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Bioactive compounds from discarded mushroom beds

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Abstract: Discarded dried button, dried oyster, and fresh button mushroom beds were examined for their bioactive compounds and industrial enzymes. Levels of reducing sugars and total sugars were high in all mushroom beds. Carboxymethyl cellulase activity was higher (920 µg/mL) in the dried oyster mushroom bed extract. *Schizophyllum commune*, *Fomitopsis feei*, *Trametes gibbosa*, and *Trametes elegans* were grown on direct mushroom bed extract, 0.2% glucose-containing mushroom bed extract, and common production medium for determination of growth and lignolytic activities. Since lignolytic activities were high with the direct mushroom bed extract, this medium was further diluted to 1:1 and 1:2 ratios and again tested for growth and lignolytic activities with *F. feei*, *T. gibbosa*, and *T. elegans*. It was determined that the 1:1 ratio gave good results for all these organisms. The button mushroom bed extract was concentrated using a rotary evaporator and compared for growth and lignolytic enzymes with dried powder extract using *F. feei*, *T. gibbosa*, and *T. elegans*. Growth was high with these 3 organisms in the dried powder extract, but lignin peroxidase activity was the highest with the rotary evaporator extract using *T. gibbosa*. The results indicate that these waste mushroom bed extracts can be used as cost-effective media for the growth of microorganisms and for the production of bioactive compounds and industrial enzymes.

Key words: Discarded mushroom beds, bioactive compounds, industrial enzymes, white rot fungi

Introduction

Today mushrooms are becoming more and more popular amongst people as a continental or Chinese delicacy. As the number of diabetic patients is increasing steadily in India, mushrooms can supplement a good diet for these patients with low calories and high protein value. Due to their fast growth and simple cultivation without need of any chemical fertilizers or pesticides, mushroom farming is becoming a very popular cottage industry. The government is encouraging it as a small-scale industry and giving subsidiaries for mushroom cultivation. Using agricultural waste materials, rural women are cultivating mushrooms in their spare time and even in small extra spaces in their homes. Hence, this has developed as a village industry and is earning respectable amounts of money to support rural families. It is becoming one of the important activities of the Development of Women and Children in Rural Areas groups formed in the villages to help themselves. This program has been revolutionary in all districts of the state of Andhra Pradesh and is becoming very popular in other states of India.

This mushroom production technology is considered a very attractive and useful program because it not only recycles agricultural wastes such as paddy/jowar/maize straw, cotton waste, groundnut waste, and oil cake but also produces highly nutritious

mushrooms for human consumption. It also gained significance because it is low-tech and manageable in normal conditions. With a simple 2-day training process, illiterate woman can easily practice the preparation of mushroom beds with agricultural wastes, their sterilization methods, spawning, casing, cropping, and so on. Previously, villagers were afraid of the mushrooms and never ventured to touch them. Their reasons were, first, that they did not know how to distinguish between edible and poisonous mushrooms, and, second, that the appearance of poisonous mushrooms (colored or spotted) in the house or agricultural fields was considered a bad omen. Now in the changed conditions, people are fascinated with the production and are cultivating mushrooms, for which they are receiving attractive payment.

The use of mushrooms for their bioactive compounds has been in practice for a long time, since these mushrooms can provide compounds that have nutritional, medicinal, and biological importance (1-4). However, the isolation of bioactive compounds from discarded mushroom beds is not in common practice. When our scientific group visited a village, we found huge piles of discarded mushroom beds on the outskirts of the village and saw that their disposal was becoming a problem. To solve this disposal problem and to get "wealth out of waste" (WOW), the present investigation was undertaken. Collection of these discarded beds from many villages and extraction of industrially important enzymes and bioactive compounds is the topic of this paper.

Material and methods

Collection of waste mushrooms beds

After the completion of mushroom harvesting (button and oyster), the discarded beds were collected from the villages in and around the city of Warangal and were used in the present investigation.

Preparation of extracts

The preparation of the extract from the discarded beds was done by following the methods of earlier reports, slightly modified in the present study (5,6). From the discarded dried mushroom bed, 3 g of solid fermented material was collected in the zone where spawn had spread and fruit bodies had grown. This was extracted with 40 mL of distilled water using a pestle and mortar. After homogenization, the extracts were filtered and centrifuged. The supernatant obtained was used for further studies and compared with fresh button mushroom bed extract.

