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An eco-friendly fungal dry retting technology for fibre extraction of Grewia optiva (J. R. Drumm. ex Burret)

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ABSTRACT:

Grewia optiva (J. R. Drumm. ex Burret) (bhimal) is one of the most popular agroforestry trees in western Uttarakhand. Besides providing fodder for the livestock's, natural fiber extracted from its bark is used for various purposes. The traditional method of extraction of G. optiva natural fibres require immersion of twig bundles for more than 90 days in water, for retting. After this, fibres can be easily removed from twigs, washed and sundry. In present study, an eco-friendly and water-saving retting technology has been developed, harnessing the power of pectinolytic fungi naturally present on the bark of G. optiva. By leveraging a dry fermentation process, this innovative method overcomes the limitations of traditional retting, offering a sustainable solution for the textile industry while reducing environmental footprint. Four strains of filamentous fungi, namely Aspergillus niger, Penicillium sp., Pestalotiopsis sp. and Rhizopus sp. were isolated from the bark of G. optiva and purified. Of these, A. niger was mass cultured and tested for its ability towards extraction of fiber from the lignocellulose material. Twigs of G. optiva in four replications were kept at ambient room temperature and spore suspension (106 cfu/ml) of A. niger was sprayed, maintaining >85% humidity during July–August 2020. Complete retting was observed within 13 days in dry and aerobic conditions. The retted bark was removed manually from the twigs. Bark was washed in running fresh water to obtain fibres and dried. Based on these findings, it is recommended to use A. niger for dry retting technology to obtain fibres from the bark of G. optiva. This approach can save 85.6% time as compared to the conventional methods of G. optiva fibre extraction.

Keywords: Natural fibre, filamentous fungi, *Aspergillus niger*, *bhimal*

Introduction :

The global freshwater crisis has reached a critical point, largely due to excessive and unsustainable use. Natural water sources like rivers, lakes, and ponds are depleting rapidly, while groundwater reserves are also being drained quickly (UNDP, 2006). Compounding the issue, global warming is causing erratic weather patterns, including delayed monsoons and scarce rainfall. In this context, the fibre industry and other stake holders which relies heavily on water-intensive processing, is particularly vulnerable. Therefore, technological interventions that reduce water usage could have a significant impact to combat water resources.

Grewia optiva is a significant forestry tree species known for its natural fibres. Locally known as bhimal, biul or bihu in India, it is a small to medium size deciduous tree with 9–12 m height. This species is mainly found in the Himalayan region of India (Uttarakhand, Himachal Pradesh, Jammu and Kashmir) up to 2000 m amsl (Brandis, 1906, Verma *et al.*, 2002). It is a multipurpose tree and used by growers on the boundaries of their fields and homesteads, for fodder. Negi (1986) reported that some major fodder trees on which hill farmers fall upon during the lean period are *G. optiva*, *Celtis australis* (khirak), *Bauhinia variegata* (kachnar), *Morus alba* (shahtoot), *Quercus leucotricophora* (ban), *Robinia pseudocacia* (black locust) and others. Of these, *G. optiva* excels in overall nutritive value and high digestibility. It provides quality fodder for animals, fuel wood, edible fruits, natural fiber and natural shampoo which is medicinally tested for dandruff control (Arora, 2011, Kumar *et al*., 2021).

Fibre retting is a complex process, and its properties are highly dependent on the type of retting and its parameters. Under-retted and over-retted fibres make inefficient fibre separation and fibre weakening, respectively (Preisner *et al*., 2014). During the retting process, phloem-derived fibre bundles are loosened from hemicellulose, lignin, and pectin. Leftover fibres are rich in cellulose contents and hence performing in high-strength properties. Retting is the most important post-harvest operation for quality of *G. optiva* fibre production and the fibre quality depends to a large extent on the bio-chemical process of retting. The fate of a good fibre production depends fully on the proper retting carried out in good quality water. If retting is properly carried out, the extracted fibre would exhibit genetic quality of the variety. Improper retting may lead to inferior quality of fibre in spite of good crop which ultimately may face lower price in the market and lower net return to the farmers (Ranjan *et al*. 2021).

