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DEVELOPMENT OF LIGNOCELLULOLYTIC MICROBIAL CONSORTIUM FOR THE PRODUCTION OF ORGANIC MANURES FROM AGRICULTURAL WASTES

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Abstract: Organic farming systems are emerging in India. Indian Government declared states like Sikkim as an organic state and Kerala Government declared Kasargod as an organic district. But the farmers are in dilemma that they are not getting chemical fertilizers as well as there is an insufficiency in supply of organic manures. Therefore, the farmers are in search of an alternate technology for the production of organic manures from agricultural wastes. Lignocelluloses are structural recalcitrant organic matter; its utilization could allow self-sustained production in agriculture. The huge amount of lignocellulolytic waste materials generated in agriculture can be efficiently utilized through proper decomposition. Cost effective organic manure which can be sourced from agricultural waste will help the farmers in producing crops, economically and environmentally sustainable. Bacterial consortium is more effective in treating the waste with varying composition. The present study aims at the conversion of agricultural wastes into organic manures through bacterial consortium capable of decomposing the lignocellulosic waste. Different microbes were isolated from waste materials. Efficient Cellulose and Xylan degrading isolates mainly of *Bacillus* genera were determined by the BLAST search, based on a high sequence similarity. DNSA method was used for estimating the reducing sugars. Further, the characterization and isolation of novel enzymes capable of breakdown of cellulose and other plant material could be achieved. The most abundant microbes isolated from the lignocellulose wastes are the *Bacillus* group. More number of isolates which have the efficiency to degrade lignin and cellulose was sourced from mangrove peat. The lignocellulose in mangrove detritus can be degraded by certain fungi and bacteria, but the form is indigestible by most animals. This may be the reason for getting higher number of lignocellulose degrading isolates from mangrove peat. The study will follow the development of an efficient in-vitro decomposition process through microbes with a view to convert the biomass to organic manure to mitigate the ill-effects of chemical fertilizers. Further knock out experiments has to be carried out to unravel the consortium dynamics along with isolate compatibility studies.

Key words: Organic farming, R2A agar, Cellulose, Xylan, Carboxy methyl, Cellulose agar, Monreal, Reese medium

INTRODUCTION

Organic farming is one of the best approaches to meet the objective of sustainable food production. Recently, the organic farming systems are emerging in India. Indian Government declared states like Sikkim as an organic state and Kerala Government declared Kasargod as an organic district. But the farmers are in a dilemma that they are not getting chemical fertilizers as well as there is an insufficiency of supply of organic manures. Therefore, farmers are in search of an alternate technology for the production of organic manures from agricultural wastes.

Lignocelluloses are structural recalcitrant organic matter; its utilization could allow self-sustained production in agriculture. Lignocelluloses consist of lignin (25–30%), hemicelluloses (25–30%) and celluloses (35–50%) in agricultural wastes (Ghatak, 2011). The huge amount of lignocellulolytic waste materials generated in agriculture can be efficiently utilized through proper decomposition. Cost effective organic manure which can be sourced from agricultural waste will help the farmers in producing crops, economically and environmentally sustainable. Bacterial consortium is more effective in treating the waste with varying composition. The functional and structural stabilities of microbial consortia are considered to be important factors in their biomass degradation capability, and thus their

potential for biotechnological application. The present study aims at the conversion of agricultural wastes into organic manures through bacterial consortium capable of decomposing the lignocellulosic waste. Thus, this manure can be utilized for aquaculture as well as agriculture in an integrated manner.

The objectives of the study are:

- To isolate and characterize lignocellulolytic bacteria from wastes
- To study the efficiency of culture microbes to degrade lignocellulose

METHODOLOGY

Isolation of bacteria from lignocellulosic samples using R2A

Samples were collected from waste materials of sugarcane bagasse and mangrove peat. After sterilization, samples were kept for decomposition; 1 g of each sample was suspended in 9 ml normal saline and serial dilution of the suspension was carried out with the growth medium R2A agar with the composition of (0.5 g l^{-1} yeast extract, 0.5g l^{-1} protease peptone, 0.5 g l^{-1} casamino acids, 0.5 gl^{-1} glucose, 0.5 g l^{-1} soluble starch, 0.3 g l^{-1} dipotassium phosphate, 0.5 g l^{-1} magnesium sulfate 7H2O, 0.3 g l^{-1} sodium pyruvate, 15.0 g l^{-1} agar) (Haki *et al.*, 2011).