A total of 950 g of fresh button mushroom bed was soaked in 4.5 L of distilled water for 1 day. After filtration and centrifugation, the obtained supernatant was used for further studies. This extract was used as broth for the production of lignin peroxidase and laccase by *Schizophyllum commune*, *Fomitopsis feei*, *Trametes gibbosa*, and *Trametes elegans*, comparing it with common production medium as control (1 g peptone, 2 g yeast extract, 1 g dipotassium hydrogen phosphate, 0.2 g magnesium sulfate heptahydrate, 5 g ammonium sulfate, 20 g glucose, and 1000 mL distilled water at pH 6.0) and the same extract containing 2% glucose after 7 and 14 days of incubation.

Since lignin peroxidase activity was high in the direct mushroom bed extract, it was further diluted with distilled water at 1:1 and 1:2 ratios and was tested for the production of lignin peroxidase and laccase by inoculation with *F. feei*, *T. gibbosa*, and *T. elegans* after 7 days of incubation.

Using a rotary evaporator, 500 mL of the fresh button mushroom extract was concentrated under vacuum. It was then weighed (approximately 4 g) and stored. The fresh button mushroom bed was dried under shade and powdered. Two grams of this powder was extracted with 0.2% glucose solution by boiling for 30 min. After filtration, the supernatant obtained was used as broth. Quantitative estimation of the laccase and lignin peroxidase activities of this extract was done and compared with the rotary evaporator extract from *F. feei*, *T. gibbosa*, and *T. elegans* that were incubated for 7 days.

pH

The pH levels of both the button and oyster mushroom bed extracts were determined by using pH papers (BDH Chemicals Ltd., UK).

Bioactive compounds

Total soluble sugars

The amount of total soluble sugars was estimated using the phenol-sulfuric acid method (7). The

quantity of total sugars was expressed as µg/mL of extract using a standard graph (100 mg/100 mL glucose).

Reducing sugars

Estimation of reducing sugars was carried out (8) and the amount of reducing sugar was calculated using a standard curve prepared from glucose (100 mg/100 mL). The quantity of reducing sugar was expressed as µg/mL of extract.

Total lipids

The total lipid content was assayed (9) using a mixture of chloroform and methyl alcohol (2:1).

Proteins

Total protein content was estimated (10) and the amount of protein in the sample was calculated using a standard graph drawn with bovine serum albumin (10 mg/100 mL).

Enzyme assays

Laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14), and manganese peroxidase (EC 1.11.1.13)

Laccase activity was measured (11) by taking the optical density of the reaction mixture prepared by mixing 0.5 mL of distilled water, 1 mL of sodium acetate buffer (pH 4.5), and 0.5 mL of substrate solution (46 mM guaiacol) to 0.5 mL of crude enzyme extract at 440 nm for up to 90 s with time intervals of 30 s. Lignin peroxidase activity was evaluated (12) by following the same procedure as for laccase, but 0.5 mL of hydrogen peroxide was also added to the mixture. Manganese peroxidase activity was assayed (13) 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.

Lipase (EC 3.1.1.3)

Lipase activity was measured using the universal titrimetric method (14,15). The oil-water emulsion and enzyme extract (0.1 mL: 9.9 mL: 1 mL) was titrated against 0.1 N sodium hydroxide using a phenolphthalein indicator. A blank (9.9 mL water : 0.1 mL Tween 20 : 1 mL sterilized broth) was previously run to find the standard deduction in titer value. The activity was expressed as the amount of enzyme required to liberate one micromole equivalent of fatty acid per milliliter per minute.

Cellulase (EC 3.2.1.4)

Cellulase activity was assayed by determination of the reducing sugars released from carboxymethyl cellulase (CMC) (16,17). A volume of 0.5 mL of the culture supernatant fluid was incubated with 1 mL of 2% CMC in 0.05 M sodium acetate buffer (pH 4.8) at 50 °C for 10 min. The reduced sugar product was assayed using the dinitrosalicylic acid method using glucose as the sugar standard. The activity was expressed in µg/mL of extract.

