ENVIRONMENTAL SCIENCE ARCHIVES
ISSN: 2583-5092
Volume II Issue 2, 2023
www.envsciarch.com

RESEARCH PAPER OPEN ACCESS

Bacterial Inocula Package Formulation and Application for Fibre Extraction from Bark of Dried Cultivated Varieties of *Corchorus capsularis*

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Received:
2023/7/24
Accepted:
2023/08/06
Published:
2023/08/16

Abstract
In the present study, the bacterial consortia of three combinations were used and identification of unknown bacteria was carried out. The combination of 3PRRF5b+4DTF1b+10DTW2b was found to have better retting performance. Formulations showed good potential as candidates for the microbial consortium. Selected microbes were screened out by gram staining. Growth and colony characteristics were determined and the Gram-Negative and Positive selection criteria of unknown bacteria were done. The possible dichotomous identification maps and dendrogram for Gram-Negative and Gram-Positive organisms were measured for probable detection of the isolates. On the biochemical analysis, the organisms can match up to 80% till genus level but this research needs to be optimized for the identification of these consortia.

Keywords: Microbial inocula; Enzyme production; Potentiality; Bark; *Corchorus capsularis*

Introduction
Microbial consortia are elements of diverse communities of bacteria and fungi found naturally throughout the biosphere. Microbes naturally colonize plants, providing beneficial, harmful, or neutral interactions (Haque et al., 2002). Microbial populations can be obtained directly from the environment or subculturing from an available stock culture. These microorganisms may come from various sources, depending on the activities that lead to their use in a particular issue (Ahmed and Ahkter, 2001). Microbial inoculums/consortia on the basis of their enzyme production ability can be formulated (Yadi et al., 2013). The objectives of inoculum development were rapid growth rates and high biomass concentration at the beginning of a fermentation process. However, the most important objective is to obtain a pure culture of the microbial population for the purpose of fermentation (Ahmed, 2008). These inoculums may be applied on dried or preserved bark. In our previous report, an attempt was taken to investigate the formulation of bacterial consortium as a whole-cell biocatalyst for the degradation of dry jute ribbons. The microbial consortium was constructed from 7 (seven) selected isolated bacteria to become 7 (seven) combination cultures. The bacterial consortium exhibited remarkable retting efficacy due to the induction in activities enzymes. The process of separation and extraction of fibers from non-fibrous tissues and the woody part of the stem through dissolution and decomposition of pectins, gums, and other mucilaginous substances called retting, is a biochemical process carried out by the action of various retting microbes. The microbial formulation may be found suitable not only for reducing retting duration but also for improving fiber quality. Inoculum formulation is the process of preparing a population of important microorganisms from a dormant stock culture to a population of microorganisms that can be used for inoculating a final productive stage (Ahamed and Vermette, 2008). More than 90% of jute growers ret the jute and mesta plants in stagnant water following the conventional method of retting (Alam et al., 2003).

The repeated retting of jute and mesta in the stagnant water of the same natural retting tank led to the production of inferior quality fiber unless the tank is recharged with fresh water after each retting. To overcome this problem, an attempt was taken to use microbial formulation not only to reduce retting duration but also to improve fiber quality. On the other hand, our country's actual situation during a retting season was facing water scarcity. Groundwater is the only source in case of low rainfall. An artificial retting tank (cemented or polyethylene lined) is the only option to hold water for a long time. There was an urgent need to develop an improved method of retting that requires very less amount of water, lesser time than the conventional method, improves fiber quality and above all is user-friendly and eco-friendly in nature. In the present research, an attempt was taken to identify these microbial teams and was taken to formulate microbial inoculums for fiber extraction. In inocula development, the methods must aim at minimizing the loss of viable microorganisms during recovery from the dormancy stage, obtaining a genotypically identical copy of the population that was stored, increasing biomass and the development of culture to a physiological state suitable for performance in the final production stage. This developed method of retting will help to develop novel technology for fiber extraction of preserved bark of jute and can be suitable for not following obligatory seasonal retting.