200µl of each dilution was spread on the surface of agar using the standard spread-plate technique. All plates were incubated at 28°C for 24 hours before isolating individual colonies and further incubated for 48 and 72 hours to allow the growth of slow growing microbes. The observed colonies were selected based on their morphology and colour. The colonies were selected and streaked on separate R2A plates until purity. After purification, the cultures were compared by Gram-staining to eliminate those of similar morphology and colour.

Cellulose and xylan degrading activity

Methods followed was Haki *et al.*(2011) with slight modification. Isolates were grown in 10 ml of LB broth with the composition of 10.0 g l^{-1} peptone, 5.0 g l^{-1} yeast extract, 5.0 g l^{-1} NaCl, for 24hours, shaking at 28°C; 5µl of each broth culture was singly dropped onto a plastic petri dish containing Carboxy methyl cellulose agar (Composition of carboxymethyl cellulose agar per 100 ml ddH2O- 0.5g CMC, 0.1g $NaNO_3$, 0.1g K_2HPO_4, 0.1g KCl, 0.05g $MgSO_4$, 0.05 g yeast extract, 1.5g agar) incubated for 48 hours at 28°C and flooded with Congo red solution. The zone of clearance around the colony was determined for each isolate to visualize the cellulase activity. Halo diameters were measured using a ruler for a semi qualitative comparison of cellulase activity among the isolates after 48 hours of incubation. Qualitative evidence for xylanase activities of all of the cellulase-positive isolates was evaluated using the same method described for the screening of cellulase activity. However, for the xylanase activity, 0.5g of xylan spelt oats was substituted for CMC.

DNA isolation and 16S rRNA amplification

DNA extraction was done using Nucleospin Tissue DNA extraction Kit. Isolates from different substrates showing the capacity to degrade cellulose and xylan were grown in 10 ml of LB broth. After the isolation of genomic DNA, it was run on 1% agrose gel. The resulting DNA was used as a template in PCR reaction to amplify a region of the 16S rRNA gene. Universal primers within the conserved regions of the 16S rRNA gene for Eubacteria used were: HAD-1and E1115R (Haki *et al.,* 2011). The PCR products were viewed on 1% agarose gel to confirm size, quantity and purity, and sequenced. Successful results were obtained from sugarcane bagasse and mangrove peat; and were determined by the BLAST search, based on a high sequence similarity of 85-100%.

Based on the halo development in CMC and xylan plates, isolates were selected for quantitative estimation of their ability to utilize cellulose and xylan as the sole carbon source. The isolates were inoculated in Monreal and Reese medium containing CMC and oat spelt xylan as the sole carbon source individually. Assays were done by the DNSA method for estimating reducing sugars at pH 7.0. Supernatants were collected every 24 hours for a period of 7 days and were used as the enzyme source. Substrates used were 0.5% CMC and 0.5% oat spelt xylan. DNSA method is to detect the reducing sugars formed after the cellulose, hemicelluloses or xylan utilization. The amount of glucose formed is directly proportional to the amount of degradation.

RESULTS AND DISCUSSION

Major isolates identified from sugarcane bagasse using 16SrDNA sequence analysis was *Bacillus subtilis* , *Bacillus vietnamensis* , *Bacillus cereus* , *Bacillus thuringiensis and Bacillus* sp. *Bacillus cereus showed the maximum* CMC plate and Xylan CZ:CS ratio on plates. And the same species showed the maximum average cellulase activity and xylanase activity.

From the mangrove peat major isolates identified were *Bacillus pumilus, Shwanella sp, Bacillus cereus, Bacillus selenatarsanitis, Bacillus subtils, Alcaligenes feacalis, Bacillus endophyticus, Bacillus megaterium. Bacillus megaterium, Bacillus pumilus, Bacillus cereus, showed the maximum* CZ:CS ratio with diameter of clear zone in CMC and Xylan plate. And the same species including *Bacillus subtils, Bacillus endophyticus showed* the maximum average cellulase activity and xylanase activity.

