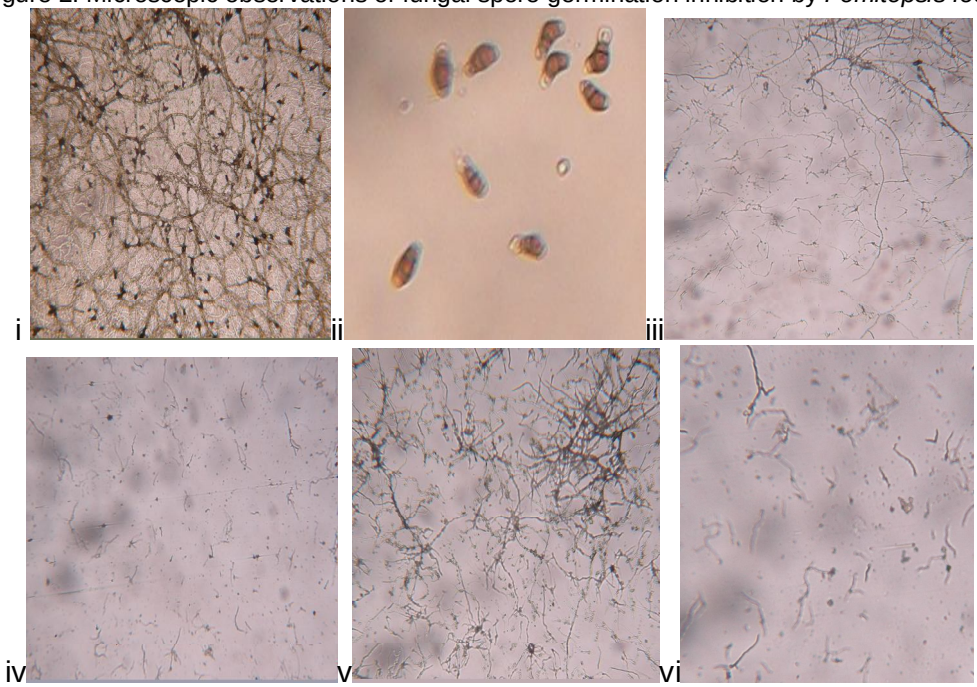
a = *Escherichia coli;* b = *Staphylococcus aureus*; c = *Enterobacter aerogens*; d = *Bacillus subtilis*; e = *Micrococcus luteus*; *f = Proteus mirabilis*.

Figure 1 and Table I showed the antibacterial activity of *Fomitopsis feei* on *Escherichia coli*, *Staphylococcus aureus, Enterobacter aerogens, Bacillus subtilis, Micrococococcus luteus* and *Proteus mirabilis*. Culture filtrates of ten days incubated rice bran extract containing medium showed good antibacterial activity on all organisms viz., *Enterobacter aerogens*, *Bacillus subtilis, Micrococococcus luteus* and *Proteus mirabilis* and the highest inhibition zone of 19 mm on *Escherichia coli* followed by 16 mm on *Staphylococcus aureus*. The report on *Lentinula edodes* also supported that rice bran may be good source for the production of antimicrobial metabolites [17]. *Enterobacter aerogens* was inhibited by all types of extracts with both ten and twenty days incubated samples. Among the substrates tested for *F. feei*, sweet sorghum extract containing medium was not suitable for producing antibacterial substances against almost all organisms. However, the results obtained were totally different from both ten and twenty days hence further work is in progress to optimize the condition for the better production of bioactive compounds.

Figure 2 showed the microscopic observations of spore germination inhibition by *F. feei* on *Curuvlaria lunata*, *Aspergillus terreus* and *Aspergillus fumigatus*. Culture filtrates of sawdust extract containing medium showed best inhibition of the germination of spores of *Curvularia lunata*. Rice bran extract containing culture filtrates showed good inhibition on all the three fungi. Ten days incubated whey extract containing culture filtrates showed maximum spore germination inhibition of *Aspergillus terreus.* Twenty days incubated sugarcane bagasse extract containing medium and rice bran extract containing medium incubated for both ten and twenty days showed maximum spore germination inhibition of *Aspergillus fumigatus* (98.5%). Highest antioxidant activity (93.5%) was recorded by ten days incubated rice bran extract containing

medium and twenty days incubated YEMB medium followed by (92.8%) sugarcane bagasse extract containing, twenty days incubated medium (Table II).

