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Development and validation of a fungal spore formulation for retting of jute

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Abstract

Traditional retting of jute requires large volume of slow moving water bodies which are sometimes not available due to scarcity of rain during the harvesting season. This hampers fibre extraction from a very well grown jute crop. Fungal retting of jute is a promising technology under such condition as it requires very little water. Here we report the development and validation of a ready-to-use fungal spore formulation for retting fungi of jute. Spores of three different fungal cultures (*Aspergillus flavus, A. niger and Sporotrichum thermophile* designated as F1, F2 and S1, respectively) reported earlier to be suitable for dry retting of jute were preserved in kaolin powder and in a mixture of kaolin and wheat bran in different ratios for different duration up to four months. Viability of the spores, activities of retting enzymes and effectiveness of the preserved spores as inocula were studied. Spores of all the three fungal cultures were viable for at least up to 4 months with culture F2 being able to maintain maximum viability (12.77%). Solid preservative: culture ratio of 1.5:1 was found to give highest viability of F1 and F2 cultures while 2:1 was found to be the best for S1. Between kaolin and kaolin+wheat bran mix, kaolin alone was found to be better in terms of survival percentage of the fungal spores. Cultures revived after 4 months were able to carry out retting of jute under field conditions producing better quality fibre (TDN 2+50%↑) as compared to conventional water retting (TDN3+40%↑). Thus, a simple kaolin based 'Ready-to-Use" spore formulation of 3 retting fungi was developed and validated under laboratory and field conditions.

Key words: Fungi, Jute, Kaolin, Preservation, Ready-to-use, Retting

Introduction

Jute is an important commercial crop and the price of jute fibre, its main commercial product, depends on its quality or grade. Retting is one of the most important factors which determine quality of jute fibre. Jute is traditionally retted with water which requires large slow moving water bodies and such water bodies are diminishing day by day due to scarce rainfall during the retting period. As a result farmers have no option but to ret the harvested jute stem in shallow water resulting in poor fibre quality. Moreover, farmers sometimes go for several charges in a season in the same water bodies which not only deteriorates fibre quality but also causes environmental pollution (Das, 2018). Water retting also causes skin infections as farmers have to stand in the polluted water for hours to extract the fibre. In order to overcome these problems several retting technologies (ribbon retting, accelerated retting, fungal retting etc.) have been evolved overtime which minimize water requirement to various extents. Among these, fungal retting technology is very promising as it allows jute retting with very little quantity of water. Fungal retting technology not only saves water to a great extent but also eliminates environmental pollution and health hazards and at the same time produces quality jute fibre. In spite of being a promising technology, there are issues with fungal retting such as availability of

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the fungal inoculum, complexity of methodology, upscaling and cost benefit ratio which are required to be looked into before farmers can harvest the benefit of this technology.

Fungi when grown in submerged culture often produce high concentrations of conidia. The ability of many fungi to produce conidia profusely in a short growth period offers good opportunity to use them in dry fungal formulation for short term storage. This article deals with development and validation of a "ready to use" fungal spore formulation for retting of jute.

MATERIALS AND METHODS

Cultures and culture conditions

Three pectinolytic fungi viz. *Aspergillus flavus (F1), A. niger* (F2) *and Sporotrichum thermophile* (S1) used in this study were isolated from different retting environments and rotten fruits by enrichment culture (Banik, 2016). The fungi were maintained in the laboratory on potato dextrose agar (PDA) medium (peeled and sliced potato-200 g, dextrose-20 g, agar-20 g and double distilled water to make the volume up to 1 L, pH 5.6 - 5.8). For isolation of conidia, inocula of actively growing fungal mycelia were aseptically transferred to 250 ml potato dextrose broth in a 500 ml flask and kept under incubation in a BOD at 32 ± 1°C for *Aspergillus flavus(F1)* and *A. niger (F2)* and at 40 ± 1°C for *S. thermophile*. Conidial concentrations were measured microscopically using a haemocytometer after 10-12 days of inoculation. For each fungal culture three replications were maintained and 3 conidial counts were taken from each flask. Suspensions containing $5x10^6$ conidia /ml were used in the preservation formulations.

