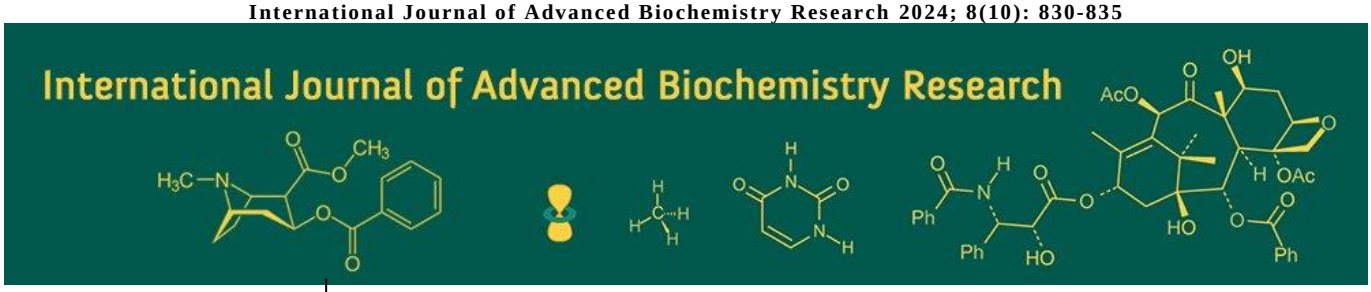

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Isolation and screening of lignocellulose degrading microorganisms

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Abstract
Large volume of agricultural residues is generated in India after every harvest season. They are mainly composed of cellulose, hemicellulose and lignin. These lignocellulosic materials are difficult to decompose because of their physical properties. Enhancing the microbial decomposition of these agricultural residues will manage the problem of its disposal and associated problems like burning and pollution. This study was aimed at isolating and screening of ligno-cellulolytic microorganisms from natural sources like forest litter, compost pit and agricultural wastes. Ten fungi and five bacteria were isolated in the form of pure colonies and screened for lignocellulosic activity using carboxymethylcellulose (CMC) and PDA and NA medium supplemented with guaiacol and methylene blue. Six fungal isolates and four bacterial isolates showed cellulolytic ability where four fungal and four bacterial isolates showed lignolytic ability. *Trichoderma* sp.(DFO-10), *Aspergillus* sp. (DFO-8), *Chaetomium* sp. (DFO 4), bacterial isolates like DBO-3, DBO-4 and DBO-5 showed promising results and can be utilized as potential degraders.

Keywords: Decomposition, lignocellulose, fungi, bacteria

1. Introduction
Large volume of agriculture residues is generated in India after every harvesting season. Being voluminous and time consuming, management of these agriculture residues is a major problem in the agriculture sector. In present condition, crop residue is customary to burnt in the field. After harvest, a lot of leftover crop residues are burned in the field, mostly to remove any remaining straw and stubble. Burning of the crop residues may add some nutrient like potassium but exert deleterious effect on micro flora and properties of soil. It results in the loss of plant nutrients like N, P, K, and S as well as the production of greenhouse gases that cause global warming. As a result, proper handling of crop residues becomes extremely important.

Crop residues are a potential source of plant nutrients, and by recycling them into the soil, their beneficial effects on soil fertility and productivity could be harnessed. Crop residues contain carbon (40%-45%), nitrogen (0.6%-1%), phosphorus (0.45%-2%), potassium (14%-23%), and micro elements, which are necessary for the crop growth. They can be used for alleviating the imbalances in the nutrient status in the soil and can reduce the overdependency on chemical fertilizers. The major components of these agriculture residues are cellulose, hemicellulose and lignin (Andlar *et al*., 2018) [2]. Cellulose and hemicellulose are macromolecules constructed from different sugars; whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors. Crystalline structure of cellulose, water resistance of lignin and protective coating of lignin-hemicellulose matrix on to the cellulose fibres makes it difficult to naturally degrade these agricultural residues (Isikgor and Becer, 2015) [15]. In general cellulose and hemicelluloses constitutes 75 to 80% while lignin constitutes only 14% of common agricultural waste but in some cases up to 99% of cellulose, 85% hemicelluloses and 40% lignin have been found in agricultural residues (Kumar *et al*., 2009) [18]. Wider C:N ratio also contribute to the slow decomposition of the agricultural waste. These causes accumulation of agriculture wastes in the field and hinders the activities of the farmer. Insufficient and improper disposal of agricultural wastes has led to acute environmental pollution (Kadarmoidheen *et al*., 2012) [17].