The retting process of *G. optiva* bark involves various biochemical, chemical and enzymatic reactions as it consists of cellulose cemented by non-cellulosic materials, such as pectin, hemicelluloses and others (Sindwani *et al*. 2017). This process utilizes a complex microbial community for dissolution process after immersion of *G. optiva* plants in water, releasing soluble constituents like sugar, glucosides and nitrogenous compounds. Retting microbes present in plant and water continue to build up their population by utilizing these soluble compounds (Chen, 2014). As these easily

soluble compounds are exhausted, the retting microbes start to utilize free sugars, pectins, hemicellulose and proteins of the plants as essential nutrients for their development and multiplication under favorable condition. These so-called cementing agents are removed by the enzymes produced by microbes in the process of retting. Generally, pectinase, xylanase and cellulase are associated enzymes found in retting microorganisms and they act on specific substrates sequentially (Das *et al*. 2012). Pectinase enzymes first attack on pectins. Next xylanase consumes easily decomposable hemicellulose, short chain xylan and softens the fibre. Cellulase enzyme is supposed to be harmful in retting process especially after completion of retting (Banik, 2016) and becomes active when all easily decomposable carbohydrates are consumed. At this stage, fibre cellulose is attacked by cellulose enzyme which happens during over retting. Given this, the microorganism having less cellulase enzyme activity may be more effective for retting purpose (Das *et al*. 2012, Shah *et al*. 2021).

As mentioned, *G. optiva* provides very good and useful natural fibre for making low cost articles, such as rope, mats, bags, boots, carpets, chapals, curtains and play a key role in the village economy. Farmers harvest the twigs during winter a lean period as other fodder not available and remove leaves from twigs of *G. optiva* for fodder of livestock. After taking the fodder from twigs, twigs are collected and made bundles for putting into pond locally known as khall or gadhera. These bundles are completely drowned in gadhera for more than 90 days (Kumar *et al*. 2017). These bundles are taken out from pond/gadhera for the manual extraction of fibre, washed in water and dried under the Sun. This conventional retting method of *G. optiva* has certain shortcomings:-

- Retting duration is very long.
- Longer retting duration encourages over retting resulting less fibre recovery from top portion of the plants.
- The strength of over retting fibre may have very poor quality.
- Because of lower fibre quality, farmers get low price for their fibre in the market.
- Most of the fibre produced by this method is unsuitable for production of high valued diversified products.
- Traditional retting process is not safe for aqua life and other dependents.
- Young generation do not very keen to follow traditional fibre extraction system as it give unfavourable smell.

In the present study, we have focused on developing a significantly faster method for fibre extraction technology from the bark of *G. optiva*. This includes retting process in dry and aerobic conditions by the action of *A. niger* in very short interval of time.

Materials and Methods

Collection of plant material

Twigs of *G. optiva* were collected from four different experimental sites viz. ABF Kalsi, Vikasnagar, Dehradun, Uttarakhand, India (N 30° 31' 16.8", E 77° 50' 32.6"), Amgaon/ Kandi, Pauri Garhwal, Uttarakhand (N 29° 56'51.7", E 078° 27' 14.5) and Walna, Ranikhet, Almora, Uttarakhand (N 29° 41" 5.7",E 79° 25' 30.2"). The samples were collected in paper bags and brought to Forest Pathology laboratory, Forest Research Institute, Dehradun, India for further studies.

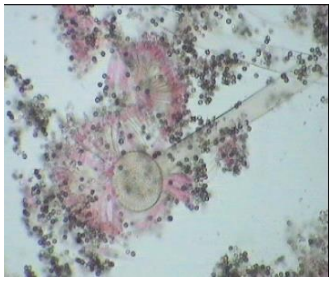
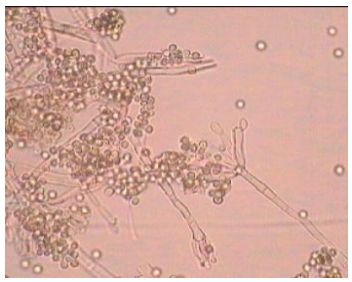
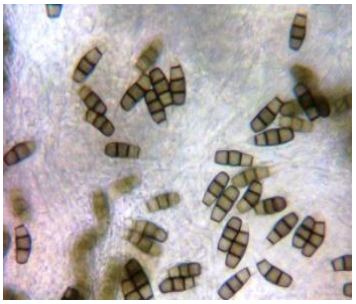
Preparation of culture medium