Material and methods

The isolated bacterium was tested for retting of dry jute ribbons in a consortium of two combinations was studied in our previous report (Ahmed and Sarkar, 2022) where primary screening was carried out. The present study was conducted in the Department of Microbiology, BJRI, Dhaka where all the chemicals and media were reagent grade (Sigma). Nutrient agar media (Sigma) was used throughout the study. Isolated bacterial sample density was homogenized using the method by Agilent-5301 where the calculator uses the extinction coefficients for bacterial cultures to calculate the cell concentrations from the optical density (OD_{600}) reading taken with a spectrophotometer. For bacterial cell cultures OD_{600} of 1.0 = 8 x 10^8 cells/ml. Out of the collected isolates from different locations of jute retting water in different periods of time, 11 (eleven) isolates were selected for this experiment which is designated as- 15DTW2b, 4DTW2b, OMEW4b, 4DTW1b, 3PRRF5b, 3PRRF2b, 4DTF1b, 10DTW2b, 10DTW3b, OMPW4b and 4DTW7b.

Application of consortia

Screening of microbes for pectinase and xylanase production was described in our previous report (Ahmed and Sarkar, 2022). Moreover, inoculum preparation, formulation designing, treatment, preparation of microbial consortia, inoculum development and culture condition followed in present research were the same as described in our previous report. In the case of combinations of microbial consortia, three combinations of the isolates were used in the present research whereas, in our previous report, single and two combinations were already described (Ahmed and Sarkar, 2022). Microbial consortia of selected microbial broth of 15 different combinations using three microbial samples were applied on cultivated varieties of white jute (*Corchorus capsularis* L.; CVL-1) dry fiber with water where 5ml distilled water was added in 5ml each of three microbial broths for 2g dried fiber. In the present research, the identification of unknown bacterial samples and application of inoculums in three different possible combinations was carried out on preserved jute bark.

Fig. 1. Treatment of preserved CVL-1 dry fiber with the selected microbial broth of three combinations with water

Results and Discussion

Selection of bacteria for consortium formulation

The collection of selected microbes was screened out and formulated different three possible combinations and applied in preserved CVL-1 dry fiber of jute (Figure 1).

In three combinations of microbial consortia, the combination of 3PRRF5b+4DTF1b+10DTW2b was found better retting performance followed by 15DTW2b+OMEW4b+OMPW4b combination (Table 1).

Table 1. Treatment of preserved CVL-1 dry fiber with selected microbial broth of three combinations

Sl.	Selected microbes	Selected microbial combination$	Fineness* (Soft & touch method)	Brightness** (Soft & touch method)	Smoothness/ Softness *** (Soft & touch method)
1	15DTW2b	15DTW2b+OMEW4b+3PRRF5b	3	2	2
2		15DTW2b+OMEW4b+4DTF1b	2	1	1
3	OMEW4b	15DTW2b+OMEW4b+10DTW2b	1	2	2
4		15DTW2b+OMEW4b+OMPW4b	3	3	3
5	3PRRF5b	15DTW2b+OMEW4b+4DTW7b	1	2	2
6		OMEW4b+3PRRF5b+4DTF1b	2	1	1
7	4DTF1b	OMEW4b+3PRRF5b+10DTW2b	1	1	1
8		OMEW4b+3PRRF5b+OMPW4b	2	3	3
9	10DTW2b	OMEW4b+3PRRF5b+4DTW7b	2	1	2
10		3PRRF5b+4DTF1b+10DTW2b	4	4	4
11		3PRRF5b +4DTF1b+OMPW4b	2	2	1
12	OMPW4b	3PRRF5b+4DTF1b+4DTW7b	2	1	1
13		4DTF1b+10DTW2b+OMPW4b	3	3	2
14	4DTW7b	4DTF1b+10DTW2b+4DTW7b	1	1	2
15		10DTW2b+4DTW7b+15DTW2b	3	2	3

* 1=Poor, 2=Fair, 3=Good, 4= Very good, 5=Excellent
** 1=Poor, 2=Fair, 3=Good, 4= Very good, 5=Excellent
*** 1=Poor, 2=Fair, 3=Good, 4= Very good, 5=Excellent
$Using a web-based calculator to give the concentration of bacterial cell cultures based on spectrophotometer readings at OD_{600}.