CONCLUSIONS

This work will follow the efficiency study of various bacteria with knockout experiments. The most abundant microbes isolated from the lignocellulose wastes are *Bacillus* group. The organisms like *Bacillus megaterium, Bacillus pumilus, Bacillus cereus, Bacillus endophyticus* and *Bacillus subtils* will be selecting for their efficacy study within the consortium for field level experiments. More number of isolates which have the efficiency to degrade lignin and cellulose was sourced from mangrove peat. The lignocellulose in mangrove detritus can be degraded by certain fungi and bacteria, but the form is indigestible by most animals. This may be the reason for getting higher number of lignocellulose degrading isolates from mangrove peat.

Future holistic applications of these isolated microbes in agricultural waste decomposition and industrial process could extend to Consolidated Bioprocessing. These microbes have the potential to perform the decomposition of structural recalcitrant compounds like lignocellulose by enzymatic and genetic modifications. The characterization and isolation of novel enzymes capable of breakdown of cellulose and other plant material could be achieved. Further knock out experiments has to be carried out to unravel the

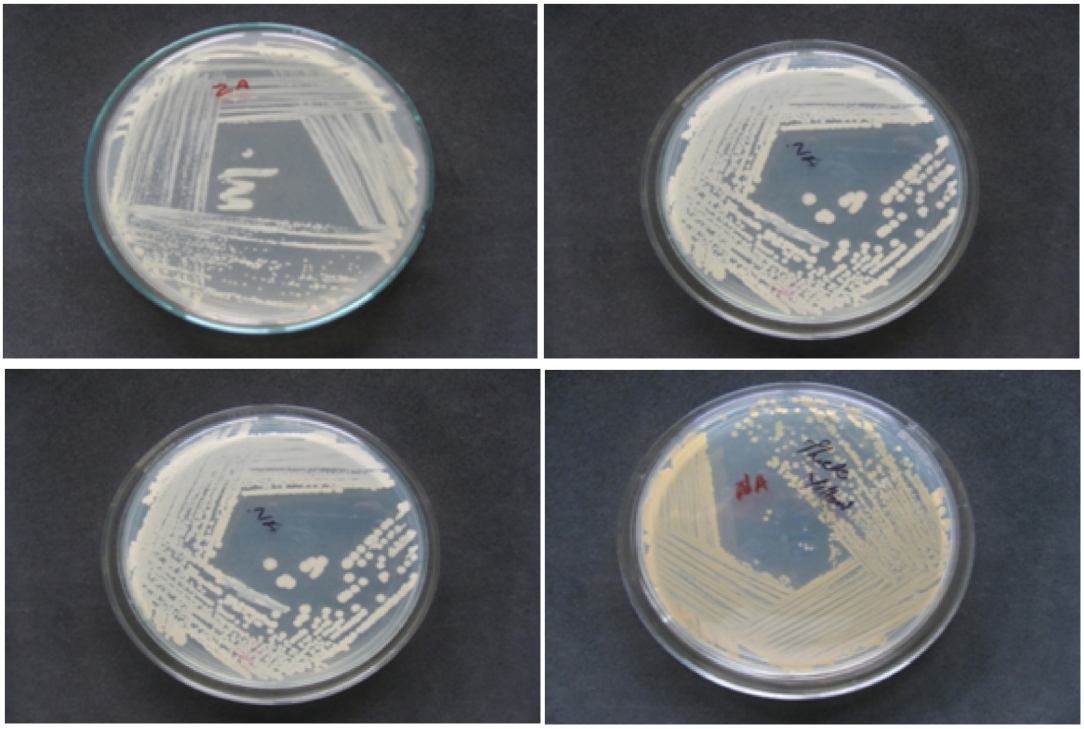

Fig. 1. Plates showing pure isolates of Bacteria from different substrates

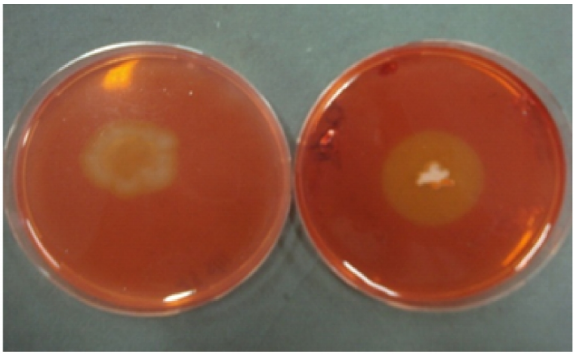

Fig. 2. Plates showing a zone of degradation on CMC plates stained by Congo red