A viable option for managing and recycling the lignocellulosic waste is composting of these agriculture residues through the action of lignocellulolytic microorganisms. Both fungi and bacteria with their ability to produce variety of cellulases and lignases are the most used microorganisms. The recycled wastes by microbic degradation improve soil health and fertility when applied to soil. Microorganisms produce enzymes which can hydrolyse the lignocellulosic materials. A series of enzymes are produced which act synergistically to completely hydrolyse cellulose into simple sugars. The product of one enzyme act as a substrate for another enzyme and in this way whole cellulose system is degraded into simple sugars. For degrading lignin biomass microorganisms secrete extracellular lignin degradation oxidases which are primarily classified into laccase, manganese peroxidase and lignin peroxidase. By utilizing the decomposing potential of these microorganisms, agricultural residues can be recycled into good quality compost which can improve the organic matter, physico-chemical properties and biological properties of soil along with release of macro and micronutrients. It will also save the environment from being polluted by burning and will maintain the sustainability of crop production.

This study was conducted with the hypothesis that microorganisms obtained from natural sources such as forest litter, compost pit and agricultural field can enhance the degradation of lignocellulosic residues. Major focus of this work was on isolation and screening of lignocellulolytic fungi and bacteria from natural sources, which can be used for degrading the lignocellulosic residues.

2. Materials and Methods

2.1 Collection of samples

Partially decomposed samples were collected from natural lignocellulosic sources such as forest litter, compost pit and agricultural wastes. The samples were collected in sterilized polythene bags and transported to the laboratory and stored in refrigerator at 4 °C.

2.2 Isolation of microorganisms

After processing of collected samples (sorting of gravels, large sized leaves parts, woody pats etc.) fungi and bacteria, were isolated by using serial dilution method. Potato dextrose agar plates were used for fungal isolation. The plates were incubated for 7-8 days at 25-30 °C. Morphologically different fungi were isolated. Pure fungal cultures were obtained by subsequent subculturing on sterile potato dextrose agar plates. Nutrient agar medium was used for plating bacteria at pH 7 for 48 hours of incubation at 30^0 C. Bacterial colonies were purified by repeated streaking.

2.3 Screening for lignocellulosic activity

Purified fungal and bacterial isolates were screened for their cellulosic potential using Carboxy Methyl Cellulose (CMC). Lignolytic fungal and bacterial isolates were screened by inoculating on PDA medium supplemented with 0.02% guaiacol and NA medium supplemented with 0.25 g L^{-1} methylene blue.

2.3.1 Ligno-cellulolytic activity of isolated fungal cultures

The cellulolytic activity was tested on CMC agar plate. A 5-mm fungal inoculum disc was cut from the hyphal edges of a 5-day old fungal culture and placed in the centre of the CMC agar plates and incubated at 30 °C for 7 days. After incubation, the plates were flooded with 0.1% aqueous solution of congo red for 15-20 minutes followed by destaining with 1 M NaCl for 15-20 minutes. Degradation of cellulose was visualized as a clear zone of hydrolysis around the colony. Diameter of the clear zone and colony was measured. Isolates were selected on the basis of hydrolysis capacity value (HC value). (Choudhary *et al*. 2016) [7].

$$\text{HC value} = \frac{\text{Total diameter (zone + colony)}}{\text{Colony diameter}}$$