Identification of selected microbes

Selected microbes were screened out by gram staining in order to know their identifications (Table 2). Growth and colony characteristics were also determined of these isolates (Tables 3 & 4; Fig. 2). The Gram-Negative and Positive selection criteria of unknown bacteria were shown in Tables 5 and 6. The possible dichotomous identification maps for Gram-Negative and Gram-Positive organisms were shown in Fig. 3. Lastly, a dendrogram was also done for the probable detection of the isolates (Fig 4).

Table 2. Gram staining of the isolated microbes

Bacterial Isolates	Gram Positive	Gram Negative
15DTW2b	-	+
10DTW2b	+	-
3PPRF5b	+	-
4DTW7b	-	+
4DTF1b	+	-
OMEW4b	+	-
OMPW4b	-	+

Table 3. Characterization of unknown bacteria

Bacterial Isolates	Media					
	MSA	SS	MAC	TCBS	EMB	Cetrimide
15DTW2b	-	Pink	Pink	Yellow	Pink	-
10DTW2b	-	Colorless	Pink	-	Colorless	-
3PPRF5b	-	Light Pink	Pink	Yellow	Pink	-
4DTW7b	-	Light Pink	Pink	Yellow	Pink	-
4DTF1b	-	Light Pink	Pink	Green	Colorless	-
OMEW4b	-	Pink	Colorless	Yellow	Pink	-
OMPW4b	-	Pink	Pink	Yellow	Pink	-

MSA = Mannitol salt Agar, SS = Salmonella-Shigella Agar, MAC = MacConkey Agar,
TCBS = Thiosulfate-citrate-bile salts-sucrose Agar, EMB = Eosin Methylene Blue Agar

Table 4. Isolated colony characteristics on Blood Agar media

Isolates	Pigmentation	colony size	Hemolysis	Margin	Elevation	Consistency
15DTW2B	Gray	small	Negative	entire	raised	plaster
10DTW2B	Gray	pinpoint	Negative	entire	raised	plaster
3PPRF5B	Gray	small	Negative	undulate	raised	mucoid
4DTW7B	Gray	small	Negative	entire	raised	mucoid
4DTF1B	Gray	pinpoint	Negative	entire	raised	plaster
OMEW4B	Gray	small	Negative	entire	raised	plaster
OMPW4B	Gray	small	Negative	undulate	raised	mucoid

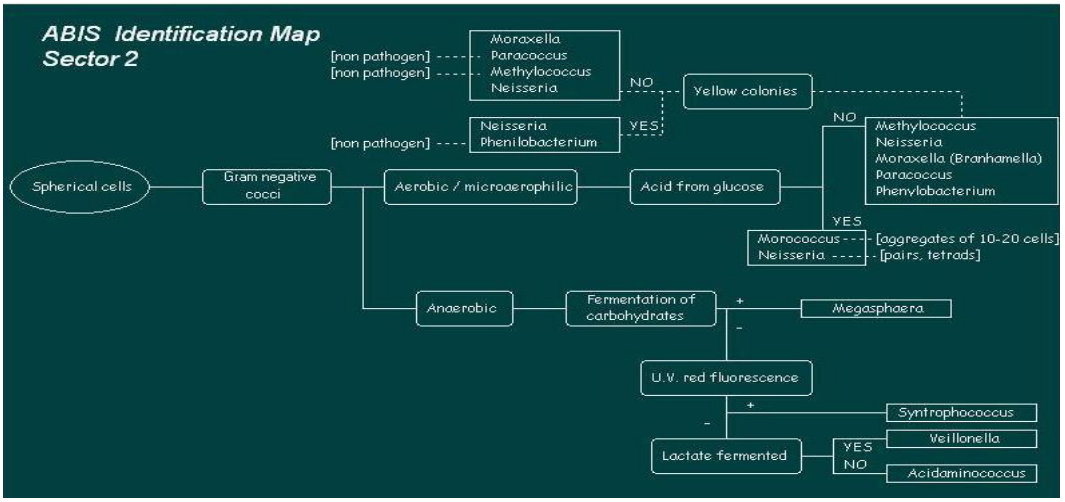
Fig. 2. Growth of isolated colony characteristics on Blood agar media