Figure 2: Microscopic observations of fungal spore germination inhibition by *Fomitopsis feei.*



i ii iii iv v vi

i Control of *Curvularia lunata spores* ii Inhibition of *C.lunata* spores by culture filtrate of ten days incubated culture medium with saw dust extract iii Control *Aspergillus terreus spores;* iv Inhibition of *A.terreus* spores by culture filtrate of ten days incubated culture medium with rice bran; v Control of *Aspergillus fumigatus* vi) Inhibition of *A.fumigatus* spores by culture filtrate of twenty days incubated culture medium with sugarcane bagasse.

Table I: Antibacterial activity of *Fomitopsis feei* (Inhibition zones in millimeter)

Agricultural Wastes	B.s.(mm)		M.l.(mm)		S.a.(mm)		P.m.(mm)		E.c.(mm)		E.a.(mm)	
Days of incubation	10	20	10	20	10	20	10	20	10	20	10	20
Whey	0	5	0	4	4	6	0	9	6	7	7	9
Sugarcanebagasse	0	0	0	0	7	0	0	4	7	5	9	4
Groundnut bran	0	0	0	0	3	0	0	0	0	2	7	1
Ricebran	13	3	10	5	16	7	12	7	19	6	14	8
YEMB	0	0	0	0	6	6	0	4	4	6	8	3
Sawdust	11	0	0	0	13	5	10	5	13	5	12	2
MEB	0	3	0	5	5	5	0	5	0	10	7	3
Sweet sorghum	0	0	0	0	3	0	0	0	0	0	6	0
Mean	3.00	1.38	1.25	1.75	7.13	3.63	2.75	4.25	6.13	5.13	8.75	3.75
SD	5.58	2.00	3.54	2.43	4.82	3.07	5.12	3.11	6.88	3.04	2.82	3.20
SE	1.97	0.71	1.25	0.86	1.71	1.08	1.81	1.10	2.43	1.08	1.00	1.13
t-Value	0.77		0.32		2.02		0.7		0.376		3.32	
P-Value	>0.05		>0.05		<0.05		>0.05		>0.05		<0.01	
Result	Not significant		Not significant		Significant		Not significant		Not significant		Significant	

B.s. = *Bacillus subtilis* (ATCC-6633); M.l.= *Micrococcus luteus* (ATCC-10054) ; S.a.= *Staphylococcus aureus* (ATCC-29737) ; P.m.= *Proteus mirabilis* (NCIM-2241); E.c.= *Escherichia coli* ATCC-2343); E.a.= *Enterobacter aerogens* (NCIM-5139); 0 = No activity.

Fomitopsis feei showed higher lignin peroxidase activity among the lignolytic enzymes (Table III). Culture filtrates of ten days incubated medium containing saw dust extract given highest lignin peroxidase activity (302 U/ml) followed by twenty days incubated whey supplemented medium (280 U/ml). Laccase activity was high with the culture filtrate of twenty days incubated medium containing whey. Manganese peroxidase activity was less with this fungus compared to laccase and lignin peroxidase activities and was high with filtrates of ten days incubated whey (40 U/ml)

and twenty days incubated sugarcane (40 U/ml) extracts containing media. Production of laccase and lignin peroxidase was best in liquid medium with this result compared to solid state fermentation on *Fomitopsis feei* from our earlier research [18].