Potato dextrose agar (PDA) medium (Hi Media) was used for isolating and growing fungi. PDA medium (39g PDA per liter of distilled water) was poured in conical flasks, plugged and sterilized at 15 lbs psi pressure and 121ºC temperatures for 20 minutes. After sterilization, PDA was poured into sterile plates when cooled to room temperature for isolation and growth of fungi.

Isolation and identification of the fungi

The twigs samples were cut into small pieces of 3-4 cm long and put in the conical flask containing sterilized distilled water and plugged under aseptic conditions. It was allowed to mix and settle for 24 h at room temperature. Next day, the stock solution was shaken properly and 1 ml was poured onto PDA medium plates with the help of micropipette. It was then spread over with the help of a sterile spreader on plate, sealed with parafilm and incubated at 25±2ºC for 3–5 days to obtain fungal colonies over the plate. Single colonies were picked and purified on fresh PDA plates. Identification of fungal colonies was done by mounting a small amount of fungal mycelia on a clean glass slides in cotton blue for microscopic observations. Fungi were isolated and purified following standard microbiological procedures and were identified following standard literature (McClenny, 2005, Maharachchikumbura *et al*., 2014 & Navi, *et al*., 1999). Based on microscopic features, four different fungi were identified as *A. niger*, *Penicillium* sp., *Pestalotiopsis* sp. and *Rhizopus* sp. (Table 1).

Table 1. Pure cultures and microscopic images of isolated fungi

Organisms	Culture Image	Microscopic Image
Aspergillus niger		
Penicillium sp.		
Pestalotiopsis sp.		
Rhizopus sp.		

Mass culturing of Aspergillus niger

A beaker of 500 ml was taken and potato dextrose broth (PDB) was prepared by adding 39 g in 1 L of purified/distilled water. After sterilization by autoclaving at 15 lbs pressure (121˚C) for 15 minutes, mycelial discs of 5-mm-diameter were taken from 7–10 days old culture of *A. niger* with the help of a sterile cork borer and inoculated onto broth. The flasks were incubated at 25±2˚C for 7–10 days till a thin film of mycelia spread over the media. After 10 days, the inoculum was ready to use. It was filtered through what man filter paper no.1 and the spore concentration was adjusted to 10^6 cfu/ ml with the help of a haemocytometer.

Inoculum of Aspergillus niger

Degradation and Severity Tests

The twigs were taken and inoculated separately with the spore suspensions of the fungi (*Aspergillus niger*, *Penicillium* sp., *Pestalotiopsis* sp. and *Rhizopus* sp.) made in sterile distilled were sprayed (10^6 cfu/ml) over the twigs until run off. The inoculated twigs samples were placed in covered plastic trays and incubated at ambient room temperature with 80 to 100% RH for 13 days in four replications including control. Severity test was carried out and wood samples were evaluated as per Horsfall and Barratt (1945) and observations were recorded (Table 2).

Experiment for fibre extraction from bark of *Grewia optiva* through dry retting technique with the action of *Aspergillus niger*

Based on degradation and severity tests results employing the dry retting technique, further studies were carried out during July-August, 2020. A matured tree of *G. optiva* was selected for the extraction of fibre and 50% branches were chopped properly. Green leaves were removed from the branches and branches were cut into the 30 cm in length. The twigs of *G. optiva* were collected and put under the sunlight for drying. After this, an artificial chamber size 5'*4'*3' fit covered with transparent plastic sheet was prepared for the retting of twigs. Twigs of *G. optiva* were dipped into water and kept for overnight in this chamber at room temperature for maintaining the moisture in sticks. Next day, twigs were taken out from water. The spore suspension was sprayed on these sticks of *G. optiva* samples (100 sticks in each) in four replications {R_1, R_2, R_3, & R_4} everyday (morning and evening). The control trays consist of uninoculated sticks which were kept in chamber at ambient room temperature (30 ± 2^0C) while humidity was maintained >85% by putting extra two trays of water. Spraying of distilled water only was done on control. Humidity was measured by hygrometer during experiment period.