Preparation of formulations

Preservative materials viz. kaolin and wheat bran were sterilized at 121°C and 15 psi pressure for 1h in an autoclave. Appropriate amounts of conidial suspensions were aseptically mixed in 0.5:1. 1:1, 1.5:1 and 2:1 ratio with solid matrix (sterilized kaolin powder alone or kaolin+wheat bran mixture (in 1:1 ratio) so as to get a final concentration of $7x10^9$ conidia per gram.

The conidia-solid matrix mixtures were air dried in a pre-sterilized oven for three consecutive days and brought to a moisture level of about 4%. The respective conidial formulations were poured into 20 ml culture tubes, screw capped and sealed with a plastic tape. The tubes were stored in the laboratory in a dark place under ambient conditions (30 - 34°C).

The number of viable conidia in each formulation was tested before storage, following serial dilution technique using PDA medium. The number of colonies formed after 24 h was used for determining viability percentage.

Test for viability

Four months old formulation (10 mg each) was suspended in sterile water (1ml). A small aliquot was taken and serially diluted for plating on an PDA plate for checking viability.

Enzyme Assay

Fungal mother culture was developed in potato dextrose broth from freshly grown fungal culture revived from different formulations. The cultures were maintained at 32 ± 1°C (for *A. niger* and *A. flavous)* and at 40 ± 1°C (for *S. thermophile*) for 10 days. Ten millilitre of the culture (actively growing fungal mass was disintegrated with a blender) was inoculated in solid matrix (200g) containing rice husk and wheat bran (1:1 ratio) in polypropylene bags (18 cm x25 cm size) and allowed to grow for 8-10 days. Culture filtrate was collected in 50 ml centrifuge tubes and centrifuged at 10000 rpm for 10 min at 4°C. The supernatants obtained after centrifugations were used as crude enzyme for assay and quantification of protein content (Bradford, 1976).

Pectinase assay was carried out by the procedure of Okafor et al., (2010) using 1% pectin as substrate. Reaction mixture contained 0.5 ml of substrate

solution (1% pectin solution prepared in citrate buffer, 0.05M; pH 5.0) and 0.5 ml of crude enzyme. A blank was also run using 0.5ml enzyme extract and 0.5 ml citrate buffer. Reaction was continued for 30 min at 50ºC in a water bath. Amount of reducing sugar released was quantified by dinitro salicylic acid (DNS) reagent (Miller, 1972) using galacturonic acid as standard. One unit of enzyme activity was defined as the amount of enzyme, which liberates 1 µmole of reducing sugar per ml per minute under assay conditions.

Xylanase activity was measured following the procedure of Bailey et al., (1992). The substrate solution was 1.0% birch wood xylan in 0.05 M Na-citrate buffer at pH 5. The enzyme assay was performed taking 1.80 ml of substrate solution in a 15 ml test tube. The sample was allowed to equilibrate in the water bath to 50.0 °C. Two hundred microliter of enzyme, diluted appropriately in citrate buffer was added and mixed. The reaction was incubated for 30 min at 50°C. Amount of reducing sugar released was quantified by dinitro salicylic acid (DNS) reagent (Miller, 1972) using D-xylose as standard. One unit of enzyme activity was defined as the amount of enzyme, which liberates 1 µmole of reducing sugar per ml per minute under assay conditions.

Retting of green jute stem

Ability of the three fungal cultures to carry out laboratory level retting was carried out with 100g of green jute stem (1ft length) cut from the middle portion of 90 days old defoliated jute plant. One gram each of fungal formulations (after 4 months of storage) was suspended in sterile distilled water and sprayed on the green jute stem packed in a sterile polypropylene bag. The bags were closed with a cotton plug for aeration and visually checked for the extent of fungal growth and loosening of fibre.

For field retting experiment, fungal mother culture in PD broth was developed from freshly grown fungal cultures revived from different formulations after 4 months of storage. Polypropylene bags (18 cm x25 cm size) containing solid matrix (200g) of rice husk and wheat bran (1:1 ratio) were inoculated with actively growing fungal mass (disintegrated with a blender) and allowed to grow at 32 ± 1°C (for *A. niger* and *A. flavous)* and at 40 ± 1°C (for *S. thermophile*) for 8-10 days. Fungal growth along with solid matrix was ground into pieces and mixed with water in 1:10 ratio and made a suspension with the help of a mechanical stirrer. The suspension was sprayed on the green jute stems for inoculation and the stems were bundled (50 Kg) and covered with a polythene sheet (40 micron) and left on a flat surface. Polythene covers were opened everyday to monitor fungal growth. As retting progressed, the stem turned from green to dark brown and finally blackish with visible growth of fungus. At an optimum time of retting, fungal growth on the stem was removed by rubbing with a piece of gunny bag and fibres were extracted from the stems by loosening it at the base of the stem and then pulling it with hand. The extracted fibres were then gently washed in water to remove the adhering debris and gummy substances and then dried in the sun. Strength, fineness and fibre grades were determined following standard CACP guidelines (2019).