Table II: Antioxidant and antifungal activities of *Fomitopsis feei*

Agricultural Wastes	Antioxidant (%)		C.I.(%)		A.t.(%)		A.f.(%)	
Days of incubation	10	20	10	20	10	20	10	20
Whey	0	72.6	22.3	99	0	98.5	96	0
Sugarcanebagasse	92	92.8	98.5	44	0	44	98	98.5
Groundnut bran	87	0	88.9	22.3	0	44.5	88	97.5
Ricebran	93.5	0	98	77.8	97.5	94.4	98.5	98.5
YEMB	0	93.5	88	44.5	0	55.6	97.5	98
Sawdust	84.8	0	99.5	66.7	97	44.5	0	97.5
MEB	34.5	34.5	0	0	0	0	97	55.6
Sweet sorghum	84.8	51.7	22	44.5	11.5	0	0	0
Mean	59.58	43.14	64.65	49.85	25.75	47.69	71.88	68.20
SD	41.32	40.71	42.08	31.21	44.31	36.66	44.49	44.57
SE	14.61	14.39	14.88	11.03	15.67	12.96	15.73	15.76
t-Value	0.8		0.798		0.91		0.165	
P-Value	>0.05		>0.05		>0.05		>0.06	
Result	Not significant		Not significant		Not significant		Not significant	

C.l. = *Curvularia lunata* (KUCCC-C-12) ; A.t. = *Aspergillus terreus* (KUCCC-A-25) ; A.f. = *Aspergillus fumigatus* (KUCCC-A-9) ; 0 = No activity.

Lipase activity (Table IV) was more (7.4 µm) with ten days incubated extract of rice bran containing medium followed by ten days incubated extract of saw dust containing medium (6.1 µm). Growth (Table V) of mycelia increased remarkably with incubation time and the highest mycelial growth (9.6 g/lit.) was recorded in media supplemented with the extract of whey after twenty days of incubation. Since fungal organisms takes at least 5-6 days for adequate growth, the present study was carried out for comparing the objectives for ten and twenty days. During mycelial growth, culture broth pH dropped from 4.0-6.0 to 2.0-3.0. This low pH measured after mycelial growth may indicate acid production by *Fomitopsis feei*. Exopolysaccharide production (6.6 g/lit.) was high in medium supplemented with the extract of sweet sorghum after twenty days of incubation. Production of exopolysaccharides by optimizing the medium composition in submerged culture by other white rot fungi was reported earlier from our laboratory [19, 20].

Growth pattern of *F.feei* was compared both on solid and on different submerged media [21]. The production of extracellular polysaccharide (87% glucose) was stimulated by wheat-straw on the ligninolytic fungus *Pleurotus eryngii* grown in liquid medium [22]. The liquid cultures of the white-rot fungi *Bjerkandera adusta* and *Phanerochaete chrysosporium* grown on wheat straw-containing glucose–peptone–corn steep liquor medium possessed significant levels of the pro-oxidant activity [23]. Production of exopolysaccharides by optimizing the medium composition in submerged culture was reported earlier from our laboratory.

Suspended cultures of white-rot fungus, *Trametes versicolor,* supplemented with bagasse powder showed a concentration dependent enhancement in the ligninolytic enzymes activity in liquid shake cultures [24]. The optimum pH is 3.5 and the optimum temperature is 40°C for maximum lignolytic enzymatic activity. Lignin peroxidase, which is immunological and structural different from manganese II dependent peroxidases, has been shown to be able to oxidize a number of important bonds in lignin. In recent years, agro-residues have been experimented for activity enhancement of the ligninolytic enzymes of various white-rot fungi [25]. The addition of bagasse powder (1% w/v) in stationary liquid cultures of *Pleurotus ostreatus* enhanced laccase production by 6- fold on seventh day [26].

There were many reports concerning rice bran as substrate for the production of many biological compounds such as alpha amylase [27]. In liquid basal medium, 1% (w/v) rice bran as a carbon source was found to be the most efficient substrate for laccase production compared to 1% (w/v) glucose, wheat bran and rice straw meal. The highest laccase productivity with rice bran in liquid medium was 22 U/g substrate at 15 days cultivation. This was 11 times higher than the maximum activity obtained at thirty days on solid substrate cultivation [28]. The increase growth of *Lentinus edodes* mycelium on rice bran and wheat

bran supplemented wheat straw and high activity of cellulose and xylanase enzymes in these treatments suggest that such combinations could be evaluated for cultivation of *Lentinus edodes* in regions of the world where sawdust is not readily available [29].

