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A Commercial-Scale, Circular-Economical Bio-Refinery Model for Sustainable Yields of Mushrooms, Cellulase-Complex, Bio-Priming Agents, Bio-Ethanol, and Bio-Fertilizer

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ABSTRACT

In the present study, 126-day-long commercial-scale demonstration of a circular-economical bio-refinery, its sequential operational stages, employing 96 kg of a substrate mixture comprising urban vegetative waste and e-commerce packaging waste, resulted in yields of: 36 kg of mushrooms; 124,800 IU of exoglucanase; 1.2 x 10^{13} seed bio-priming agents; 1.08 L of bio-ethanol; 6.76 kg of bio-compost/bio-fertilizer. The first stage of oyster mushroom cultivation resulted in degradations of 73.8% lignin and 88.2% hemicellulose. Remnant biomass from the mushroom cultivation phase was subjected to solid-state fermentation (SSF) employing *Trichoderma koningii* Oudemans Tk-7 for the production of in-house cellulases. Post SSF, the harvested spores of *T. koningii*, when used as a seed bio-priming agent, resulted in a 26% average increase in the percentages of germination. Remnant biomass from SSF, after an in-house cellulase-based saccharification and fermentation, resulted in a bio-ethanol yield of 0.43 g/g of glucose. The unsaccharified biomass fraction, when subjected to cow dung-based aerobic windrow composting, resulted in a bio-compost, which exhibited a 242.5% increase in seed germination indices. Significant variables related to bio-process and substrate utilization have been analysed. With a ~70% process-related profit margin, the proposed approach could prove to be a promising agripreneurial proposition.

Keywords: Bio-compost; Biomass pre-treatment; Bio-refinery; Germination indices; Saccharification; Seed bio-priming.

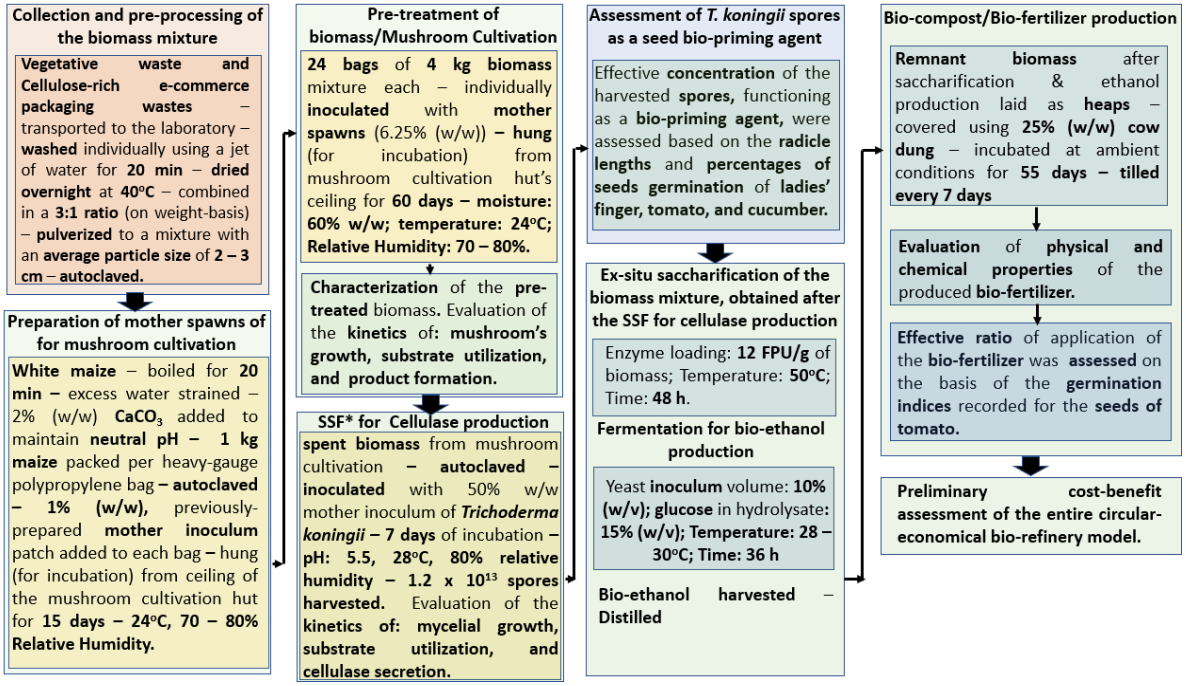
INTRODUCTION

Sky-rocketing prices of conventional 2nd generation biomass ($ 7.5 per kg (Singh *et al*., 2016)), and heavy competition between their usage in fodder and fuel industries has resulted in exploration of their alternate sources (Fradj *et al*., 2016). Globally, around 6.72 x 10^7 kg of paper-based, recyclable e-commerce-related packaging wastes, such as corrugated/non-corrugated cardboards, cushioning/stuffing paper were generated in the year 2019 ("How Much Waste Does E-commerce Create?", 2021). In India, a meagre 33% of such accumulated wastes enters the recycle chain ("Solid Waste Management in Urban India: Imperatives for Improvement," 2020). Per year, a world-wide accumulation of close to 21 x 10^8 tons ("What a Waste 2.0: A Global Snapshot of Solid Waste Management to 2050", 2022) of urban vegetative waste, such as dried leaves, twigs, and pruning has been reported. In and around the Pondicherry University's campus, annually, around 2 tons of such tree-based wastes are generated. Usually, the above-mentioned wastes are incinerated/buried as landfills, posing a threat to the environment and ecosystem. *Pleurotus ostreatus* VS-6 (Division: Basidiomycota; family: Pleurotaceae), commonly known as the edible oyster mushrooms/hiratake, can be cultivated over a mixture of such waste biomass, which doubles up as a biomass pre-treatment step as well, as elucidated in a previous work of ours (Ramamoorthy *et al*., 2023a). *Trichoderma koningii* (Division: Ascomycota; family: Hypocreaceae), known for its improved sporulation (Mulatu *et al*., 2021), is a well-reported bio-control agent, plant symbiont, and an efficient rhizosphere inhabitant (Xue *et al*., 2017); it finds significant application in around 250 commercial bio-pesticide formulations in India and around 8 such registered formulations in Brazil (Mulatu *et al*., 2021). To ameliorate seed health, bio-priming, a fairly new technique, involves pre-conditioning of seeds of plants using a stipulated quantity of fungal spores to result in improved: tolerance to abiotic/biotic stress; hydration; nutrient-uptake; seedling vigour (Singh *et al*., 2020). In the present demonstration, the spores of *Trichoderma* spp. collected after the cellulase production SSF would serve as bio-priming agents and promote microbial colonization, proliferation, and defense against phyto-pathogens (Singh *et al*., 2020). Bio-compost/bio-fertilizer, produced using remnant biomass from ethanol production, would improve soil health by enriching it with nitrogen, phosphorous, carbon,

organic matter, and would increase water-holding and moisture-retention (Mandal *et al*., 2014). Intellectual property rights have been secured for the proposed self-sustainable bio-refinery model (Pallam *et al*., 2023 DE202022107273), and the present manuscript is an in-depth demonstration of the concept. The bio-refinery, employing a waste biomass mixture-based substrate in a stage-wise process, consolidates: (i) biomass pre-treatment and cultivation of mushrooms; (ii) production of cellulase and a seed bio-priming agent; (iii) ethanol production; (iv) bio-compost/bio-fertilizer production.

