

Microbial Retting of Banana Pseudostem

Sonia Sharma, Neeraj Wadhwa



Abstract: Fibrous wastes correspond to approximately 54.3 wt.-% of a banana plant. The waste weight is mainly of bunches, stems, and leaves. These ligno-cellulosic fibre waste can be designed and tailored to yield value added products which can be exploited by the farmers, exporters and weavers. Pectin (plant cement) and hemicelluloses (22-25%) found in plant cell walls in the plate (middle lamella). These macromolecules have needs to be hydrolyzed faster without affecting the quality of fibre. The chemical and mechanical processes adopted for fibres actually affect color uptake, strength of fibre thus fetching lower price. We report a biological ecofriendly method of retting where pectinases produced by the endophytic microorganisms of banana pseudostem were used to separate the fibre bundles of cellulose.. Endophytes are microorganisms which live in close relationship with living plant tissues in a cooperative relationship delivering a few auxiliary metabolites and proteins with the possibility to hydrolyse a few plant-determined macromolecules of the host. They spend the entire or part of their lifecycle colonizing within sound tissues of the host plants. The endophytic pectinase helps to break down the cell walls and can find commercial application in extraction of fibres. Seven bacterial isolates were screened and isolated in pectinase screening agar medium [PSAM]. Pectinase producing endophytic bacteria is isolated from corm of *Amorphophallus paeoniifolius* reported by us as *Staphylococcus sciuri*, *Exiguobacterium acetylicum*, *Exiguobacterium indicum* which are good pectinase producers having specific activity of 8.26, 12.61, 6.81 respectively. Treatment of banana pseudostem with these microbes showed 91%, 94%, 96.7% loss in the total weight of stem leaving behind pure cellulose fibres.

Keywords: Banana pseudostem, Endophytes, Pectinase, Cellulose fibres, 16s RNA, Retting

I. INTRODUCTION

Retting process of plant material involves mechanical, chemical and/or enzymatic reactions. Ecofriendly retting processes utilize complex microbial community for accelerating the release of soluble constituents like sugar, glycosides and nitrogenous compounds from complex polymers of the plant material from where the cellulose fibres need to be extracted. This processes of release of simple monomers also helps in further growth of microbes. Biofibres are natural polymers, they are generally biodegradable but they do not possess the necessary thermal and mechanical properties desirable for engineering plastics. Literature also shows some reviews on cellulosic as well as

ligno-cellulosic fibre based composites. which can be tailor made to make versatile products like rugs, fabrics etc. Since bio-fibres are cheap and biodegradable, the bio composites from bio-fibre reinforced biodegradable polymers can render a major contribution in solving waste disposal which is serious environmental problem of 21st century [1] Vegetable and fruit plants waste are one of the main environmental problems that exist today and there is need for developing value added products from this waste which will benefit the farmers and the weavers. In the banana cultivation industry it is found that there is generation of high amount of organic waste in form of leaves, pseudo-stem. It is the fruit that is exported and this only represents 11.16 wt.-% of the plant total weight. All other waste residues correspond to fibrous and non-fibrous materials. Fibrous wastes corresponding to 54.3 wt.-% of the total plant weight are mainly bunches, stems, and leaves. From all these parts of the plant, fibre bundles can be extracted. Different methods can be implemented to separate fibre bundles from vegetable resources like employing mechanical, chemical procedures alone or in combination. Both biological and chemical processes are traditionally employed to extract soft fibres such as flax and jute by retting procedures. Fermentation of vegetable source can be performed by anaerobic or aerobic ways, generating soluble components and gases such as methane. In general, this procedure consists in soaking a vegetable waste source for some days in water containing a concoction of microorganisms. Fibre bundles are thus obtained by the biological action of microorganisms present in the bath [2]. Retting is a fermentation process, where certain bacteria (e.g., *Clostridium*, *Bacillus*) and fungi (e.g., *Aspergillus*, *Penicillium*) release the fibre by decomposing pectin present in the bark[3]. Pectin and hemicelluloses (22-25%) are the plant cement and are rich in the primary wall and middle lamella. The predominant task for extraction of fibre is to remove these non-cellulosic substances without causing damage to the fibre cellulose. Pectinases are the group of enzymes that helps to break down the cell walls cementing material. They have been utilized to increase the volume of fruit and vegetable juices, lowering the viscosity of the juice, reducing the cloudiness of the juice, caused by suspended pieces of cell wall [4].