Results and Discussion

Fungal cultures were efficiently utilized for retting of green ribbon of jute (Haque et al., 2001; Banik et al., 2003, 2016). However, in order to supply large quantities of fungal inoculants to the jute farmers during retting season, a ready-to-use formulation is highly essential. In the current work, three fungal cultures, previously reported from our lab to be effective in jute retting (Banik, 2016), were assessed for short term preservation in spore form. Spore viability, culture regeneration, retting enzyme activity and retting of green jute stems were assessed for development of ready-to-use formulation of retting fungi.

Development of spore formulation

The different fungal cultures preserved in *kaolin* and '*kaolin + wheat bran*' mix were viable for at least

Table 1: Conidial survival percent* of different fungal cultures after different periods of preservation in kaolin formulations.

Cultures	Solid Matrix	Culture/Solid Matrix Ratio	Conidial survival % after different days of storage			
			30 Days	60 Days	90 Days	120 Days
F1	Kaolin	0.5:1	2.038062284_{ef}	0.931296_{de}	0.320313_{c}	0.117188_{c}
		1:1	4.614705882_{def}	2.448317_{cde}	2.19375_{c}	1.583333_{c}
		1.5:1	19.1235119_{bcdef}	17.7388_{abc}	5.108333_{bc}	0.741699_{c}
		2	6.215625_{def}	4.113281_{cde}	2.262931_{c}	0.74533_{c}
	Kaolin+Wheat bran	0.5:1	0.45546875_{f}	0.246094_{e}	0.17301_{c}	0.093041_{c}
		1:1	1.61328125_{ef}	1.060938_{de}	0.635973_{c}	0.177885_{c}
		1.5:1	8.287760417_{def}	6.279948_{cde}	0.607493_{c}	0.559524_{c}
		2	4.67625_{def}	2.65625_{cde}	0.690148_{c}	0.319557_{c}
F2	Kaolin	0.5:1	14.39144737_{bcdef}	5.463913_{cde}	5.093847_{bc}	5.105882_{abc}
		1:1	29.109375_{abcde}	11.24361_{bcde}	10.86798_{abc}	10.43618_{abc}
		1.5:1	57.45844_{a}	23.6648_{ab}	19.72181_{ab}	17.69559_{ab}
		2	42.96523086_{ab}	29.91051_{a}	20.53676_{a}	19.20368_{a}
	Kaolin+Wheat bran	0.5:1	3.654411765_{ef}	2.676471_{cde}	2.571756_{c}	1.92847_{c}
		1:1	20.390625_{bcdef}	8.14048_{cde}	4.2016_{c}	8.023922_{abc}
		1.5:1	26.51367188_{bcdef}	10.28257_{bcde}	9.366789_{abc}	7.806373_{abc}
		2	39.34168544_{abc}	16.33985_{abcd}	15.20204_{abc}	10.11346_{abc}
S1	Kaolin	0.5:1	9.533928571_{def}	2.801393_{cde}	2.708978c	1.660465_{c}
		1:1	12.9_{cdef}	5.768884_{cde}	5.653604_{bc}	2.789876_{bc}
		1.5:1	22.79464286_{bcdef}	8.678012_{cde}	6.827928_{abc}	5.861928_{abc}
		2	32.67678571_{abcd}	9.389098_{bcde}	8.719197_{abc}	5.425084_{abc}
	Kaolin+Wheat bran	0.5:1	2.15546875_{ef}	1.530994_{de}	1.396091_{c}	1.146284_{c}
		1:1	3.880434783_{ef}	3.574532_{cde}	1.381696_{c}	1.332207_{c}
		1.5:1	4.913896333_{def}	4.792688_{cde}	3.311486_{c}	2.8125_{bc}
		2	16.4644566_{bcdef}	15.10334_{bcde}	8.9375_{c}	5.158277_{abc}

Note: Mean ((n = 3) survival percentage of three fungal cultures after different days of storage in different formulations followed by common letter(s) within the same row are not significantly different (P< 0.05) by Duncan's multiple range test (DMRT).