MATERIALS AND METHODS

A sketch of the workflow followed in the study along with significant operational parameters has been presented in fig. 1. The procedure for collection, pre-processing, proximate analysis of biomass, preparation of mother spawns, bed spawns, mushroom cultivation bags, and characterization of the remnant biomass from mushroom cultivation are as described in a parallel study of ours (Ramamoorthy *et al*., 2023a). The remnant biomass was subjected characterization using (i) Scanning electron microscopy (SEM, Jeol, JSMIT800, Tokyo, Japan) to visualize morphological changes post a platinum sputtering-based pre-coat of the biomass; (ii) Fourier-transform Infrared Spectroscopy (FT-IR) (Bruker Alpha-II, USA) to assess variations in compositions on the basis of changes in characteristic functional groups (absorption spectra in the range of 500 - 4000 cm^{-1}); (iii) X - Ray Diffraction (XRD, Bruker D8 Advance, USA) to analyze differences in crystallinity (Eq. 1).



SSF* - Solid-state fermentation was employed for the production of cellulases using *Trichoderma koningii* Oudemans Tk-7.

Figure 1: A summarized workflow of the proposed circular-economical bio-refinery model

Cellulase production from remnant biomass obtained after mushroom cultivation

Primary inoculum for SSF was prepared using minimal media, and *Trichoderma koningii* Oudemans Tk-7's spore concentration of: (3 x 10^{10})/mL, and incubated at 28°C, for 5 days. The minimal media components are as mentioned in (Ramamoorthy *et al*., 2023b) and the reagents were procured from Hi-Media laboratory reagent manufacturers, Maharashtra, India. Biomass mixture, collected after mushroom cultivation, was autoclaved and loaded on to aerated trays (2 kg per reactor) of the SSF reactor; the biomass was homogenously mixed with 50% (v/w) of minimal media. The moist 5-cm substrate bed, incubated using 50% (w/w) primary inoculum, was maintained at pH 5.5, incubated at 28°C, for 7 days, at 50% moisture (w/w) content with an RH of 80%, and an aeration rate of 1 - 0.5 vvm. Pre-calibrated DO probes were introduced at 4 different points of the substrate bed to record the average DO values during the course of the SSF.

Estimation of enzyme activities of the cellulase complex and kinetics of nutrient utilization

The IUPAC-recommended enzyme assays were used for measurement of the individual enzyme activities of the cellulase complex (Ghose 1987). During mushroom cultivation process, every 5 days, sample volumes, each of 3 cm^2, from 6 different locations (mycelia-dense regions) of the cultivation bags were collected to record the average values of enzyme and nutrient utilization kinetics. During cellulase production SSF, every 24 h, to record average values, 6 cubes (3 cm x 3 cm x 5 cm) were excised from 3 different regions of the SSF bed.

Seed bio-priming using *T. koningii spores* harvested during cellulase production SSF

Prior to total cellulase harvest from the SSF, the koji's bed surface was flooded using 0.9% NaCl, and the collected liquid was centrifuged at 5000 RPM for 10 minutes; the pellets contained the spores of *T. koningii*. 0.9% NaCl was used to dilute the spore suspension to various concentrations of (per mL): 10^4, 10^5, 10^6, and 10^7. Seeds of tomato, cucumber and ladies' finger, procured from Krishi Vigyan Kendra, India, were repeatedly bio-primed (dispersed by spraying) with 1 mL of the stipulated spore suspension, every 4 h, and incubated at ambient temperature (28^{o}C) until dry. Post bio-priming, the seeds, then sown in soil, were incubated at ambient temperatures of 30^{o}C and 28^{o}C, during days and nights, respectively, in an open environment not directly exposed to rainfall; a moisture content of 25 - 30% (w/w) was maintained at all times. 5- 6 days, 3 - 4 days, and 12- 14 days were required for germination of the seeds of tomato, cucumber, and ladies' finger respectively. The radicle lengths and the percentages of germinations (Eq. 4.) were calculated for each seed and each trial.

Ex-situ enzyme-based saccharification, fermentation for the production of bio-ethanol

In a temperature-programmable stirred tank reactor (STR), per gram of biomass, a loading of 10 exoglucanase units of the previously-produced and harvested cellulases, along with 50mM citrate buffer (1:10 ratio (biomass : buffer)), 0.01% (w/v) sodium azide, and 0.25% (w/v) Tween-80 were introduced and saccharified at 50^{o}C for 48-h. A stipulated volume of 15% (w/v) sugar-containing saccharified hydrolysate, and 10% (w/v) of *Saccharomyces cerevisiae* RD-33 were subjected to fermentation in an STR under micro-aerophilic conditions for 30 - 36 h, at 30^{o}C. 166-proof-pure (83% w/v) bio-ethanol was obtained after distillation. The sugar consumption profiles throughout the courses of the operations were assessed using a semi-preparative thin-layer chromatographic separation-assisted colorimetric quantification. The Miller's DNSA protocol was employed for the spectrophotometric quantification of sugars (Miller 1959).

A spectrophotometric procedure was used for the quantification of bio-ethanol. 10% (w/v) of potassium dichromate was introduced into a solution of 5M sulphuric acid. To 250 µL of the fermentation sample (containing bio-ethanol), 100 µL of the potassium dichromate-sulphuric acid mixture was added and incubated for 15 min at ambient temperature. Post the development of a colour ranging between brown to bluish-green, the overall reaction volume was brought to 1 mL using distilled water. The absorbances of the fermentation samples were recorded at 595 nm using a UV-vis spectrophotometer.