Table 5. Gram-negative selection criteria of unknown bacteria

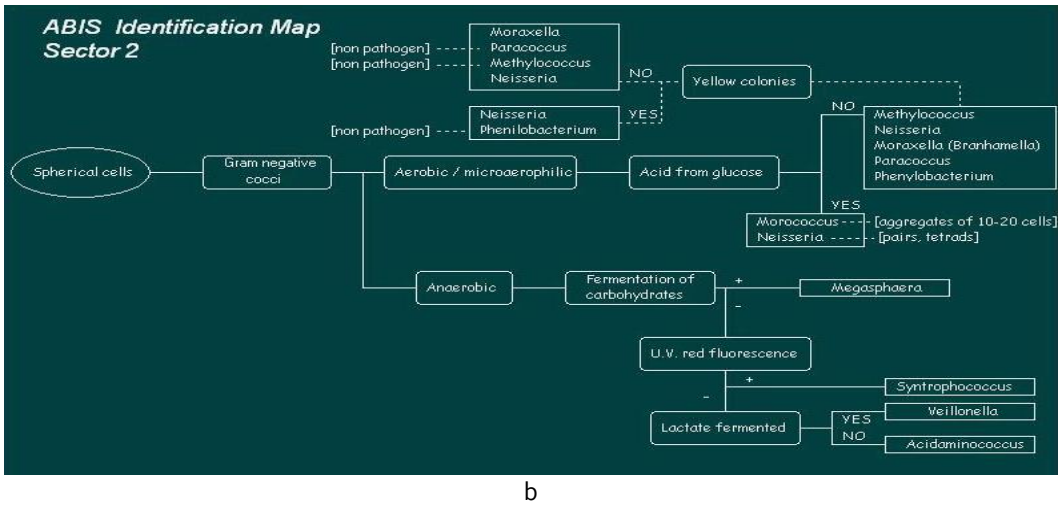
Isolates	Motility	Indole	MR	VP	Citrate	Urease	Catalase	Oxidase	Glucose	Lactose	Sucrose	Sorbitol	Mannitol	H_2S	Gas	TSI	Hemolysis
15DTW2b	-	-	+	+	+	+	+	-	+	+	+	-	+	-	+	+	-
4DTW7b	-	-	+	+	+	-	+	-	+	-	-	1	+	-	-	-	-
OMPW4b	-	-	-	+	+	+	+	-	+	+	+	-	+	-	+	+	-
Chromobacterium rhizoryzae	-	-	-	-	+	-	+	-	+	+	-	-	+	-	+	+	-
Chromobacterium alkanivorans	-	-	+	+	+	-	+	-	+	-	+	-	+	-	+	-	-
Moraxella sp	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	+	-
Aeromonas sp	-	-	-	-	-	-	+	+	+	+	+	-	-	-	+	+	-
Photobacterium damselae subsp. *damselae*	-	-	+	+	-	-	+	+	+	+	-	-	-	-	+	+	-
Photobacterium damselae subsp. *piscicida*	-	-	+	+	-	-	+	+	+	+	-	-	-	-	+	+	-
Vibrio vulnificus bio group 1	-	-	+	+	+	+	+	+	+	+	-	-	+	-	+	+	-