The screening test for lignolytic activity in fungi was done using PDA supplemented with 0.02% guaiacol to analyse qualitatively the lignolytic capability through the assay for laccase activity. A 5- mm fungal inoculum disc was cut from the hyphal edges of a 5-day old fungal culture and placed in the centre. The fungi were incubated and observed for the reddish dark colour zone around the colonies. Zone of clearance (ZOC) was calculated. Fijai *et al*. (2019) [10]

$$\text{ZOC} = \frac{\text{Total diameter (zone + colony)}}{\text{Colony diameter}}$$

2.3.2 Ligno- cellulolytic activity of isolated bacterial cultures

Isolated bacterial cultures were spot inoculated on CMC agar plates and NA plates supplemented with 0.25 g L^{-1} methylene blue for screening of their cellulolytic and lignolytic activity respectively. Similar to fungal cultures, the bacterial plates were also flooded with 0.1% aqueous solution of congo red for 15-20 minutes followed by destaining with 1 M NaCl for 15-20 minutes and the HC value was recorded. For lignolytic activity plates were observed for halo zone around the colonies as a measure of lignin peroxidase activity and zone of clearance was recorded (Umashankar *et al.* 2018) [29].

3. Results and discussion

From the different partially decomposed samples, fungal and bacterial colonies were obtained by serial dilution plating on PDA and NA medium, respectively. Ten fungal and five bacterial cultures were isolated in the form of pure cultures by repeated subculturing. Ten fungi were identified as *Fusarium* sp. (DFO 1), *Trichoderma* sp. (DFO 2), *Aspergillus* sp. (DFO 3), *Chaetomium* sp. (DFO 4), *Trichoderma* sp. (DFO 5), *Alternaria* sp. (DFO 6), *Mucor* sp. (DFO 7), *Aspergillus* sp. (DFO 8), unidentified fungi (DFO 9) and *Trichoderma* sp. (DFO 10). Five bacterial isolates were named as DBO 1, DBO 2, DBO 3, DBO 4 and DBO 5. Details of various isolates and their types are provided in the Table 1.

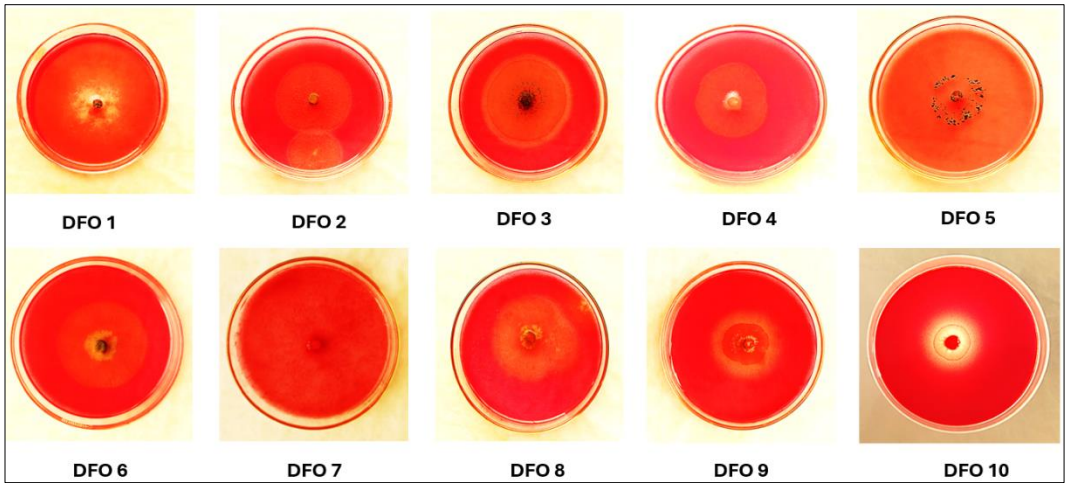

Fig 1: Cellulosic activity and clear zone formation of isolated fungal cultures

3.1 Screening for ligno-cellulosic microorganisms

Cellulase producers created clear zones on the plates. The ratio of diameters of clear zone and colony is expressed as Hydrolytic Capacity value (HC value), more the ratio; more is the cellulolytic activity of an isolate. Six decomposing fungal organisms (DFO) such as *Trichoderma* sp. (DFO-2), *Aspergillus* sp. (DFO-3), *Chaetomium* sp. (DFO-4), *Aspergillus* sp. (DFO-8), Unidentified fungi (DFO-9) and *Trichoderma* sp. (DFO-10) showed HC value.