II. MATERIAL AND METHODS

A. Screening of pectinase producing bacteria

Seven bacterial isolates from the corm were cultivated in pectinase screening agar medium [PSAM]. Nutrient agar containing 1.5% pure pectin. Media is sterilized by autoclaving at 121°C for 15 min. bacteria were streaked on plates. pectin utilization was detected by flooding the culture plates with freshly prepared iodine-potassium iodide solution

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[Iodine-1.0 g, potassium iodide-5.0g in 330ml distilled water] this solution gives color to the medium containing pectin resulting in a translucent halo region where pectin is degraded, which indicated the presence of pectinolytic activity[5].

B. Secondary screening of pectinase

All pectinase producing endophytes were inoculated in media of composition peptone 3g, yeast extract-0.5g, KH₂PO₄-0.15g, CaCl₂-0.001g, pectin -0.5g, Na₂CO₃-0.5g at pH 8[6].

C. Identification of bacteria by using 16S rRNA

All the seven endophytic bacteria were classified by 16S rRNA identification. Sequencing was done by Sanger method in ABI 3500xL genetic analyzer (Life Technologies, USA) at NCIM Pune

D. Determination of extracellular enzyme activity

Determination of reducing sugars was done by (DNS) method, 1959 utilizing pectin as substrate. For enzyme assay, 5ml of freshly grown culture was taken and centrifuged at 10,000 rpm for 5min. The culture broth was centrifuged at 10,000 rpm and was served as the source of the enzyme. In addition, substrate was prepared by mixing 0.5% (w/v) citrus pectin in 0.1M of pH 7.5 phosphate buffer. 900 μ l of substrate was added to two clean labeled test tubes; enzyme blank and test sample, 100 μ l of distilled water and crude enzyme was added to the respective test tubes. Reaction was stopped by adding DNS reagent (2 ml) to each test tube after 10 minutes of incubation at 50 °C. Test tubes were placed in a boiling water bath (92°C) for 10 minutes so that color develops. Finally, the tubes were cooled and freshly prepared sodium potassium tartarate (1ml) was added. Absorbance values were measured at 540nm using spectrophotometer (JENWAY 6300 UV/Vis). Enzyme activity was measured against enzyme blank. One enzyme unit was defined as the amount of enzyme that catalyzes μ mol of galacturonic acid per minute (μ molmin⁻¹) under the assay conditions [7].

E. Determination of amount of protein

The amount of protein produced was assayed by Bradford test. 100 μ l of sample was diluted with 900 μ l of water and mixed with 2ml Bradford reagent. Absorbance was taken at 595nm using spectrophotometer (JENWAY 6300 UV/Vis) [8].

F. Production of pectinase by submerged fermentation

All pectinase producing endophytes were inoculated in 500 ml Erlenmeyer flask having 250ml of production media comprising of composition peptone 3g, yeast extract-0.5g, KH₂PO₄-0.15g, CaCl₂-0.001g, pectin -0.5g, Na₂CO₃-0.5g at pH 8 with 1% (v/v) inoculum (106 spores/ml) and incubated at 30°C under shaking condition (150 rpm) for 10days. The culture broth was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was used to evaluate enzyme activity and weight of remaining stem at different days of retting mentioned below.

G. Retting of pseudo-stem

Retting was performed in presence of bacterial strains producing extra cellular pectinase under two conditions

(i) Bacterial retting in media and

(ii) Bacterial retting in water. Bacterial inoculum were added to specific media in bacterial retting while in water bacterial inoculum was added in water at the beginning of the process. Each 500 ml Erlenmeyer flask was used to ret 2-3 stems of approximately the same weight. After retting, stems were washed in running water and placed to dry in a petridish at room temperature [9].

H. Loss in weight

There is also reduction in weight of fibre after 10 days of experiment. The reduction in weight was calculated by measuring the weight before and after retting.

The retting of banana stem again carried out using *Staphylococcus sciuri*. About 5 cm and 1cm wide banana stem fibre sticks were incubated in presence and absence of EDTA (0.01 M) composition of the reaction mixtures was as follows;

i Glycine -NaOH buffer pH 9.0 (100 ml of 100 mm) of Cultural broth supernatant (100 μ l) showing the presence of enzyme activity was added in both the sets and the reaction mixtures were incubated for 24 hrs at 37°C in water bath followed by vigorous shaking and washing with hot water (10 ml) for 60 minutes. Water was drained off and the treated fibres were dried for 72 hr. and their dry weight was calculated. Heat inactivated supernatant was considered as control.