Protease (EC 3.4.21.99)

Protease enzyme activity was assayed by following a spectrophotometric method (18,19). The activity of the reaction mixture, which was prepared by incubating the enzyme extract with casein, was terminated by using 110 mM trichloroacetic acid. Next, the filtrate obtained after filtration through Whatman No. 1 filter paper was added to sodium carbonate and Folin-Ciocalteu reagent and incubated at 37 °C for 30 min, and its optical density was taken at 660 nm. A standard curve was prepared using tyrosine and expressed as the quantity of the enzyme that releases soluble fragments of trichloroacetic acid giving a blue color equivalent to 0.5 mg/mL tyrosine under the conditions of the assay.

Alpha amylase (EC 3.2.1.1)

Amylase activity was calculated by the following formula (20,21), and 1 unit of alpha amylase is the amount of protein that will hydrolyze 10 mL of starch/ min under specific conditions. The reaction mixture (3 mL), containing 1 mL of 0.1 M acetate buffer (pH 4.8), 0.5 mL of extract, and 1 mL of starch solution, 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 acid. Absorbance was recorded at 620 nm. A decrease in absorbance was a measure of amylase activity.

$$\text{mg of starch hydrolyzed} = \frac{\text{Abc} - \text{Abd}}{\text{Abc}} \times \text{mg of starch initially present,}$$

where Abc = absorbance of control and Abd = absorbance of digested sample.

Beta amylase (EC 3.2.1.2)

The beta amylase activity was assayed (8) using a reaction mixture contained 200 µL of soluble starch in a phosphate buffer (0.5 M, pH 7.5), 200 µL of enzyme extract, and 300 µL of phosphate buffer. The reaction was incubated for 15 min at 30 °C, then 300 µL of dinitrosalicylic acid solution was added and the mixture was boiled for 15 min. Before cooling, 100 µL 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 milligram of reducing sugar as glucose per milliliter per minute under the assay conditions.

Cellobiohydrolase (EC 3.2.1.91)

Exoglucanase or cellobiohydrolase activity was measured (22) using the reaction mixture for a reducing sugar assay containing 0.5 mL of enzyme extract, 1.5 mL of 0.05 M citric acid buffer (pH 4.8), and 0.05 g of cellulose substrate. After incubation for 1 h at 50 °C, the reaction was stopped by the addition of 2 mL of 3,5-dinitrosalicylic acid reagent. The resulting mixture was boiled for 15 min and the reduced content was measured by absorbance at 575 nm and expressed in terms of milligrams of reducing groups (as µg glucose/mL) liberated using a 100 mg/100 mL glucose standard graph.

Xylanase (EC 3.2.1.8)

Xylanase activity was assayed (23) by preparing a reaction mixture containing 0.5 mL of 0.1% (w/v) substrate in a 0.1 M sodium acetate buffer (pH 5.0) and 0.1 mL of enzyme extract. The mixture was incubated at 40 °C in a water bath with agitation for 30 min. The reducing sugar released was measured using 3,5-dinitrosalicylic acid. The color was developed by boiling in a water bath for 5 min. Absorbance was read at 540 nm and 10 µM xylose was used to prepare a standard graph. The enzyme activity was expressed in units/mL.

Catalase (EC 1.11.1.6)

Catalase activity was determined (24) using a reaction mixture containing 40 mM H_2O_2 in a 50 mM phosphate buffer (pH 7.0) and 0.1 mL of enzyme extract in a total volume of 3 mL. The absorbance of H_2O_2 was taken at 240 nm and the activity of the enzyme was expressed in units/mL of extract.

Results

Of the 3 mushroom bed extracts, the first and second were button and oyster mushroom beds collected 15 days after their harvest, while the third was a button mushroom bed collected immediately after its harvest. These mushroom bed extracts were assayed for bioactive compounds and enzymes and the results are shown in Table 1. The pH levels of the dried button, dried oyster, and fresh button mushroom bed extracts were 5, 4.5, and 4, respectively. The amounts of total sugars were 830, 390, and 720 µg/mL from the dried button mushroom bed extract, the dried oyster mushroom bed extract, and the fresh mushroom bed extract, respectively. The reducing sugar content of the dried button, dried oyster, and fresh button mushroom extracts was 720, 630, and 1330 µg/mL, respectively. The total lipid content of the dried button mushroom bed extract was 20 mg/g, while that of the oyster mushroom bed extract was 10 mg/g and the fresh button mushroom bed extract's was 70 mg/g of sample. The total protein content of the dried button, dried oyster, and fresh button mushroom bed extracts was 180, 131, and 375 µg/mL of extract, respectively.