Bio-compost production using remnant biomass obtained after saccharification

Post-saccharification, while the produced soluble sugars entered into fermentation stage, the remnant unsaccharified/partially-saccharified biomass, was collected, heaped, uniformly plastered with 25% (w/w) fresh cow dung and incubated at 28^{o}C (ambient temperature) in an open environment free from rainfall, for 55 days. During the aerobic windrow composting, tilling was performed using shovels every 7 days; the dimensions of the triangularly-shaped pile was (in cm): 60 x 60 x 60 (L x B x H). The produced compost was mixed in various ratios (as mentioned in fig. 9) with soil, and its efficiency was evaluated on the basis of germination indices (Eq. 5) recorded for the seeds of tomato. The bio-compost's physical and chemical properties were evaluated and compared against compost standards prescribed by Fertilizer Control Order (FCO), Ministry of Agriculture and Rural Development, government of India.

RESULTS & DISCUSSION

Analysing the efficacy of biomass pre-treatment/mushroom cultivation, and substantiating the observations on the basis of the kinetics of substrate utilization and mushroom formation during the process

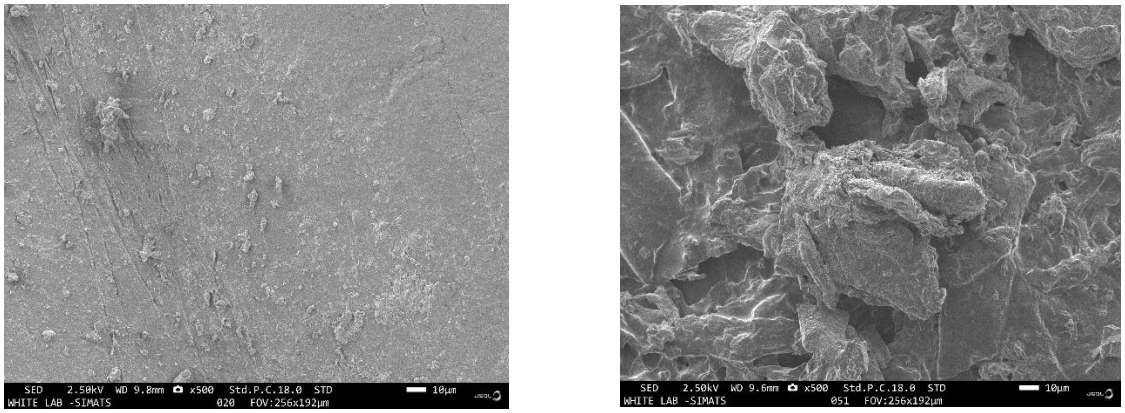
The 60-day-long mushroom cultivation of the biomass mixture resulted in (in w/w) 82.8% cellulose, 88.2% hemicellulose, and 73.8% lignin degradations (tab.1). Once introduced into mushroom cultivation bags, during the adaptation period between 0-5 days, the growing mycelia of mother spawns preferentially-consumed and depleted the maize kernel's starch before adapting to the biomass mixture-based substrate (Zhang *et al*., 2015).

Table 1: A table summarizing variations in the substrate mixture's composition during every phase of the proposed circular-economical bio-refinery model

PHASE I - MUSHROOM CULTIVATION PROCESS								
Proximate Analysis of a mixture of urban vegetative waste and e-commerce packaging waste								
Total weight of the biomass mixture - 96 ± 0.5 kg								
S. No.	**Total Reducing Sugars** (% w/w)				**Lignin** (% w/w)		**Others** (% w/w)	
	Glucose[c]	**Xylose[h]**	**Arabinose[h]**	**Starch**	**Acid Soluble Lignin**	**Klason Lignin**	**Protein**	**Ash**
1.	35 ± 1	6.5 ± 0.2	5.5 ± 1	2 ± 0.5	5 ± 0.1	16 ± 2	19 ± 1	6.3 ± 0.8
	After the entire phase of the 60-day-long mushroom cultivation process							
	6 ± 0.5	1.2 ± 0.2	0.22 ± 0.1	0.1	0.5 ± 0.3	4.2 ± 1	8.2 ± 0.6	3.2 ± 0.4
	Weight of the harvested mushrooms - 36 ± 2 kg							
PHASE II - CELLULASE PRODUCTION BY SOLID-STATE FERMENTATION								
2.	**Proximate analysis of the spent biomass collected after mushroom cultivation**							
	Total weight of the spent biomass mixture - 24 ± 1.1 kg							
	24 ± 1	4.8 ± 0.6	0.9 ± 0.1	0.4	2 ± 0.25	16.8 ± 0.2	32.8 ± 1	12.8 ± 0.1
	After the solid-state cellulase production process							
	18 ± 0.5	1 ± 0.2	0.74 ± 0.3	-	1.2 ± 1	10 ± 0.4	28 ± 1.1	9.6 ± 0.2
	T. koningii spores (bio-priming agents) collected after the process: 1.2×10^{13}							
PHASE III - SACCHARIFICATION AND BIO-ETHANOL PRODUCTION								
3.	**Proximate analysis of the biomass mixture collected after cellulase production**							
	Total weight of the biomass mixture - 18 ± 0.6 kg							
	24 ± 1	1.3 ± 0.2	1 ± 0.1	-	1.6 ± 0.1	13.3 ± 0.1	37.3 ± 1	13 ± 0.7
	Weight of sugars released from the biomass mixture, during ex-situ saccharification (% w/w)							
	Glucose		**Xylose**			**Arabinose**		
	16.7 ± 1		1.3 ± 0.1			1 ± 0.1		
	Bio-ethanol production from the saccharified hydrolysate							
	Weight of sugars in the saccharified hydrolysate - 3.42 ± 0.3 kg							
	Weight of bio-ethanol produced (% w/w)		**Weight of unconsumed sugars (% w/w)**					
			Glucose		**Xylose**		**Arabinose**	
	31.6 ± 0.2		26.3 ± 0.5		6.84 ± 0.3		5.26 ± 0.1	
PHASE IV - COMPOST/BIO-FERTILIZER PRODUCTION								
4.	**Proximate Analysis of the remnant biomass, after ex-situ, enzyme-based saccharification**							
	Total weight of the biomass mixture - 14.7 ± 0.5 kg							
	Glucose[c]	**Xylose[h]**	**Arabinose[h]**	**Starch**	**Acid Soluble Lignin**	**Klason Lignin**	**Protein**	**Ash**
	7.3 ± 0.2	-	-	-	2 ± 0.4	16.3 ± 0.8	45.6 ± 1	16 ± 0.6
	Weight of the compost/bio-fertilizer produced - 6.76 ± 0.7 kg							

Glucose[C] : The quantity of glucose converted to cellulose equivalents using an anhydro correction factor of 0.90; **xylose[h] and arabinose[h] :** The quantities of xylose and arabinose converted to hemicellulose equivalents using an anhydro correction factor of 0.88.