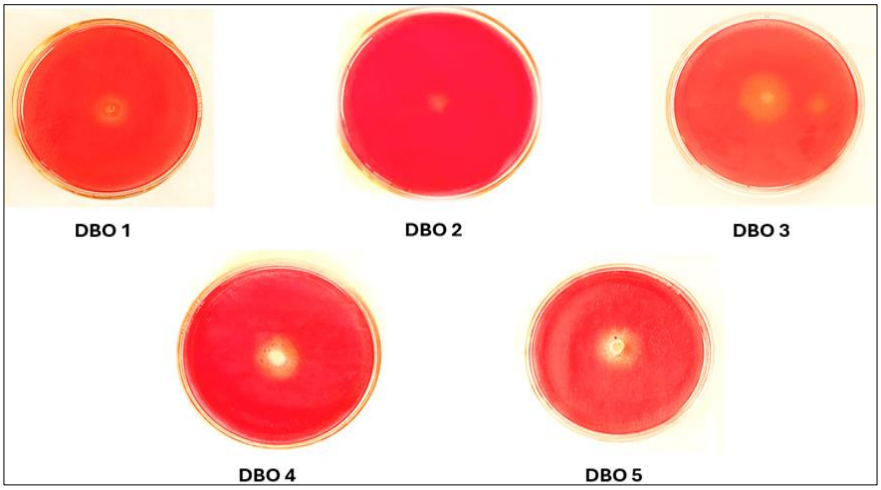

Fig 2: Cellulosic activity and clear zone formation of isolated bacterial cultures

Trichoderma sp. (DFO-10) showed highest HC value of 1.86 followed by Unidentified fungi (DFO-9) with HC value of 1.52 in the screening of cellulolytic fungi. Four decomposing bacterial organisms (DBO) such as, DBO-1, DBO-3, DBO-4 AND DBO-5 showed halo zone around their colonies. DBO-3 showed maximum HC value with 3.30 followed by DBO-5 (2.38), DBO-4 (2.24) and DBO-1 (1.62).

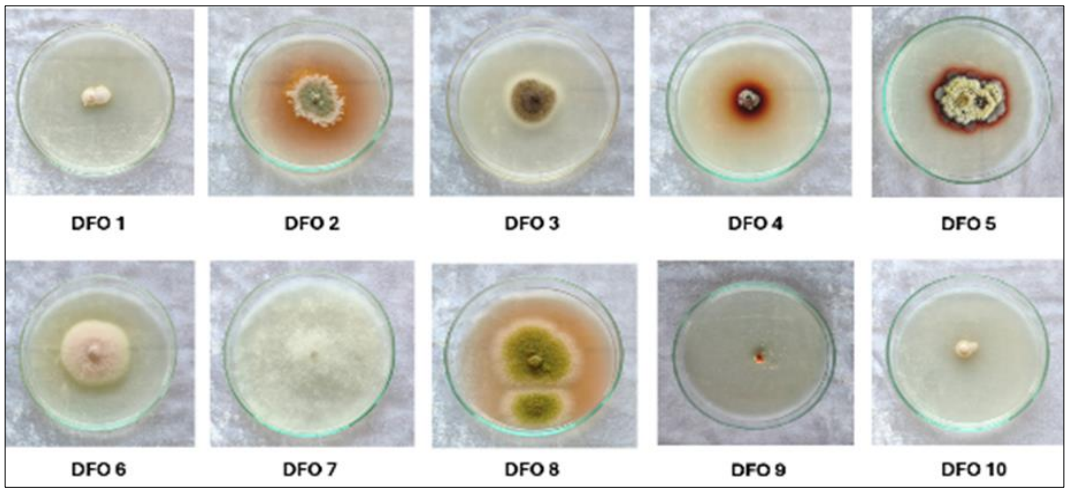

Fig 3: Lignolytic activity and zone formation of isolated fungal cultures

No hydrolysed zone was observed in DBO-2.