In terms of cellobiohydrolase activity, 20 µg/mL of activity was noticed in the dried button mushroom bed extract, but there was no activity in the oyster mushroom bed extracts, and 790 µg/mL was shown in the fresh mushroom bed extract. Catalase activity was recorded from the dried button, dried oyster, and fresh button mushroom bed extracts (0.15, 0.12, and 0.5 U respectively). The oyster mushroom bed extracts did not show a positive result for protease and beta amylase activity, but 340 and 310 µg/mL of beta amylase activity was recorded from the dried button and fresh button mushroom bed extracts, respectively. Neither the dried button nor the oyster mushroom bed extract showed a positive result for the lignolytic enzyme assays, such as laccase, lignin peroxidase, and manganese peroxidase, but 48 U of laccase activity was recorded from the fresh button mushroom bed extract. In terms of xylanase activity, 8.4 U/mL was shown in the dried button mushroom

Table 1. Bioactive compounds and enzymatic activities of 3 mushroom bed extracts.

Parameters	Dried button mushroom bed extract	Dried oyster mushroom bed extract	Fresh button mushroom bed extract
pH	5	4.5	4
Total sugars (μg/mL)	830	390	720
Reducing sugars (μg/mL)	720	630	1330
Total lipids (mg/g)	20	10	70
Lipase (μm)	0.5	1.5	0.5
Proteins (μg/mL)	180	131	375
Cellobiohydrolase (μg/mL)	20	-	790
Xylanase (U/mL)	8.4	5.7	24.3
Carboxymethyl cellulase (μg/mL)	200	920	100
Protease (μg/mL)	40	-	47
Catalase (U/mL)	0.15	0.12	0.5
α-amylase (U)	1.18	9.0	6.37
β-amylase (μg/mL)	340	-	310
Manganese peroxidase (U/mL)	-	-	-
Laccase (U/mL)	-	-	48
Lignin peroxidase (U/mL)	-	-	-

- = not detected

bed extract, 5.7 U/mL was given by the oyster mushroom bed extract, and 24.3 U/mL was found in the fresh button mushroom extract. Carboxymethyl cellulase activity was 200, 920, and 100 μg/mL in the dried button, dried oyster, and fresh button mushroom bed extracts, respectively.

Table 2 shows the dry weight and the lignin peroxidase and laccase activities of *S. commune*, *F. feei*, *T. gibbosa*, and *T. elegans* in direct mushroom bed extract, extract prepared by mixing with (0.2%) glucose, and common medium after 7 and 14 days of incubation. Dry weight was high in the glucose-containing extract for all organisms. Lignin peroxidase activity (1668 U/mL) was high with the mushroom bed extract when compared to the glucose-containing extract and the common production medium for *T. gibbosa*. Laccase activity was high with the glucose-containing extract for *T. gibbosa* (1980 U/mL).

Since the mushroom bed extract gave remarkable results for lignin peroxidase and laccase activity compared to the glucose-containing extract and the common production medium, it was further diluted to 1:1 and 1:2 ratios and tested again for the same after 7 days of incubation with *F. feei*, *T. gibbosa*, and *T. elegans*. The results are given in Table 3. Dry weight, lignin peroxidase, and laccase activities were high in the 1:1 ratio medium compared to the 1:2 ratio medium.

Lignin peroxidase and laccase activity from both rotary evaporator and dried powder extracts containing broths inoculated with *F. feei*, *T. gibbosa*, and *T. elegans* were assayed after 7 days (Table 4). The dry weight of all of the organisms was high in the dried powder extract medium (4, 3.2, and 2.4 g/L, respectively), but *T. gibbosa* showed high lignin peroxidase activity (376 U/mL) and laccase activity (414 U/mL) in the rotary evaporator extract medium.