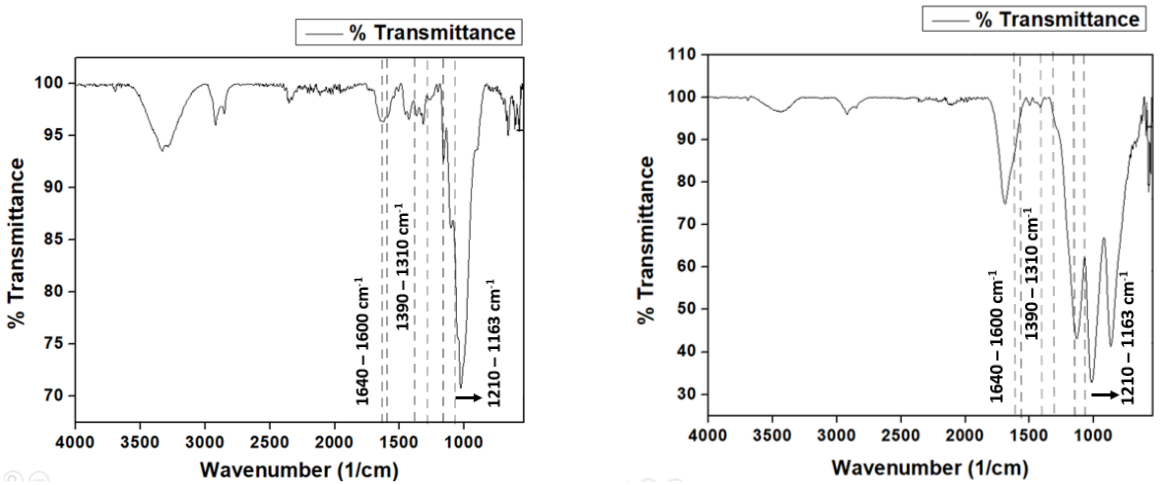
Once the mycelia sense the outermost, lignin-rich region of the biomass, based on a chemotropic response (Adamatzky *et al*., 2018), a plethora of lignin-deconstructing oxidative enzymes, such as manganese peroxidases, lignin peroxidases, versatile peroxidases, and laccases are secreted from the hyphal tips (Sonnenberg *et al*., 2022); the same was confirmed in a previous, parallel study of ours (Ramamoorthy *et al*., 2023a). The enzyme-bearing hyphal tips of the mushroom degrade lignin at the point-of-contact on the biomass surface; the degradations are visualized as surface lesions on the pre-treated biomass as observed in the scanning electron micrographs (SEM) (fig. 2 (b)).



(a) **(b)**

Figure 2: Scanning electron micrographs (SEM) of the biomass mixture (a) prior to and (b) after the mushroom cultivation process.

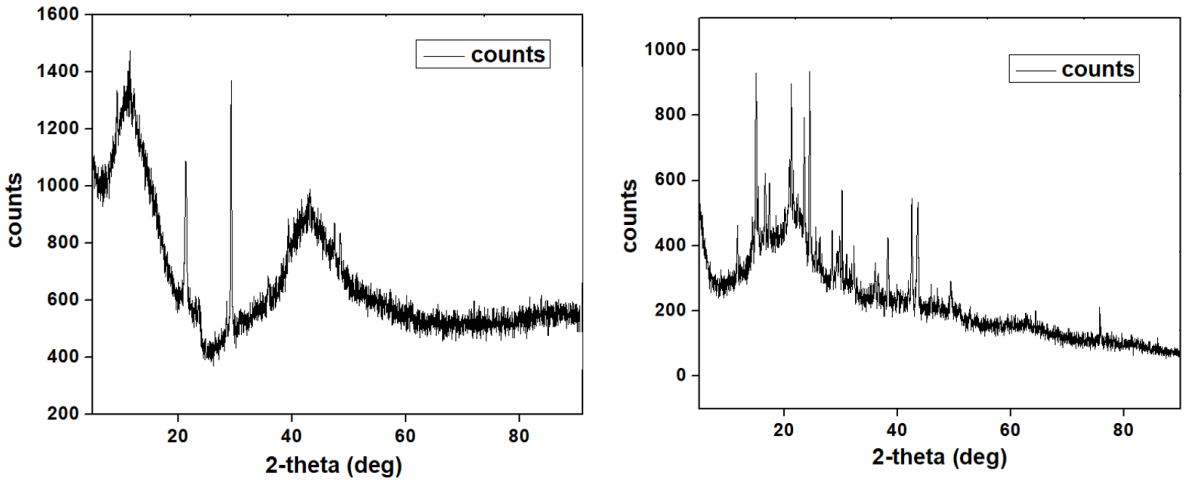
Lignin removal/depolymerization could also be attributed to the observation of an uneven/rough surface of the biomass sample in SEM post pre-treatment (fig. 2(b)) (Ramamoorthy *et al*., 2023a). Significant reductions of FT-IR spectral intensities between the untreated (fig. 3(a)) and pre-treated biomass mixture (fig. 3(b)) within the characteristic regions: (a) 1390-1310 cm^{-1} - O-H bending of phenolic groups of lignin (Awoyale *et al*., 2021); (b) 1640-1600 cm^{-1} -lignin's C=C aromatic ring vibrations (Romao *et al*., 2022). After lignin degradation, the hyphae, based on a thigmotropic/topographic-effector response (Bowen *et al*., 2007), gain entry into the hemicellulose/cellulose-rich interior of the biomass. Owing to its amorphous and readily-assimilable nature, 80% of hemicelluloses was consumed until 15 days (fig. 5). Simultaneously, gradual consumption of around 1.2% cellulose was visualized until 10 days (fig. 5). A sharp 50.5% decline in DO% until 10 days (fig. 5) elicited an oxygen limitation-based stress and a favourable cellulase production environment (Ramamoorthy *et al*., 2023a), resulting in a rapidly-ascending cellulase-production profile until 15 days. Between 15-20 days, as the mushroom's fruiting bodies were produced, the DO% gradually started increasing to 60 ± 5% until the completion of mushroom cultivation (fig. 5) at 60 days. Once the fruiting bodies began budding, the mycelia differentiated into dedicated, nutrition-providing appendages, while the fruiting bodies, containing gills begin to function as oxygen-providing appendages of the mushroom (Ramamoorthy *et al*., 2023a). With cellulolytic-mycelia extending to around 20-50 cm within the cultivation bag, accelerated cellulose consumption was visualized until the end of the process (fig. 5). Around 36 kg of mushrooms were harvested at the end of the pre-treatment/mushroom cultivation process (tab. 1). In the SEM (fig. 2(b)), a comparatively larger number of elevated, clustered, spherical mounds, and lesions over the pre-treated biomass's surface explains considerable deconstruction of cellulose and hemicellulose (Awoyale *et al*., 2021), in-turn resulting in mitigated fiber integrity and structural deformation of cells (Romao *et al*., 2022). Consequentially, crystallinity of the biomass was altered (Montalbo-Lomboy *et al*., 2015), and it was concluded based on variations in the FT-IR spectral peak intensities of the untreated and pre-treated biomass (fig. 3 (a) and (b)) between the regions: (a) 1373-1319 cm^{-1} - vibrations attributed to C-H deformations of the construction of cellulose and hemicellulose (Ramamoorthy *et al*., 2020); (b) 1710-1718 cm^{-1} - stretching vibrations among C=O of carboxylic acid or acetyl groups, which make up hemicelluloses (Awoyale *et al*., 2021); (c) 1210-1163 cm^{-1}- attributed to ester groups' O-C=O stretching, unique to bonds of hemicelluloses (Awoyale *et al*., 2021). As the above-indicated spectral regions relate to the hemicellulose, it elucidates that considerable lignin content had degraded. An 18.1% decrease of the percentage of crystallinity, as indicated in the pre-treated biomass's XRD spectra (fig. 4 (a) and (b)), elucidates significant reduction in the overall crystallinity of the biomass mixture post pre-treatment; this observation differs significantly from reports by contemporary researchers, who have recorded an increased crystallinity post pre-treatment of biomass (Imman *et al*., 2018, Romao *et al*., 2022). Conventionally, during pre-treatment, the typically amorphous polymers, lignin and hemicelluloses (Jiang *et al*., 2013), when deconstructed and solubilized, would result in the crystalline polymer cellulose (Jiang *et al*., 2013) being left behind in the biomass. While characterizing pre-treated biomass, the FT-IR spectral peaks and the XRD spectra would correspond to the presence of the retained, crystalline cellulose fraction (Imman *et al*., 2018), thereby recording increased biomass crystallinity. However, in the present biological, mushroom cultivation-based pre-treatment, due to enzyme-driven cellulose deconstruction and consumption, the crystallinity of cellulose was reduced; in other words, the enzymes had degraded cellulose to easily-assimilable oligosaccharides in order to facilitate consumption by the growing mushrooms. Hence, comparatively amorphous cellulose/partially-digested cellulose, which had lost its innate crystallinity, was left behind in the biomass mixture.