III. RESULT

A. Screening of pectinase producing bacteria

Out of seven endophytes three bacterial strains gave positive pectinase test. Degradation of substrate pectin was visible in form of clear halo zone around the bacterial colony.

B. Secondary screening of pectinase and Production of enzyme in cultivation media

Yeast extract lactose, Tryptone media at alkaline pH (8-10) was the optimal media composition and condition for *Staphylococcus sciuri* growth and pectinase production; Yeast extract, pectin and KNO₃ media at alkaline pH (8-10) was preferred by *Exiguobacterium acetylicum*; *Exiguobacterium indicum* preferred pectin and KNO₃ media at acidic pH 4 (Unpublished –lab data). The above mentioned three cultures were grown in secondary screening media and specific activity for the enzyme was calculated please refer to Table I

Table- I: Comparative analysis of specific activities of pectinase producing bacteria confirming that *Exiguobacterium acetylicum* being a good producer of pectinase

S.no	Culture broth	Total enzyme (μ mol ml ⁻¹ min ⁻¹)	Total protein mg/ml	Specific Activity (μ mol min ⁻¹ mg ⁻¹)
1	<i>Staphylococcus sciuri</i>	6.94	0.84	8.26
2	<i>Exiguobacterium acetylicum</i>	4.16	0.33	12.61
3	<i>Exiguobacterium indicum</i>	2.58	0.38	6.81

The culture broth was centrifuged and in the cell free supernatant pectinase activity was measured .We report that *Exiguobacterium acetylicum* showed maximum specific activity of 12.6 Units nits followed by *Staphylococcus sciuri* which showed 8.26 Units and *Exiguobacterium indicum* 6.81 Units

C. Retting experiment:

When the Pseudostems were incubated along with the endophytes in their optimized media as mentioned above for seven days good separation of the fibres was seen when *Staphylococcus sciuri* was used. The texture and color of the separated cellulose fibre was far better. (seeFig. I). However when pseudo stems were incubated with endophytes presence of water no separation of fibres was seen (see Fig.II)

We report that there is approx. 95% to 98% loss in weight in both media and water samples containing the microbes. (See TableII) Loss in weight of the pseudo-stem was calculated by the formula. Loss in weight= Initial weight- Final weight *100/ Initial weight

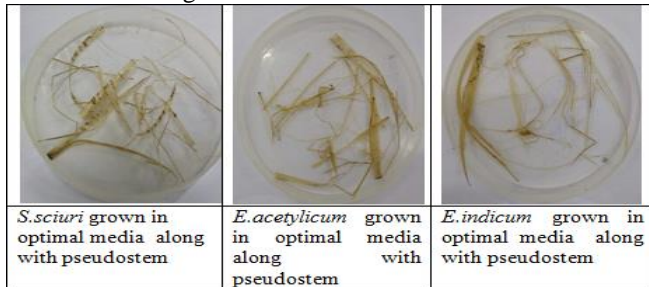


Figure I Banana Pseudo stems were incubated with the identified endophytes in their optimized media: Yeast extract lactose , Tryptone media at alkaine pH (8-10) for *Staphylococcus sciuri*; Yeast extract ,pectin and KNO3

media at alkaline pH (8-10) for *Exiguobacterium acetylicum* and pectin, KNO3 media at acidic pH 4 for *Exiguobacterium indicum* for seven days . Separation in the fibres is clearly seen .Coarser fibres are visible for *Exiguobacterium acetylicum* treated and *Exiguobacterium indicum* treated fibres however the fibres were more finer and smoother for *Staphylococcus .sciuri* treated pseudostem in optimal media

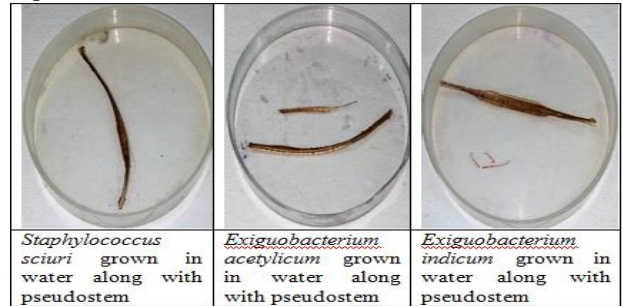


Figure II. Pseudo-stems were incubated with the endophytes in water for seven days and no separation of fibres was seen for *Staphylococcus sciuri*, *Exiguobacterium acetylicum*, or *Exiguobacterium indicum* treated pseudostems

Table- II: Depicts the maximum percentage loss in weight of Banana Pseudo stem on retting. *Staphylococcus sciuri* being the most efficient in retting.