Table 2. Growth and lignolytic enzyme activity of 4 white rot fungi in 3 media.

Type of media	Type of activity	Days of incubation	*S. commune*	*F. feei*	*T. gibbosa*	*T. elegans*
Direct mushroom bed extract	**Dry weight** (g/L)	7	12.8	4.8	5.6	5.2
		14	5.2	4.8	4.8	4.6
	Lignin peroxidase (U/mL)	7	-	-	1668	-
		14	324	-	444	-
	Laccase (U/mL)	7	-	-	1860	-
		14	-	-	602	600
Mushroom bed extract + 0.2% glucose	**Dry weight** (g/L)	7	12.8	13.6	12.8	5.6
		14	18.4	10.8	14.8	6.8
	Lignin peroxidase (U/mL)	7	-	-	796	-
		14	-	-	388	-
	Laccase (U/mL)	7	-	-	1980	-
		14	-	-	1690	-
Production medium (control)	**Dry weight** (g/L)	7	4.8	6.8	9.6	7.2
		14	11.6	6.9	8.4	5.6
	Lignin peroxidase (U/mL)	7	-	56	12	-
		14	-	-	-	32
	Laccase (U/mL)	7	-	96	-	-
		14	-	-	36	-

- = no activity

Table 3. Dry weight and lignolytic enzyme activity of 3 white rot fungi using 1:1 and 1:2 ratios of button mushroom bed extract after 7 days of incubation.

Organism	Dilution of media with distilled water	**Dry weight** (g/L)	**Lignin peroxidase** (U/mL)	**Laccase** (U/mL)
F. feei	1:1	2.8	20	42
	1:2	1.2	6	-
T. gibbosa	1:1	2.4	588	602
	1:2	1.2	262	360
T. elegans	1:1	2.0	-	-
	1:2	1.2	-	-

- = No activity

Table 4. Comparison of rotary evaporator and dried powder extract media containing 3 white rot fungi for growth and lignolytic enzymes after 7 days of incubation.

Organism	**Type of media**	**pH**	**Dry weight** (g/L)	**Lignin peroxidase** (U/mL)	**Laccase** (U/mL)
F. feei	RE	3.5	1.6	6	-
	DPE	3.5	4	-	-
T. gibbosa	RE	5.0	2.4	376	414
	DPE	5.5	3.2	284	282
T. elegans	RE	5.0	0.8	-	-
	DPE	5.0	2.4	-	-

RE = media prepared from mushroom bed extract using rotary evaporator
DPE = media prepared from dried mushroom bed powder
- = no activity

The growth of these 3 organisms was better on the dried powder extract than on the rotary evaporator extract when grown on agar plates (Figure).

Discussion

Similar to our present observations, the wastes produced by mushroom forms for making compost have previously been studied (6). Furthermore, *Pleurotus* spp. have been analyzed for possible utilization as valuable bioactive compounds. The separation of sugars, lipids, and diversified enzymes from mushroom bed extracts is a novel concept for the creation of WOW. Reutilization of spent mushroom substrate (SMS) and the separation of bioactive compounds with soil properties like bulk density, stability, surface crust, temperature changes, infiltration rate, aeration, and water retention capacity have been utilized (25,26). It was also noticed in our study that temperature and water content played an important role in the extraction of compounds and enzymes.

The addition of straw to the soil caused an increase in the number of total bacteria, actinomycetes, and fungi of the rhizosphere (27). The yield of green gram increased in plots previously supplied with mushroom spent rice straw (28). Recently, a report (29) stated that *Pleurotus* waste was adequate to sustain the growth of *Salvia officinalis* by improving the air porosity and mineral content of the soil. Earlier research reported that mushroom compost waste can be used as an alternative fertilizer to farm yard manure in strawberry growing (30). Many reports have shown that mushroom compost waste can be a good culture substrate when it is mixed with soil as a farm yard fertilizer or used alone in order to replenish the physical condition of the soil (31-33). The results obtained in the present study clearly indicate that improvement in the physical and chemical status of the soil is closely associated with the compounds released by spent mushrooms and the subsequent discharge of a variety of enzymes during the decomposition process. Hence, SMS is an added advantage for the improvement of soil fertility. Recycling of *Pleurotus* waste from the cultivation of *Pleurotus sajor-caju* gave a significant yield of *Pleurotus sajor-caju* on starch-, peptone-, and wheat bran-supplemented SMS (34).