(a) **(b)**

*Only significant spectral positions have been marked to avoid overlaps

Figure 3: Fourier-transform Infrared spectra of the biomass mixture (a) prior to and (b) after mushroom cultivation



Crystallinity percentage (% Cr): 33.3% **Crystallinity percentage (% Cr):** 15.21%

(a) **(b)**

Figure 4: X - Ray Diffraction (XRD) spectra of the of the biomass mixture (a) prior to and (b) after mushroom cultivation

Lignin degradation during mushroom cultivation & its impact on enzyme titers during SSF

The SSF for cellulase production using *T. koningii*, utilizing the remnant biomass mixture from the mushroom cultivation/biomass pre-treatment process, was relatively straight-forward, unlike the complex submerged fermentation systems (Ramamoorthy *et al.*, 2023c). During the adaptation phase between 0-2 days, spores of the fungi germinate, mature, and differentiate into monopodial, substrate/penetrative/primary mycelia (Shabiha *et al.*, 2020), which majorly secrete cellulase in their hyphal tips (Sonnenberg *et al.*, 2022) and serve as nutrition-absorbing hyphae. Owing to two reasons, 47% hemicellulose consumption was recorded during the adaptation phase (fig. 6): (i) the foremost reason was enhanced accessibility of *T. koningii*'s hyphae to the interiors of the biomass, due to 73.8% lignin degradation during pre-treatment (Mulatu *et al.*, 2021); (ii) the secondary reason was the thigmotropic/topographical-sensing-based effector response, resulting in preferential consumption (Ramamoorthy *et al.*, 2023c) of the amorphous, easily-digestible hemicellulose (Ramamoorthy *et al.*, 2019b) after glucose exhaustion. As a result, the mycelial growth showed a rapid 80% increase until 2 days (fig. 6). As hemicelluloses are rapidly consumed, the growing hyphae establish contact

with cellulose, and on the basis of chemotropic signals, between 2-3 days, the hyphae began secreting cellulases, marking a nutritional switch-over/diauxic growth phase, as observed as a mild-plateauing of the mycelial weight curve (fig. 6). From 3-5 days, increased production of cellulase was observed, and the mycelial weight was on an increasing trend (fig. 6). Until the end of the process at 7 days (fig. 7), copious quantities of the cellulase complex were produced: 5.3 ± 0.1 exoglucanase/g. ds, 21.3 ± 0.9 endoglucanase/g. ds, 209 ± 23 cellobiase/g. ds (fig. 7). However, due to a decrease in DO% and K_La - mass transfer co-efficient, owing to oxygen transfer resistances caused by aerial/secondary mycelia (Shabiha *et al*., 2020) growing atop the substrate bed (Ramamoorthy *et al*., 2023b), the enzyme secretion pattern hit a plateau. In summary, from the 24 kg substrate mixture, approximately 124,800 exoglucanase units of the cellulase complex were harvested; furthermore, an average 10-fold increase of enzyme activities was recorded after repeated cycles of partial-purification/concentration using tangential-flow filtration. The remnant biomass mixture harvested after the SSF process showed reductions of (in % w/w): 25% cellulose; 70% hemicellulose; 15% lignin (tab. 1).