S.No	Weight of pseudostem after 7 days of Bacterial retting in optimized media				Weight of pseudostem after 7 days Bacterial retting in distilled water		
	Culture Name	Initial weight in gms	Final weight in gms	Percentage (%) of weight loss	Initial weight in gms	Final weight in gms	Percentage (%) of weight loss
2	<i>Staphylococcus sciuri</i>	2	0.04	98	2	0.064	96.8
3	<i>Exiguobacterium acetylicum</i>	2	0.05	97.5	2	0.084	95.8
4	<i>Exiguobacterium indicum</i>	2	0.056	97.2	2	0.083	95.85

Further we also report that the bacterial strain pectinases also affect texture of fibre produced. Texture of *Staphylococcus sciuri* treated pseudo stems fibres was smoother and also dried faster. From our studies we propose that treatment of banana pseudo stem with *Staphylococcus sciuri* in presence of Yeast extract lactose , Tryptone media at alkaine pH (8-10) for 7 days followed by drying at 37 degrees for 72 hours and mechanical separation of fibre bundles i.e mechanical combing alone produces a good textured cellulose fibres (see Fig. IIIa,III b and Table III).

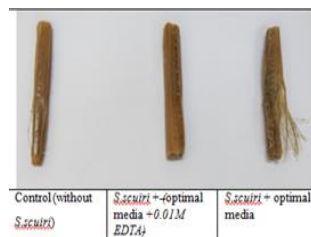


Figure 3a Status of fibres after 24hrs of drying



Figure 3 b Status of fibres after 72 hrs of drying



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Figure 3 a & b Effect of drying on texture of retted fibres : The fibres showed separation after heating and drying at 37 °C for 72 hrs. Retted fibres taken for the experimental studies are the ones produced when *Staphylococcus scuiiri* was used to treat banana pseudostem in its optimized media. The control here are the fibres where *S.scuiiri* is not used, Second set depicts the effect on fibres when treated with enzyme produced from *Staphylococcus scuiiri* in presence of 0.01 M EDTA and third set of experiment is when pseudostem is treated *Staphylococcus scuiiri* without presence of EDTA in the optimized media. As seen in the figure the fibres can be easily separated on drying if they are incubated with *Staphylococcus scuiiri* which is grown in presence of optimal media without EDTA. Requirement for drying temperatures and time are also very low which is 37 °C for 72 hrs see fig 3b.

Table3 Effect of treatment on weight loss of Pseudo-stem

S. No	Type of treatment for Pseudostem	Initial weight	Weight after 24 hrs.of drying	Weight after 72 hrs. of drying	Percentage (%) of weight loss
1	Control(without <i>Staphylococcus scuiiri</i>)	2.87	0.277	0.234	91
2	<i>Staphylococcus scuiiri</i> + optimal media +0.01M EDTA)	3.48	0.219	0.172	94
3	<i>Staphylococcus scuiiri</i> + optimal media	3.66	0.198	0.12	96.7

IV. DISCUSSION

Banana fibre because of its fineness, spinnability and light in weight, is preferred by the textile industry. It has low elongation value and strong moisture absorption property also permits dyeability thus enzymatically treated banana fibre has high potential to become a sustainable raw material for textile industry. Microbial pectinases account for 25% of the global enzymes sales. Microbial pectinases have found application in production of natural fibres during retting which is fast replacing other methods like mechanical extraction of fibres, chemical methods to remove the gummy material from fibres which reduce the fibre quality, density, and strength [11],[12]. Use of crude enzyme formulations is preferred over purified enzymes during retting as the cost of retting process is lowered. For further reduction in cost of production of enzymes researchers have used inexpensive substrates like konjac flour, wheat bran [13],[14]

V. CONCLUSION

We report a biological ecofriendly method of microbial retting method where pectinases produced by the endophytic microorganisms isolated from corm of *Amorphophallus paeoniifolius* caused 95% to 98% loss in weight of banana pseudostems in both media and water. The texture, color and separation of fibre bundles of cellulose was far better when *Staphylococcus scuiiri* was used for retting in its

optimized media conditions. Other pectinase producing endophytic bacteria isolated by us are *Exiguobacterium acetylicum*, *Exiguobacterium indicum*

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CONFLICT OF INTEREST

There are no conflicts of interest between the authors

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