consortium dynamics along with isolate compatibility studies. The study will follow the development of an efficient in-vitro decomposition process through microbes with a view to convert the biomass to organic manure to mitigate the ill-effects of chemical fertilizers.

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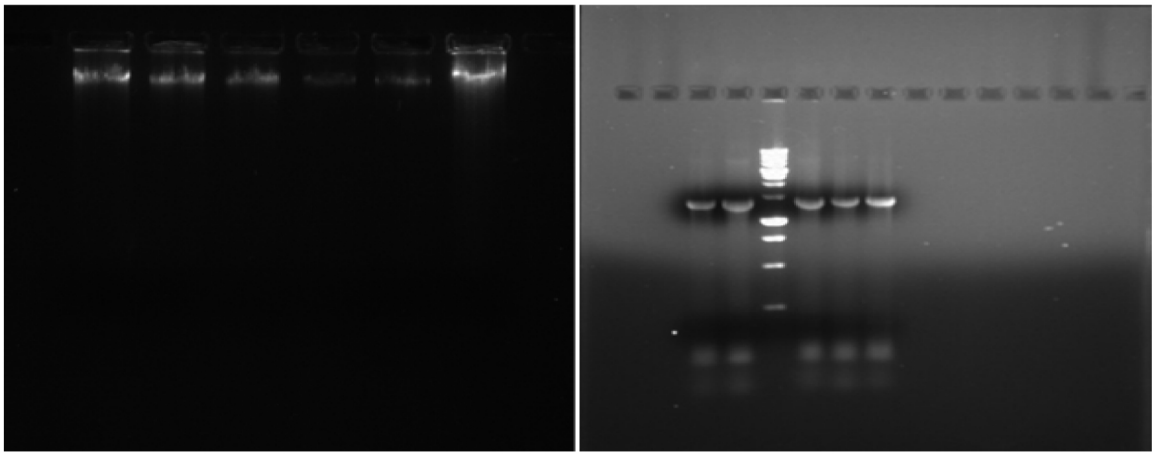

Fig. 3. Genomic DNA of isolates extracted from sugarcane bagasse, 16S rDNA gene amplified using eubacterial universal primers; Lanes 3, 4, 6, 7 and 8 show the isolates. Lane 5 is 1-kb DNA ladder

Table 1. Major isolates from sugarcane bagasse identified using 16SrDNA sequence analysis and with diameter of clear zone

Closest match on genebank	Identity percentage	CMC Plate CZ:CS ratio	Xylan plate CZ:CS ratio	ACA (µg GL/ml SP	AXA (µg XL/ml SP
Bacillus subtilis	100%	09	2.5	285	130
Bacillus vietnamensis	97%	11	05	277	146
Bacillus cereus	95%	12	10	287	321
Bacillus thuringiensis	97%	00	02	78	123
Bacillus sp	98%	05	09	282	234

Table 2. Major isolates from mangrove peat identified using 16SrDNA sequence analysis and with diameter of clear zone

Closest match on genebank	CMC Plate CZ:CS ratio	Xylan plate CZ:CS ratio	ACA (µg GL/ml SP	AXA (µg XL/ml SP
Bacillus pumilus	13	7	85	913
Shwanella sp	1.06	3	77	146
Bacillus cereus,	13	9	87	921
Bacillus selenatarsanitis	2.75	0.9	78	135
Bacillus subtils	12	1.8	182	934
Alcaligenes feacalis	6	1	65	128
Bacillus endophyticus,	5	.9	134	137
Bacillus megaterium	14	9.5	116	156

ACA-Average cellulase activity AXA- Average xylanase activity GL- glucose, XL- xylanase SP-supernatant

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