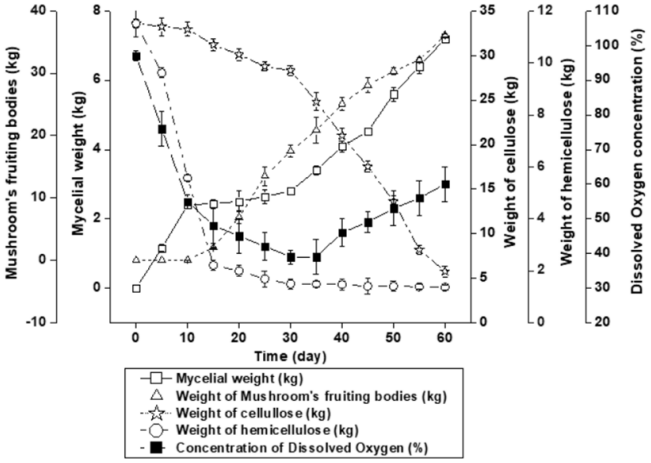
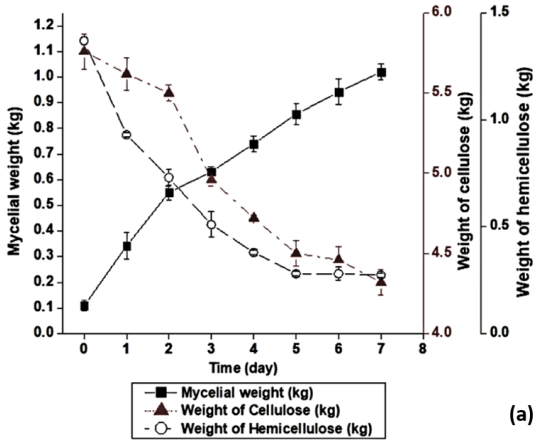


Figure 5: The trends of utilization of cellulose, hemicellulose, dissolved oxygen, and the production of mushrooms during the mushroom cultivation process of the circular-economical bio-refinery model



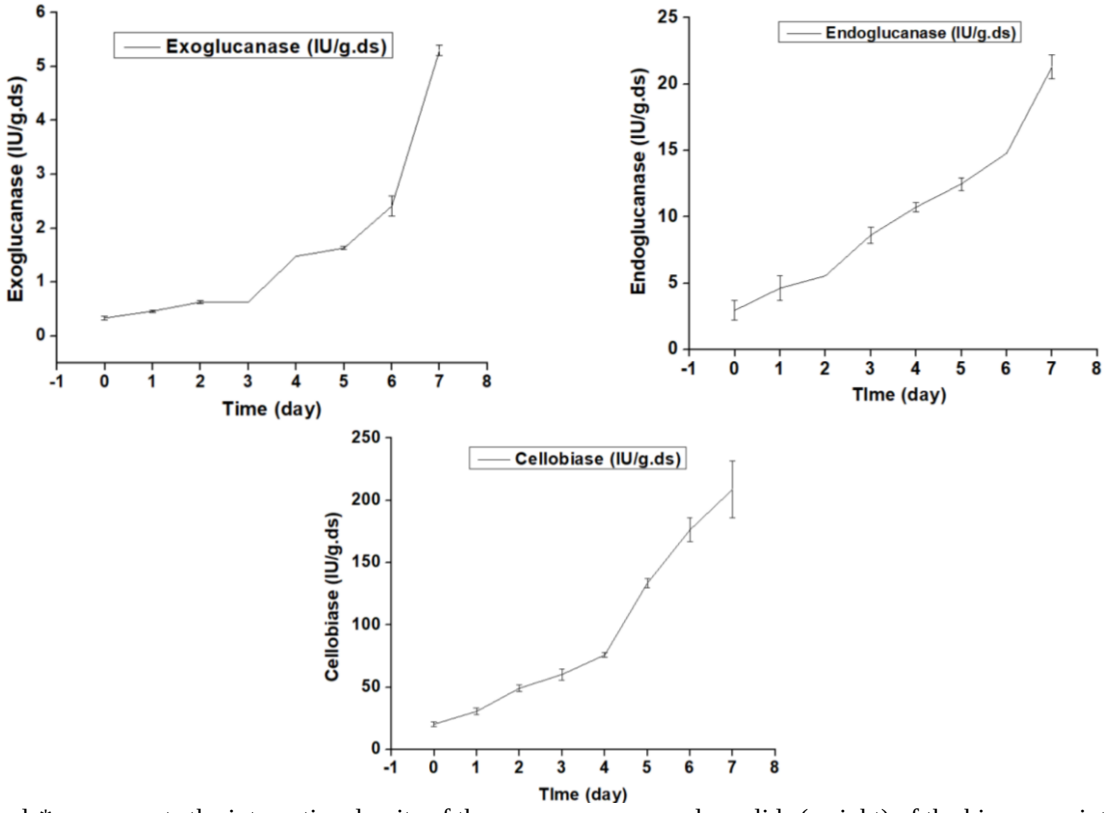
The spent biomass mixture from mushroom cultivation was used as the substrate for the cellulase production process, employing *T. koningii* Oudemans Tk-7 as the cellulolytic fungus.

Figure 6: The kinetics of (a) microbial biomass formation and substrate utilization during solid-state fermentation of the spent biomass mixture used for cellulase production

Effect of bio-priming agents on seed germination

An overall increase in crop yield is a direct function of combinatorial increases in radicle lengths and percentages of germination of seedlings, and the optimal seed bio-priming spore dosage is concluded on this basis (Singh V *et al*., 2016). Accordingly, a spore dosage of 10^5 was found to be optimal for the seeds of ladies' finger

as increases of 25% of the germination and 3.24 cm of radicle length were observed during experimental trials (fig. 8). Similarly for the seeds of cucumber, a spore dose of 10^6 was found to be optimal as it resulted in increases of 33.3% of germination and 5.93 cm of radicle length (fig. 8). Seed bio-priming improves germination rates as it initiates the secretion of certain essential metabolites, enzymes (Singh *et al*., 2020), and promotes photosynthetic rates (Singh *et al*., 2016). In the host system, while bio-priming helps in increasing the total phenolic content and unform lignin deposition in vascular bundles, it also aids in accumulation of shikimic acid, gallic acid and re-programming of its cellular defence mechanism (Singh *et al*., 2016). However, for the seeds of tomato, though a 16% increase of germination was observed at a spore concentration of 10^5, the radicle length was 1.5 cm lesser than that recorded for a spore concentration of 10^6; but, the germination for a spore dose of 10^6 was 16% lesser than that recorded for a spore dose of 10^5 (fig. 8). Yet, considering a larger surface area of exposure of lengthier radicles and better nutritional uptake (Wendling *et al*., 2016), 10^6 was concluded to be the optimal spore dose for the seeds of tomato (fig. 8). At a spore dose of 10^7, the seeds of tomato and ladies' finger suffered a decrease in their percentages of germination (fig. 8). In a similar phenomenon, while bio-primed using *T. longibrachiatum* SMF2, Shi *et al*., reported the inhibition of primary roots of *Arabidopsis* spp., caused by the bio-priming agent-induced chokonin VI (peptaibol) (Shi *et al*., 2016).