Ligninolytic fungi decolourise the PDA medium supplemented with 0.02% guaiacol to produce brown coloured zones around the colonies. Out of 10 isolates, 4 showed ligninolytic activity by producing decolourisation zone around their colonies. Four fungal isolates; *Trichoderma* sp. (DFO-2), *Chaetomium* sp. (DFO-4), *Trichoderma* sp. (DFO-5) and *Aspergillus* sp. (DFO-9) showed zone of clearance. Maximum zone of clearance was observed in *Aspergillus* sp. DFO-8 (1.92) followed by *Chaetomium* sp. DFO-4 (1.85) and *Trichoderma* sp. DFO-2 (1.58).

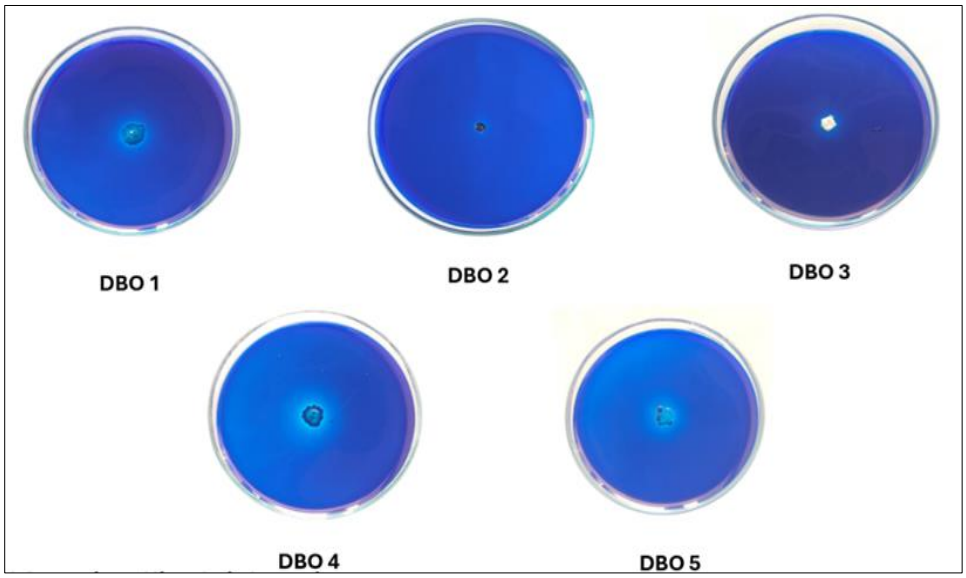

Fig 4: Lignolytic activity and clear zone formation of isolated bacterial cultures

Ligninolytic fungi decolourise the PDA medium supplemented with 0.02% guaiacol to produce brown coloured zones around the colonies.

Out of 10 isolates, 4 showed ligninolytic activity by producing decolourisation zone around their colonies. Four fungal isolates; *Trichoderma* sp. (DFO-2), *Chaetomium* sp. (DFO-4), *Trichoderma* sp. (DFO-5) and *Aspergillus* sp. (DFO-9) showed zone of clearance. Maximum zone of clearance was observed in *Aspergillus* sp. DFO-8 (1.92) followed by *Chaetomium* sp. DFO-4 (1.85) and *Trichoderma* sp. DFO-2 (1.58). Lignolytic bacteria decolourise the NA medium supplemented with methylene blue (0.25 g/L) to produce a colourless zone around the colonies.The decolourization of methylene blue has been used previously as an indicator of lignin peroxidise enzyme activity which is one of the enzymes responsible for lignin degradation. Four bacterial isolates out of five showed lignolytic ability by decolourising the medium. Four bacterial isolates; DBO-1, DBO-3, DBO-4 and DBO-5 showed zone of clearance. Maximum zone of clearance was observed in DBO-4 (2.45) followed by DBO-5 (1.81), DBO-1 (1.30) and DBO-3 (1.22). DBO-2 showed negative reaction.