Table 6. Gram Positive selection criteria of unknown bacteria

Isolates	oxygen requirement	Indole	MR	VP	Citrate	Urease	Catalase	Oxidase	Glucose	Lactose	Sucrose	Sorbitol	Mannitol	Gas	TSI	MSA	Hemolysis
10DTW2b	Aerobic	-	-	+	+	-	+	+	+	-	-	-	+	-	A/K	-	-
3PPRF5b	Aerobic	-	-	+	+	-	+	-	+	+	+	-	+	+	A/A	-	-
4DTF1b	Aerobic	-	-	-	+	-	+	+	+	-	-	-	+	-	A/K	-	-
OMEW4b	Aerobic	-	-	+	+	+	+	-	+	-	-	+	+	+	A/K	-	-
Micrococcus	Aerobic	-	-	-	+	-	+	+	+	-	+	-	-	-	A/A	-	-
Staphylococcus	Facultative	-	+	+	-	+	+	-	+	+	+	-	+	-	A/A	+	V
Brevibacterium	Aerobic	-	-	+	+	-	+	-	+	-	-	-	-	-	AL/AL	-	-
Microbacterium	Aerobic	-	-	-	-	-	+	-	+	-	-	-	-	-	AL/AL	-	-
Corynibacterium	Facultative	-	-	-	-	-	+	-	+	-	+	-	+	-	AL/AL	-	-
propionibacterium	Facultative	-	-	+	+	-	+	-	+	-	-	-	-	-	AL/AL	-	-



a

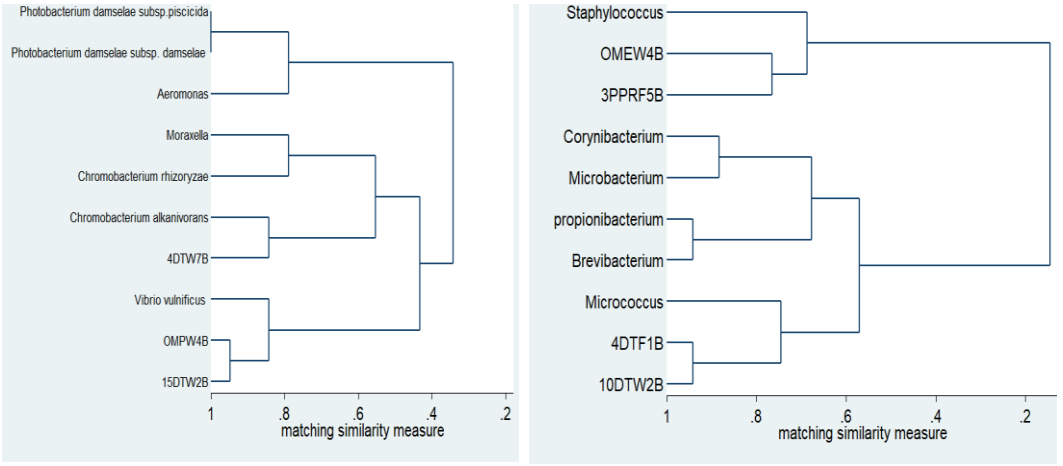


b

Fig. 3. Dichotomous identification maps (a) Gram Negative organisms, (b) Gram Positive organisms

Microorganism found in retting water is postulated to have the potential to degrade proteins and starch in the organic material found in dry jute ribbon (Banik et al., 2003, Das et al., 2012). A microbial retting consortium has been developed by Ranjan et al. (2011) characterized by the production of pectin and xylan degrading enzymes where they used a microbial consortium for fast retting of bast fiber comprising three *Bacillus pumilus* strains containing *Bacillus pumilus*

IMAU80221, *Bacillus pumilus* GVC11 and *Bacillus pumilus* SYBC-W mixed in a ratio of 1:2:1 or 1:1:2. or 1:1:1. In this ongoing research, opined that these breakthrough findings will help to further the knowledge on the unique microbial retting process in jute and will accentuate the improvement in this microbial formulation. Based on the biochemical, the organisms can match up to 80%, till the genus level. But certain key biochemical tests- such as hemolysis, and utilization of certain carbohydrates are confusing. More biochemical results such as ONPG, LDC, and Ornithine are unavailable which might have been a great help. It can presume in the case of gram-negative cocci that the isolates have similarities.