IU/g. ds* - represents the international units of the enzyme per gram dry solids (weight) of the biomass mixture.

Figure 7: The secretion profiles of the enzymes: exoglucanase, endoglucanase, and cellobiase throughout the 7-day-long solid-state fermentation for cellulase production using *Trichoderma koningii* Oudemans Tk-7, and the spent biomass mixture

Assessment of the bio-priming efficiencies of various concentrations of *T. koningii* spores

A. Germination of the seeds of *Abelmoschus esculentus* (ladies' finger)

Control
Spore concentration: N/A
Radicle length: 1.86 ± 0.5 cm
Percentage of seed germination: 41.66 ± 1 %

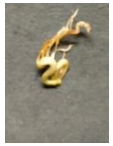
Trial - 1
Spore concentration: 10^4
Radicle length: 2.9 ± 1 cm
Percentage of seed germination: 58.33 ± 1.2 %

Trial - 2
Spore concentration: 10^5
Radicle length: 5.1 ± 1 cm
Percentage of seed germination: 66.66 ± 1.5 %

Trial - 3
Spore concentration: 10^6
Radicle length: 4.7 ± 0.8 cm
Percentage of germination: 58.33 ± 2%

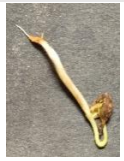
Trial - 4
Spore concentration: 10^7
Radicle length: 2.2 ± 1.5 cm
Percentage of germination: 33.3 ± 2%

B. **Germination of the seeds of *Solanum lycopersicum* (tomato)**



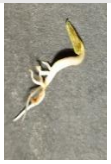
Control
Spore concentration: N/A
Radicle length: 2.63 ± 0.5 cm
Percentage of seed germination: 33.33 ± 2%

Trial - 1
Spore concentration: 10^4
Radicle length: 3.73 ± 1 cm
Percentage of seed germination: 50 ± 1%



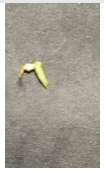
Trial - 2
Spore concentration: 10^5
Radicle length: 4.56 ± 1.5 cm
Percentage of seed germination: 50 ± 2%

Trial - 3
Spore concentration: 10^6
Radicle length: 6.06 ± 0.9 cm
Percentage of seed germination: 41.66 ± 1.5 %

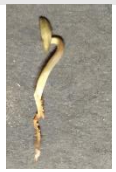


Trial - 4
Spore concentration: 10^7
Radicle length: 2.5 ± 1 cm
Percentage of seed germination: 25 ± 1.6 %

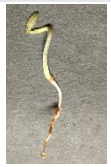
C. **Germination of the seeds of *Cucumis sativus* (cucumber)**



Control
Spore concentration: N/A
Radicle length: 0.9 ± 0.1 cm
Percentage of seed germination: 33.33 ± 3 %



Trial - 1
Spore concentration: 10^4
Radicle length: 4 ± 1 cm
Percentage of seed germination: 41.6 ± 1.4 %



Trial - 2
Spore concentration: 10^5
Radicle length: 6.33 ± 0.8 cm
Percentage of seed germination: 50 ± 2 %

Trial - 3
Spore concentration: 10^6
Radicle length: 6.83 ± 0.25 cm
Percentage of seed germination: 66.6 ± 1%

Trial - 4
Spore concentration: 10^7
Radicle length: 0.93 ± 0.3 cm
Percentage of seed germination: 33.3 ± 1%

Radicle length* - Mean radicle lengths of the germinated seeds. The number of seeds germinated from the total number seeds sowed, is expressed as the percentage of germination.
The control and experimental trials contained 12 seeds each; experiments were conducted in triplicates; the provided representational images are from one of the triplicates.

Figure 8: Assessment of seed bio-priming potential of the harvested *Trichoderma koningii* spores.

Fermentation of the residual biomass from the cellulase-producing SSF

The enzymes, produced to digest cellulose during the pre-treatment/mushroom cultivation process, along with the SSF-based cellulase production process, had efficiently de-crystallised the biomass mixture. A higher proportion of amorphous cellulose content was deduced in the remnant biomass obtained after the SSF process; higher endoglucanase titers between 6-7 days of the process confirm the same (fig. 7). Consequentially, during ex-situ saccharification, it reduced cellulase loading to 10 exoglucanase units/g of biomass to efficiently hydrolyze the substrate mixture. Reductions of 49% and 21% of ash content of the biomass during pre-treatment and cellulase production, respectively (tab. 1), enhance its (biomass's) moisture absorptivity (Awoyale *et al*., 2021) and enable effective enzyme penetration (Hinkle *et al*., 2015) during saccharification. During the biological pre-treatment (mushroom cultivation), due to 73% lignin degradation (tab. 1) and non-acidic operational conditions, undetectable quantities of lignin/lignin break-down products/pseudo-lignin were observed (Wan *et al*., 2019); due to hydrophobic non-specific binding, such breakdown products hamper cellulolytic enzymes and their saccharification yields by competing for the enzymes' active sites (Ramamoorthy *et al*., 2022b). Furthermore, due to moderate pressures and temperature regimes, pre-treatment inhibitors, such as acetic acid, formic acid, levulinic acid, furfurals, acetic acid, and 5-Hydroxy-Methyl-Furfural (5-HMF), which hinder *Saccharomyces cerevisiae*'s metabolic and fermentative pathways (Jönsson *et al*., 2016, Casey *et al*, 2010), decrease. A saccharification percentage (Eq. 2) of 17.1% was recorded for ex-situ saccharification while, during fermentation, the bio-ethanol yield was around 31.6 ± 0.2% (w/w) (tab. 1); the obtained ethanol was 166 (83% w/v) proof-pure. During ethanol production phase, a cellulose bio-conversion of 2.45% was recorded for the circular bio-refinery model (Eq. 3). While pre-treating a mixture of woody biomass using the mushroom, *Lentinula edodes*, and subsequent ethanol production, Feng and co-workers reported 67.6% of lignin degradation (Lin *et al*., 2015) and 14-17 g of ethanol production (Chen *et al*., 2022). The substrate, spent during the cultivation of oyster mushroom, contained 4.8% residual lignin; after a secondary stage of 2% sodium hydroxide pre-treatment, enzymatic saccharification and fermentation, a bio-ethanol yield of 29.14 g/L was reported by Grover and team (Grover *et al*., 2015). Peter and co-workers used the spent mushroom compost derived from chaff of Sorghum and millet, subjected it to hydrothermal pre-treatment, fermentation, and reported ethanol yields of 63.9 g/kg of dry matter (from chaff of millet) and 186.9 g/kg of dry matter (from chaff of sorghum) (Ryden *et al*., 2022).