Results and Discussion

Retting is the process of separation and extraction of fibres from non-fibrous tissue and woody part of the stem through dissolution and decomposition of pectin, gum and other mucilaginous substance. As mentioned in Table 1, four different fungi were isolated and identified from the bark of G. *optiva*. Degradation and severity tests were conducted by applying all pure fungi on wood samples of *G. optiva* and observations were recorded as shown in Table 2. *Penicillium* sp. was identified by its characteristics i.e. developed blue green colour colonies in the plates and having brush like **conidiophores**. Black colonies were produced by *A. niger*, while white colonies were produced by *Pestalotiopsis* sp. and grey colonies by *Rhizopus* sp. It was interesting to note that the pectinolytic fungi A. niger was commonly found on wood samples collected from all the study sites and observed significantly higher as compared to others fungi observed (Table 2). Similar results were recorded by Haque et al. (1992) and Banik (2016) in Jute. However, contrary results have been reported by Gustavo et al. (2012) when they tested the degradation capacity of fungi (Colletotrichum sp., Penicillium sp. and Rhizopus sp.) on mangoes and oranges and reported incidence and severity were greatest in mangoes inoculated with Colletotrichum + Rhizopus. It was concluded that degradation was greater in mangoes and oranges inoculated with a mixture of Colletotrichum and Rhizopus, and Penicillium and Rhizopus, respectively. All three fungi have optimum growth temperature at around 25°C. Sporotrichum thermophile has a different optimum growth temperature of 45°C. However, this fungus is able to grow at wider range of temperature, i.e. 30° - 50°C. If retting has to be conducted at higher temperature Sporotrichum thermophile will be more effective (Banik, 2016).

Table 2. Organisms isolated from *G. optiva wood* samples

Organisms	Location of sample collected	Appearance on agar plate	Degradation and severity Observed
Aspergillus niger	Kalsi, Dehradun	Black colonies	Faster
Penicillium sp.	Kalsi, Dehradun	Blue Green colour colonies	Not significant
Penicillium sp.	Yamkeshwar, Pauri Garhwal	Blue Green colour colonies	Not significant
Pestalotiopsis sp.	Yamkeshwar, Pauri Garhwal	White colonies	Not significant
Aspergillus nigar	Yamkeshwar, Pauri Garhwal	Black colonies	Faster
Penicillium sp.	Ranikhet, Almora	Blue Green colour colonies	Not significant

Aspergillus niger *Rhizopus* sp.	Ranikhet, Almora Ranikhet, Almora	Black colonies Grey colour colonies	Faster Not significant