(a) **(b)**

Fig 4. Dendrograms (a) Gram Negative (b) Gram Positive cluster analysis

Therefore, it cannot be detected because there is some ambiguity that needs Genome sequencing. It is believed that this will also open up an avenue to characterize the enormous diversity of the retting microbial population at the metagenome scale and incorporate other strains to complement the consortium. This will establish a correlation between microbial diversities and regional differences in fiber. In inoculum development, the methods must aim at minimizing the loss of viable microorganisms during recovery from the dormancy stage, obtaining a genotypically identical copy of the population that was stored, increasing biomass and the development of culture to a physiological state suitable for performance in the final production stage. Many researchers reported that even though microorganisms are highly adapted specific microbial types are associated with different niches or samples within a variety of ecosystems (Banik et al., 2007) Therefore, microorganisms isolated from different locations of sampling will be different from one another. Microorganism isolated that is isolated from one location may not be able to ret jute from another location, owing much to environmental factors such as humidity, temperature, etc. An example of cellulolytic and amylolytic bacteria that had been successfully isolated and screened for its ability to degrade cellulose and amylase is the Bacillus amylolique / adens. This bacterium had been isolated from sago pith waste. It has both cellulolytic and amylolytic activities in the decomposition of sago pith residue. The result so far obtained has a positive indication and has potential future; therefore, in-depth genomic analysis is needed. Genome sequencing also confirms that retting bacteria degrades pectin, hemicellulose and other non-cellulosic materials, non-harmful for fiber. The bacterial strains are also non-toxic and thus the retting water with microbial strains can successfully be used for irrigation purposes. It has a long way future plan to introduce a microbial consortia package that can be added market value.

Conclusion

In the present experiment, 4" long and 20g dry ribbons were used as lab limitations; but in the case of a large-scale experiment for getting long fiber from dry ribbon, the experiment should be conducted using long dry ribbons having more weight in the pilot and industrial scale. As the ribbon is dry, it shouldn't be twisted during the experiment; instead, spread straight in any experimental container. In case of a combination experiment, precaution should be taken during mixing in sterile conditions. In the present situation, to identify the unknown microbes it needs

lots of specific microbial media, reagents and chemicals that are inadequate. To test the physical properties of processed fibers, actually it requires at least 3.0g fibers but as the experiment was conducted with 2.0g; the partial physical property (brightness) was recorded and in the future, the total physical properties of processed fibers will be done by meeting the requirements. Slow-moving soft water produces the best quality fiber, but such conditions rarely prevail in the jute-growing areas of Bangladesh. Scarcity of water or low rainfall during retting time compels farmers to ret their jute crop in stagnant muddy water resulting in low-quality jute fiber. Under such a situation, the mechano-microbial retting process proves to be suitable for farmers. By this process, farmers can ret their jute crop after ribboning within a period of 7-9 days in a very less quantity of water with improvement in fiber quality and more net income than the conventional method of retting. *In-situ* jute retting in micro-pond with microbial consortium is another suitable option left for jute farmers as an alternative to the conventional method of retting in less time, with reduced volume of water with quality improvement.

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Author Contributions

ZA conceived the concept, wrote and approved the manuscript.

Acknowledgements
This research was supported by Microbiology Department, Primeasia University, Banani, Bangladesh. The author thanks Mr. Maruf Abony, Lecturer, Microbiology Department, Primeasia University for comments that greatly improved the manuscript. The author also thanks his colleagues from Bangladesh Jute Research Institute for sharing their pearls of wisdom with us during the course of this research.

Funding
There is no funding source for the present study.

Availability of data and materials
Not applicable.

Competing interest
The author declares no competing interests.

Ethics approval
Not applicable.

Citation: Ahmed Z (2023) Bacterial Inocula Package Formulation and Application for Fibre Extraction from Bark of Dried Cultivated Varieties of *Corchorus capsularis*. Environ Sci Arch 2(2):156-163.