Table III: Lignolytic enzyme activities of *Fomitopsis feei*

Agricultural Wastes	Lac (U/ml)		Lig P (U/ml)		MnP (U/ml)	
Days of incubation	10	20	10	20	10	20
Whey	8	142	8	280	40	0
Sugarcanebagasse	6	22	12	20	0	40
Groundnut bran	2	0	0	8	0	0
Ricebran	66	58	70	62	30	0
YEMB	2	6	68	68	20	0
Sawdust	130	4	302	94	30	20
MEB	4	28	0	174	0	0
Sweet sorghum	8	0	6	0	0	0
Mean	28.25	32.50	58.25	88.25	15.00	7.50
SD	46.38	48.40	102.70	95.72	16.90	14.88
SE	16.40	17.11	36.31	33.84	5.98	5.26
t-Value	0.8		0.604		0.94	
P-Value	>0.05		>0.05		>0.05	
Result	Not significant		Not significant		Not significant	

Lac = Laccase; Lig P = Lignin peroxidase; MnP = Manganese peroxidase; 0 = No activity.

Table IV: Lipase (Lip) activity of *Fomitopsis feei*

Agricultural Wastes	Lip (µm)	
Days of incubation	10	20
Whey	1.5	1.4
Sugarcanebagasse	1.3	0.7
Groundnut bran	0.8	0.2
Ricebran	7.4	0.3
YEMB	1.5	0.9
Sawdust	6.1	0.7
MEB	2	1
Sweet sorghum	1.9	0.3
Mean	2.81	0.69
SD	2.48	0.41
SE	0.88	0.15
t-Value	2.54	
P-Value	<0.02	
Result	Significant	

Reports were there on the antimicrobial activity of culture filtrates of growth media supplemented with different agricultural wastes. Antimicrobial activity of culture filtrates has also been recorded by *Lentinus edodes* with different agricultural wastes [30]. In the present study antimicrobial activity was greater on gram negative bacteria than gram positive bacteria. It could not support the results of previous work in which mycelial-free culture of *Lentinus edodes* [31] exhibited greater antimicrobial effect against gram positive than gram negative bacteria with *Bacillus subtilis* and *Staphylococcus aureus* among the most highly inhibited. It has been reported that [17] the highest *Bacillus subtilis* growth inhibition was promoted by filtrates of growth media supplemented with the extracts of rice bran, vermiculture or molasses. The antimicrobial activity of the culture fluid of *Lentinus edodes* mycelium grown in submerged liquid culture was tested against some common bacterial species and *Candida albicans*_[32]. Xylanase production by wild-type *Aspergillus niger* ANL301, newly isolated from wood-waste, was monitored at 24 hour intervals for a period of 168 hours in media containing different carbon sources such as oat-spelt xylan (Fluka) and three

agro-wastes i.e. sawdust, sugarcane pulp and wheat bran [33].

Table V: Dryweight, exopolysaccharide (EPS) production and final Ph of *Fomitopsis feei*

Agricultural Wastes	Dry weight(g/lit.)		EPS (g/lit.)		Final pH	
Days of incubation	10	20	10	20	10	20
Whey	0.8	9.6	3.3	3.3	2.5	2.7
Sugarcanebagasse	3.8	4	0	3.3	2.5	2.7
Groundnut bran	3	3.8	3.3	3.3	2.5	2.5
Ricebran	2.8	4.2	3.3	3.3	2.5	2
YEMB	3.2	4.4	0	0	2.7	2
Sawdust	3.2	4.2	3.3	0	2	2.7
MEB	3.4	6	3.3	0	2.5	2.5
Sweet sorghum	3.4	3.8	3.3	6.6	2.7	2.5
Mean	2.95	5.00	2.48	2.48		
SD	0.92	1.99	1.53	2.33		
SE	0.32	0.70	0.54	0.83		
t-Value	2.64		0.000045			
P-Value	<0.01		>0.05			
Result	Significant		Not significant			

CONCLUSION

The results will be useful for evaluating liquid media to enhance *Fomitopsis feei* mycelial biomass and to evaluate substances of interest produced by this fungus, such as antibacterial, antifungal, antioxidant compounds and enzymes. Since this basidiomycete showed good lignolytic enzyme activities, this can be used in biobleaching and dye reduction industries. Moreover, these agricultural wastes are ecofriendly, very cheap, easily extractable hence these extracts can be used as media for the growth of microorganisms comparing to cost effective synthetic media.

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