120 days (Table 1) with decreasing count of viable condiospores with increasing duration of preservation. Viable conidiospore (seen as growth on PDA plate) per gram of freshly suspended solid matrix (kaolin alone or kaolin+wheat bran mix) decreased with time in all the cultures but extent of decline varied with matrix material as well as matrix: conidia ratio. In general, different fungal cultures showed better survival percentage when stored in kaolin alone (Table 1). There was no contamination during storage as evidenced by visual examination of fungal growth on PDA plate (Fig. 1). Three fungal cultures varied in their ability to maintain viability during the period of storage with F2 culture being able to maintain maximum viability (Table 1). Among the solid matrix: culture ratios,

Figure 1. Test of purity of F1, F2 and S1 fungal cultures on revival from four months of storage in kaolin formulation.

1:5:1 was found to be the best in F1 and F2 while 2:1 was found to be the best in S1. In all the conditions, survival percentage was highest in F2 followed by S1 and F1. Between kaolin and kaolin+wheat bran mix, kaolin alone was found to be better in terms of survival percentage of the fungal spores. Differences observed among the cultures might be due to differences in physiological characteristics of the fungi. Thus, F2 (*Aspergillus flavus*) and S1 (*Sporotrichum thermophile*) cultures were more amenable to short term storage in kaolin powder.

A number of fungi had been tried in the past for retting of green jute stem (Banik, 2016) or ribbon (Haque et al., 2001; Ahmed et al., 1999). Fungi, namely *Sporotrichum* sp., *Schizophylum communae* and *Trichoderma* sp. efficiently retted green jute ribbons in 7, 9, and 11 days, respectively (Haque*et al*. 2001). An eco-friendly water saving fungal retting technology was developed using four fungal cultures namely, *Aspergillus tamarii, A. flavus, A. niger and Sporotrichum thermophile* at ICAR-NINFET (Banik, 2016). The technology was able to produce good quality jute fibre with strong and unbroken full length jute stick as desired by the jute farmers. However, the technology could not be popularized among the farmers as it required supply of huge quantity of freshly grown cultures to the retting site during retting season. This is not only a difficult task but also impractical as this will incur huge transport cost for supply of large quantities of fresh culture to far-flung areas. Hence, there is a need to develop a "Ready to Use" fungal formulation which can be produced in large quantities and carried easily to retting sites during the retting season of jute.

Soil (Reinecke & Fokkema, 1979), talc powder (Abdel-Kader et al., (1992). kaolin powder (Bhattacharyya &Basu, 1989; Coutinho et al., 2011), saw-dust (Abdel-Kader et al., (1992). clay and sea sand (Bakerspigel, 1953), cereal grains (Singleton et al., 1992), agar strips (Nuzum, 1989) had been used earlier as carriers for fungal formulation. Bhattacharyya and Basu (1989) reported earlier about preservation of A*spergillus* sp. in kaolin powder for a period of 90 days without losing physiological and biochemical properties required for softening of jute fibre. In the present study, we report development and validation of a formulation for preservation of fungal spore in kaolin powder (Hydrated aluminium silicate, Extra pure from Himedia, ~ 300 mesh). This carrier, a complex aluminum silicate, is readily available in the market, inexpensive and nearly chemically inert.

Retting enzyme assays

Microbial cultures have been reported to undergo morphological and physiological changes during storage. Hence, in order to ascertain the use of these fungi for retting, they were tested for activities of retting enzymes (pectinase and xylanase). In fact, enzyme activities were found to be high in the culture extract of the three fungi grown on solid matrix of rice husk and wheat bran mix. Among the three cultures, F1 showed highest pectinase activity followed by S1 and F2. On the other hand, S1

Table 2: Grades* of fibre samples retted from field grown jute plants (90 days old) with fungal cultures revived after 4 months of storage.