Table 1: Ligno-cellulolytic potential of isolated microorganisms

Sr. No	Isolates	Cellulolytic Potential	Ligninolytic potential
		HC value	Zone of clearance
a)	**Fungal isolates**		
1.	*Fusarium* sp. (DFO-1)	0	0
2.	*Trichoderma* sp. (DFO-2)	1.06	1.58
3.	*Aspergillus* sp. (DFO-3)	1.04	0
4.	*Chaetomium* sp. (DFO-4)	1.08	1.85
5.	*Trichoderma* sp. (DFO-5)	0	1.21
6.	*Alternaria* sp. (DFO-6)	0	0
7.	*Mucor* sp. (DFO-7)	0	0
8.	*Aspergillus* sp. (DFO 8)	1.16	1.92
9.	Unidentified fungi (DFO-9)	1.52	0
10.	*Trichoderma* sp. (DFO-10)	1.86	0
b)	**Bacterial Isolates**		
1.	DBO-1	1.62	1.30
2.	DBO-2	0	0
3.	DB0-3	3.30	1.22
4.	DBO-4	2.24	2.45
5.	DBO-5	2.38	1.81

Hence, based on the screening for ligno-cellulolytic microorganisms best cellulolytic fungal and bacterial cultures were found to be *Trichoderma* sp. (DFO-10) and DBO-3. Most efficient lignolytic fungal and bacterial cultures were found to be *Aspergillus* sp. (DFO-8) and DBO-4. Comparing both HC value and zone of clearance

most efficient ligno-cellulosic fungal culture was found to be *Aspergillus* sp. (DFO-8), and most efficient ligno-cellulosic bacterial culture was found to be DBO-5.

Similar results were observed by Pratima Ray and Susmita Rath (2007) [24].