Recycling of waste mushroom substrate for mushroom cultivation has been carried out using the sawdust from waste shiitake bed log for the cultivation of *Pleurotus cornucopiae* (35). Pretreatment of waste mushroom beds and methods for converting the same to yield sugars and ethanol has been patented (36). In accordance with prior work (37), the determination of laccase enzymes from mushroom bed extracts is

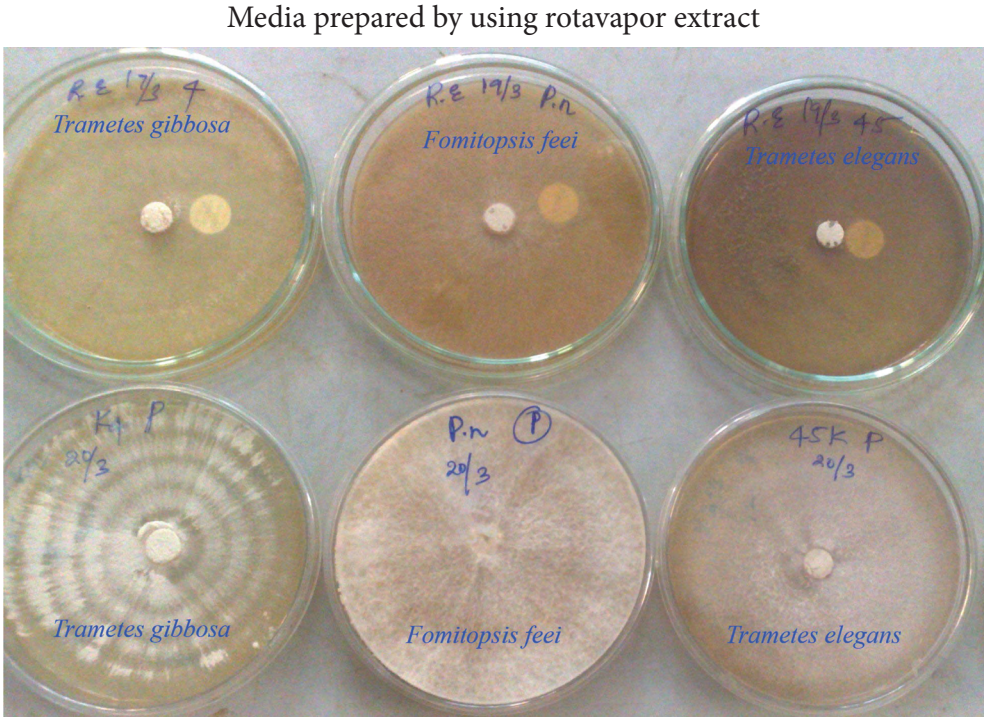

Figure. Growth pattern of 3 white rot fungi on agar plates containing media prepared using rotary evaporator and dried powder extracts.

an innovative technology for the separation of sugars from lignocellulosic materials (which are not suitable for food or fodder) for the large scale production of bioethanol to improve petrol. A change in the physicochemical properties of recycled spent mushroom compost through vermicomposting by epigeic earthworms *Eisenia foetida* and *E. andrei* was reported (38). Thermal treatment technologies were compared to determine an appropriate method of recovering energy from 2 wastes, spent mushroom compost and coal tailings (39).

As the ingredients for growth media for microorganisms are enormously increased by SMS, different bioactive compounds can effectively substitute for costly commercial media. Our present efforts were successful in the cultivation of 4 white rot fungi and establishment of production efficiencies of lignolytic enzymes during 7 and 14 days of incubation time. It has been concluded that certain bioactive compounds and industrial enzymes can be extracted from these waste beds. These waste beds can be used for the production of bioethanol, bioenergy, and biogas. Since these waste beds are ecofriendly, very cheap, easily extractable, and proven to be good media, these extracts can be used directly as media for the growth of microorganisms in comparison to cost-effective synthetic media.

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