Parameters*	Fungal Retting (Culture F1)	Fungal Retting (Culture F2)	Fungal Retting (Culture S1)	Conventional Water Retting
Bundle Strength (g/tex)	Average	Average	Average	Average
Fineness (tex)	Fine	Fine	Fine	Fine
Root Content(L, %)	>5	>10	>5	>10
Defects (% by wt.)	1.0	1.0	1.5	1.0
Colour	Good	Good	Good	Average
Total Score	70	58	62	48
Sample Grade**	TDN2+50%↑	TDN3+90%↑	TDN2	TDN3+40%↑

**Grading was made as per CACP guidelines* ** (n=10)

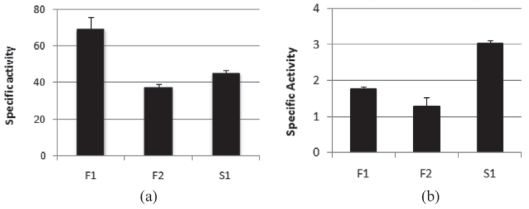

Figure 2. Pectinase (a) and xylanase (b) activities of the three freshly grown fungal cultures, F1, F2 and S1 revived from 4 months of storage in kaolin formulation.

culture showed highest xylanase activity followed by F1 and F2. F2 culture had almost equal activities of both the enzymes (Fig. 2). The enzymatic studies clearly established that these cultures can very well be utilized for retting of jute plant.

Laboratory level retting

In order to ascertain the capability of the preserved fungal cultures to carry out retting of jute fibre, 1 g each fungal formulation was suspended in sterile distilled water and sprayed on 100g of green jute stem (cut into piece of 1 ft length) and packed in a sterile plastic bag. The bags closed with a cotton plug for aeration were visually checked for the extent of fungal growth and loosening of fibre. The results were very encouraging with all the three cultures being able to complete of the green jute stem in 12 days (Figure 3).

Field level retting

Field level retting of jute plants with the three revived cultures was completed in 12 days. Table 2 shows grading of fibre as per CACP guidelines. Compared to conventional water retting (TDN3+40%↑) samples obtained with fungal retting were of better quality with F1 culture producing the best fibre (TDN2+50%↑) followed by S1 (TDN2) and F2 (TDN3+90%↑).

Thus, a very simple and cheap storage formulation of earlier identified and used retting fungi viz. *Aspergillus flavus, A. niger and Sporotrichum thermophile* was developed. The developed formulations of kaolin (culture : matrix ratio of 1.5:1

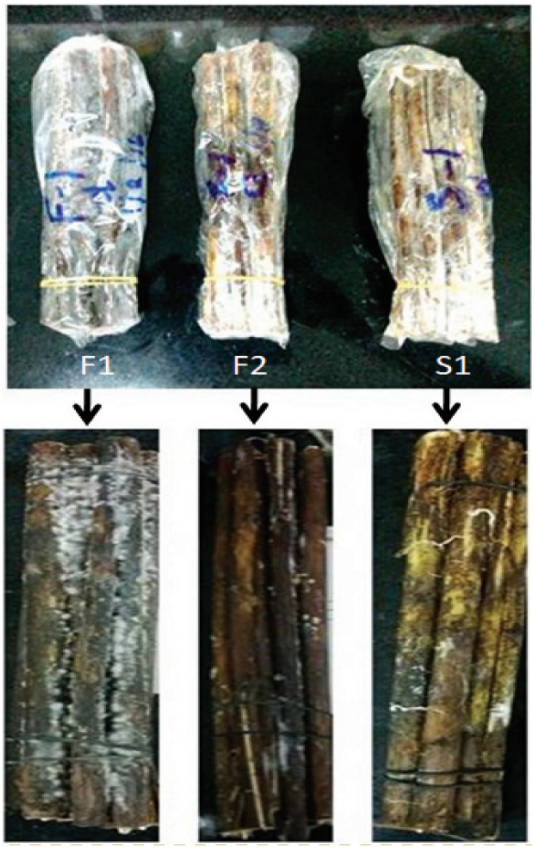

Figure 3. Laboratory level retting of jute stem with revived fungal cultures, F1, F2 and S1 after 4 months of storage in kaolin formulation.

for F1 and F2 cultures and 2:1 for S1) were able to maintain the viability of fungal spores for at least up to four month to the extent that they can be utilized as ready-to-use fungal inocula for retting of jute. Fungal retting technology of jute developed with these cultures in our lab previously (Banik, 2016) will overcome the shortcomings of supply of large quantities of ready to use inocula during the peak period of retting season of jute.

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