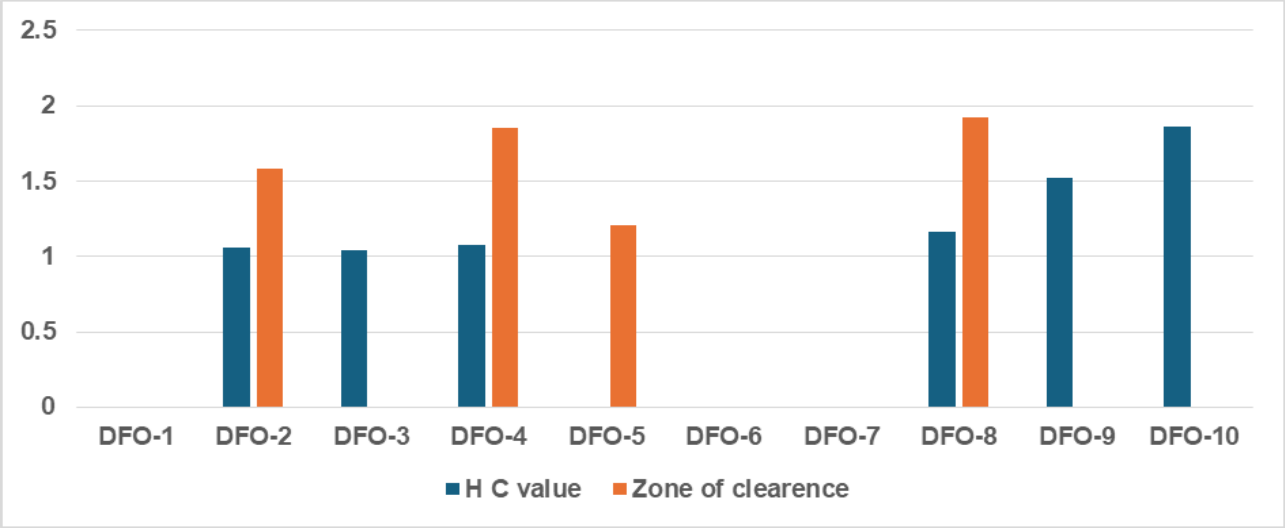

Fig 5: Lignocellulosic potential of isolated fungal cultures

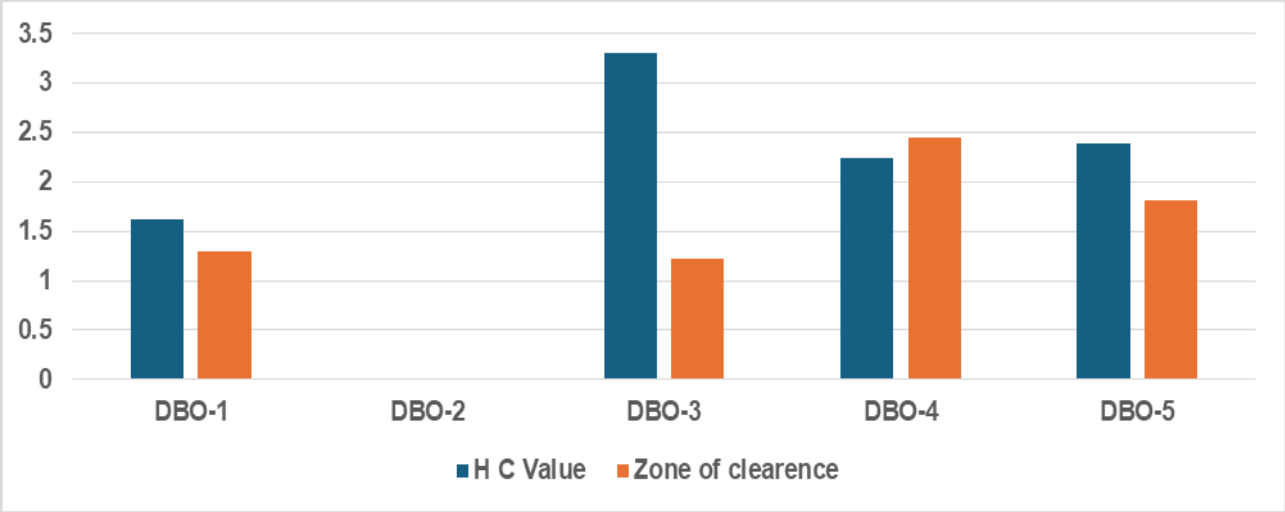

Fig 6: Lignocellulosic potential of isolated bacterial cultures

They reported highest cellulolytic activity in *Trichoderma viride* and *T. harzianum* by comparing HC values. Behera *et al.* (2014) [5] observed similar positive results in fifteen cellulose degrading bacteria based on their halo zone formation on Congo red agar medium. Observed presence of methylene blue degradation zone around the colony in the form of a clear zone which indicated the lignolytic activity of the bacteria. A. Padhiar *et al*. (2010) [22] observed zone of clearance in six fungal isolates of two white-rot fungi, *F. flavus* and *S. commune*. *F. flavus* isolates showed the highest lignin-degrading enzymatic activity up to 9cm zone of clearance whereas S. commune isolates had the lowest lignin-degrading enzymatic activity (1.5 cm zone of clearance).

4. Conclusions

The present study was undertaken with the aim of isolation and screening of microorganisms showing ligno-cellulolytic abilities. Ten fungal and five bacterial cultures were isolated from the partially decomposed samples collected from natural sources like forest litter, compost pit and agricultural wastes. Six fungal and four bacterial cultures were identified as cellulolytic based on HC value. Zone of clearance was observed in four fungal cultures and four bacterial cultures which showed their lignolytic ability. *Trichoderma* sp. (DFO-5) showed maximum cellulolytic activity whereas *Aspergillus* sp. (DFO 8) showed maximum lignolytic activity. Among bacterial cultures, DBO-3 showed maximum cellulolytic ability and DBO-4 showed maximum lignolytic ability. *Aspergillus* sp. (DFO-8) and DBO-5 showed maximum ligno-cellulolytic ability among fungal and bacterial isolates respectively. The efficiency of these cultures can be tested through more *in vitro* and *in vivo* trials to establish them as potential lignocellulose degraders.

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