Retting is a biological process, which removes noncellulosic materials attached on the fibre bundle by enzymatic activities, consequently yielding detached cellulosic fibres (Jayaramudu *et al*., 2010). Retting process utilizes a complex microbial community for dissolution process after immersion of *G. optiva* sticks in water, releasing soluble constituents like sugar, glucosides and nitrogen compounds. Retting microbes present in plant and water were continued to build up their population by utilizing these soluble compounds. The fermentative microorganisms consume the cementing materials viz., the pectins, hemicelluloses and proteins with release of galacturonic acid and sugar in retting water (Basak *et al*., 1998). Here, all microbial degradation occurs in the absence of water by the fungus *A. niger*. It is a filamentous fungus which is used for waste management and bio-transformations, in addition to its industrial uses, such as production of citric acid, gluconic acid, and enzymes such as amylases, pectinases and proteases (Godfrey and West, 1996). Several authors reported that *A. niger* species can be responsible for the postharvest decay of different fresh fruits and some vegetables (Gautam *et al*., 2011; Sharma, 2012, Gustavo *et al*., 2012). It has been demonstrated that *Aspergillus* spores are one of the most frequently identified fungal conidia in the atmosphere (Kasprzyk, 2008; Dijksterhuis, 2019). It is most commonly found in decaying vegetation, soil, or plants, but it cannot be considered particularly dangerous in comparison to *A. fumigatus*, which is the most prevalent airborne pathogen. *A. niger*, the most abundant mold found in the environment, has also been the source of several bioactive compounds and industrial enzymes (Schuster *et al*., 2002). However, allergic issues associated with humans have been noticed when exposed to spores and its vegetative forms present in air and consumable foods (Goutam *et al*. 2011). Fungal retting is conducted in dry and aerobic conditions and pectinolytic fungi *A. niger* has been chosen for fibre extraction by dry retting technique. The regular spray in morning and evening with mass culture *of A. niger* was done on sticks for retting to soften the bark. No immediate effect was observed. However, after 6-7 days, a layer of retting fungus was observed on the surface treated sticks in chamber. After regular spray of *A. niger* (mass culture) on sticks for 13 days was observed complete retting. The lignocellulose material was completely degraded and the outer bark of the *G. optiva* sticks was ready to peel off. The experiment was terminated on 13th day. The bark of each treated stick of all the replications were pulled out manually very easily, washed out properly in fresh running water and sticks were kept separately (Table 3). Finally the extracted fibres were dried under the sun for removing of moisture. Those sticks were kept as control (R_5) had intact bark that could not be removed properly for fibre extraction; however it became blackish. Similar results were also reported by Banik (2016). He applied four pectinolytic fungi for dry retting of jute, viz. *Aspergillus tamarii, A. flavus, A. niger and Sporotrichum thermophile*. Gunnar *et al*., (1997) also reported *Rhizomucor pusillus* and *Fusarium lateritium* as noteworthy retting organisms by their high level of pectinase activity, ability to attack noncellulosic cell types without attacking cellulose, capacity to penetrate the cuticular surface of the stem, and efficient fiber release from the core. Results are in accordance of Banik *et al*., (2003). They reported similar fungal dry retting of jute ribbon retting had been tried.

Table 3. Observations of application of *Aspergillus niger* on bark of *Grewia optiva* in lab for extraction of fibre. 100 sticks (size 30cm each) were taken in each replication and R_5 was kept as control.

Day	R_1	R_2	R_3	R_4	R_5 (control)
01	-	-	-	-	-
02.	-	-	-	-	-
03.	-	-	-	-	-
04.	*	*	*	*	-
05.	**	**	**	**	-
06.	***	***	***	***	-
07.	***	***	***	***	-
08.	***+	***+	***+	***+	*
09.	***+	***+	***+	***+	*
10.	***++	***++	***++	***++	*
11.	***++	***++	***++	***++	*
12.	***+++	***+++	***+++	***+++	*
13.	✓	✓	✓	✓	*

*= started infection of fungi on bark, + = magnitude of fungi increased and growth on bark, ✓= Ready to extract fibre

The fungal dry retting process has been found to be an aerobic method, distinguishing it from traditional water retting. This innovative approach offers numerous benefits, including environmentally friendly, producing no pollution, Faster processing times, significant water savings, high-quality *G. optiva* fiber production, preservation of strong, unbroken, and full-length its sticks and meeting the desires fibre to farmers. This eco-friendly and efficient method has the potential to revolutionize the *G. optiva* industry, providing a sustainable solution for fibre production while meeting the needs of farmers and producers.

Conclusion

Grewia optiva is a multipurpose agroforestry tree species. Four strains of filamentous fungi, namely *A. niger*, *Penicillium* sp., *Pestalotiopsis* sp., and *Rhizopus* sp. were identified and isolated from *G. optiva* bark that was dew retted the laboratory. Out of four isolated fungi, *A. niger* was chosen because of its high spore production and frequent isolations from G. *optiva*. Results showed that complete retting was observed within 13 days by application of *A. niger*. This dry retting technology minimizes the use of water to obtain fibre. Our findings suggest that *A. niger* may be applied to obtained fibre from bark of *G. optiva* that can save 85.6% time as compared to conventional method of fibre extraction, which takes more than 90 days.

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