Efficacy of bio-compost/bio-fertilizer in enhancing the germination indices of seeds, and its phytonutrient nature

The produced bio-compost appeared black in colour and possessed a foul odour. The pH and electrical conductivity were within the prescribed FCO standard values (tab. 2). A higher pH may result in a higher compost temperature owing to ammonia release (Mandal *et al*., 2014) and higher microbial metabolism (Mamo *et al*., 2021). The estimated moisture content was roughly 9% more than the standard, which could have been because of a lower bulk density (Mandal *et al*., 2014), lower temperature, higher total organic content (Mamo *et al*., 2021) (tab. 2), causing enhanced moisture retention/water holding within voids of the compost (Mandal *et al*., 2014). Moderately higher contents of C, N, and P (tab. 2), are usually preferred in bio-fertilizers, and they could also be attributed to a hastened decomposition of biomass by the dung-inhabiting microbial community (Mamo *et al*., 2021). As the sugars of biomass were utilized during ethanol and cellulase production processes, a C/N ratio, 6% lesser than the prescribed standard was observed (tab. 2). The application of 30% of bio-compost and 70% of soil was found to be optimal (fig. 9) during experimental assessments of germination indices of the seeds of tomato. Owing to consistent germination indices of more than 100% for each trial (fig. 9), according to the chart for standard germination indices, the compost was found to have a phytonutrient potential (Mamo *et al*., 2021).

Preliminary cost-benefit analysis

The mushroom cultivation hut/bio-refinery, inclusive of equipment, is readily-usable for other sources of biomass as well. For a complete 96 kg-batch (tab. 1), while comparing the cumulative process economy of $ 41, the returns from marketing costs of mushrooms - $ 131.7 (tab. 3) and Indian marketing cost of bio-ethanol - $ 0.86 ("Cabinet Approves Ethanol Price Hike By Up To Rs. 1.47 Per Litre For Mixing In Petrol, NDTV", 2021), signify a 70% profit (conversion from Indian National Rupees to United States Dollar, corresponding date 12th October 2022). Apart from being a prime-ingredient of several delicacies, mushrooms possess significant medicinal attributes too (da Luz *et al*., 2012).

Assessment of efficiencies of various ratios of the bio-compost

Control	**Trial - 1**	**Trial - 2**	**Trial - 3**	**Trial - 4**
100% soil	20% bio-compost and 80% soil	30% bio-compost and 70% soil	40% bio-compost and 60% soil	50% bio-compost and 50% soil
Germinated seeds: 6	Germinated seeds: 7	Germinated seeds: 10	Germinated seeds: 10	Germinated seeds: 8
*GI - N/A	GI - 162.3 ± 1.2 %	GI - 242.5 ± 1 %	GI - 240.6 ± 2.1 %	GI - 166.4 ± 1.8 %

***GI** - Germination indices calculated from differences in the number of germinated seeds between control and trial samples, and their mean root lengths.
The control experimental pot and the 4 pots for experimental trials were sown with 12 seeds each.
The results represented here are means of triplicate experimental trials.
The images are representational, and they are from one of the triplicate experimental trials.

Figure 9: Assessment of efficiency of the produced bio-compost, based on the germination indices of seeds of *Solanum lycopersicum* (Tomato)

CONCLUSIONS

As hypothesized, the consolidated bio-refinery model sequentially utilizes waste biomass mixture for sustainable yields of cellulases, ethanol, and certain other valuable by-products, which re-enter the ecological cycle during application in agriculture. Thus, the model justifies both bio-economy and circular economy, and it has a higher profit margin. Moreover, the self-sustainable nature of the model confers it to be a potential source of additional income to the economically-weak, house-keeping labourers and marginal community farmers. The present work was performed during the south-west monsoonal rains of India; during other climatical conditions, mushroom yields may decrease. Though significantly higher quantities of cellulases with a recyclable nature and a potential to saccharify several batches have been harvested, a lengthy operational cycle of 126 days, and a meagre 1.08 L of ethanol production from the processed 96 kg biomass are shortcomings of the process. Future process improvements/modifications could be directed towards mushroom and bio-ethanol production, circumventing the cellulase production stage. A mandatory proximate analysis, prior to each bio-processing, is essential to avoid batch-to-batch yield variation. The work may provide significant commercial scale-up data and future directions for process enhancements to alternate fuel researchers and process engineers.

List of Formulae

$$Percentage\ of\ Crystallinity = \left[\frac{(I_{cr}-I_{am})}{I_{cr}}\right] \times 100 \quad \textbf{.. Eq. 1.}$$

Where,

I_{cr} - XRD's spectral peak intensity at 2Θ of 22.5

I_{am} - spectral peak intensity at 2Θ of 18.5.

SP - Percentage of Saccharification

$$SP = \left[\frac{(Sugars\ in\ hydrolysate\ (g) \times 0.9)}{Quantity\ of\ biomass(g)}\right] \times 100 \quad \textbf{.. Eq. 2.}$$

CBC - Cellulose Bio-conversion

$$CBC = \left\{\frac{(yield\ of\ ethanol)}{0.51 \times (\ cellulose\ component \times biomass\ weight) \times 1.111}\right\} \times 100.. \textbf{ Eq. 3.}$$

GP - Germination percentage

$$GP = \left\{\left[\frac{Number\ of\ germinated\ seeds}{Number\ of\ seeds\ sown}\right] \times 100\right\} \textbf{.. Eq. 4}$$

GI - Germination Index

$$GI = \left\{\left(\frac{Germinated\ seeds\ in\ compost\ media}{Germinated\ seeds\ in\ control\ media}\right) x \left(\frac{Radicle\ length\ in\ compost\ media}{Radicle\ length\ in\ control\ media}\right)\right\} \times 100 \quad \textbf{.. Eq. 5.}$$

WHC - Water Holding Capacity

$$WHC = \frac{[(Wf-Wi)+MC\ x\ Wi]}{[(1-MC)\ x\ Wi]} \quad \textbf{.. Eq. 6}$$

Where,

Wi - Initial weight of the sample (g)

Wf - final weight of the sample (g)

MC - initial moisture content of the sample

$$Compost\ porosity = \left\{1 - \left(\frac{Bulk\ density}{Particle\ density}\right) x\ 100\right\} \textbf{.. Eq. 7}$$

CONFLICT OF INTEREST

The authors hereby declare that they have no conflict of interest with similar work being carried out elsewhere.

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