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Influence Of Highly Efficient Pectinolytic Microbial Consortium From A Different Agro Climatic Region On Jute Retting Vis-À-Vis That Of A Nitrogen - Fixing Pectinolytic Bacteria From The Same Region.

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Abstract: *The difference in the quality of jute fiber belonging to different agro climatic regions had been mainly attributed to plant growth and more importantly to the quality and quantity of retting water. Our study have shown that difference in the retting capability of microflora belonging to different agro climatic regions contribute significantly to the quality of jute. However, attempts to introduce highly effective microbial population from a region producing better jute fiber to a distant one producing inferior quality and having different agro-climatic conditions was not successful because of diminished growth. This was probably due to antagonism of the indigenous microbial population towards the extraneous inoculum, since under sterile laboratory condition the former showed superior retting capability in the same conditions of temperature and pH. The diminished retting due to diminished growth could be partially overcome by introducing nitrogen fixing bacteria; whereby coarseness of fibers could be reduced to the maximum by 16.6%. However, introduction of nitrogen fixing bacteria by itself, particularly when isolated from a nearby region was most successful and was found to reduce coarseness of jute fibers by 30%. This particular nitrogen fixing bacteria was found also to have pectinolytic activity and was identified as Agrobacterium tumefasciens . This report raises the possibility that possibly many bacteria accelerate the retting process by more than one function.*

Keywords: *Agrobacterium tumefasciens, jute, microbial consortia, nitrogen fixing bacteria, agro climatic regions, retting*

I. Introduction

Jute fibers obtained from plants grown in different agro-climatic zones have marked qualitative difference. In general, good quality jute is obtained where soil is fertile, annual silting occurs regularly and plenty of retting water is available. In India, broadly speaking, parts of Assam and the northern region of West Bengal consisting of the hilly region (Darjeeling, Jalpaiguri, Coochbehar), the old alluvial zone (North and South Dinajpur and Malda) and the upper part of the new alluvial zone (Murshidabad and Karimpur belt of Nadia) produces better jute fibers than the southern part of the new alluvial zone consisting of Southern Nadia, Hoogly, North and South 24 Parganas [1].

A very important aspect for production of quality jute fiber is proper retting. It has been found that, retting depends upon availability of plenty of clean water, pH and temperature of retting water, application of nutrients of retting microbes [2,3]. Occasionally there are reports on the isolation of efficient retting microbes, which could bring about retting within a short period [4,5,6]. It has also been reported that, addition of highly efficient pectinolytic bacterial inoculum to the retting water reduced the retting time appreciably [7]. However, in practice, the traditional water-retting with indigenous microbial population is still done everywhere.

There is no report on the 1) quantitative and qualitative difference of the retting microbes from different agro climatic regions and its bearing on the quality of jute fiber and 2) stability of the microbial inoculum from a different agro climatic region within the indigenous microbial population in the retting trough. In the present report, we focus on this aspect with the additional finding that a bacteria can contribute in more than one way in the retting process.

II. Materials and Methods

Root cuttings of jute from different agro climatic regions of India, particularly West Bengal was obtained from the mill floor of Reliance Jute Mill, Kolkata. Citrus Pectin (Pure, Methoxy content-Min 7%) was obtained from SRL, India and Birchwood Xylan was obtained from Sigma Aldrich, USA.

2.1 Media for isolation of microbes and for preparing microbial culture:

Nutrient broth and agar, Omeliansky's -yeast extract agar supplemented with 2% pectin or xylan and Burk's nitrogen free agar were used. All the media were prepared following Rao [8]. Soil-extract-mineral broth medium supplemented with jute extract and jute root cuttings as carbon source: Soil-extract (Stock), 9% (v/v) ,jute extract 1% (v/v) and K_2HPO_4, 0.1% w/v in tap water. In case of preparing agar medium 2% agar was added to the broth medium. Soil- extract (Stock) was prepared following Rao [8]. Jute extract was prepared by boiling 10 g of small pieces of jute root-cuttings in 100 ml tap water for 30 min. Jute root cutting pieces, 6 cm in length were sterilized in an autoclave for 30 min separately and then inserted in the above sterile broth and slant media such that the root cutting protrudes 3 cm above the broth medium and remain pressed on the surface of the slant medium respectively.

2.2 Methods:

2.2.1 Determination of the total number of bacteria vis-a-vis the number of pectinolytic bacteria of the different regions:

The root ends of the jute reeds of the different agro climatic regions were cut into small pieces of 4 cm length and then given a very brief sterile water wash to get rid of the dirt and dust of the mill floor. Root cutting pieces were subsequently immersed in sterile phosphate buffer (0.1M, pH 7.0) supplemented with 0.01%(v/v) Triton and kept for an hour. They were then subjected to vortex mixer several times to leach out the adherent bacteria The suspensions were serially diluted and plated on nutrient agar and Omeliansky's-pectin agar and incubated at 30°C for 72 h. Five replicates were kept for each dilution.

2.2.2 Determination of the softening/retting ability of the microbial consortia obtained from different agro climatic regions:

Very hard root-cuttings from the same lot were thoroughly surface sterilized with rectified spirit and immersed in sterile tap water in culture tubes. The culture tubes were inoculated with both nutrient broth culture and soil + jute-extract mineral broth culture of microorganisms from each region separately. Three replicates were kept for each region. Three uninoculated sets were kept as control. The tubes were incubated at 30°C. After 6 days of incubation the root cuttings were taken out, washed, blotted and air dried. The extent of softening (reduction in rigidity) of root cuttings was assessed subjectively by hand by three individuals. Three such experiments were carried out and the average was recorded.

2.2.3 Determination of the effect of introduction of microbial consortia obtained from two selected regions to the retting system in Kolkata:

Freshly harvested unsterilized green jute stems 120 days old were cut into pieces, 6cm in length and introduced in tap water in broad tubes. Jute : tap water ratio was kept at 1:10. The tubes were plugged with cotton plugs. The inocula were taken from nutrient agar slant cultures. Bacterial suspensions were made in sterilized tap water. Concentrations of the bacterial suspensions were kept at 10^7 per ml. Each inoculum was subsequently introduced in the retting water separately and mixed well. The inoculum : retting water ratio was kept at 1 : 100. Six replicates were kept for each type. At intervals, the extent of retting was assessed by hand and extent of bacterial growth was assessed by turbidimetric method with a spectrophotometer.

2.2.4 Isolation of nitrogen fixing bacteria:

Soil samples from the rhizosphere region of jute plants grown in a fertile plot of the districts of North and South 24 Parganas were taken for isolation of nitrogen fixing bacteria. In each case, ten gram of soil was mixed with 100 ml of sterile tap water in a ehrlenmeyer flask. The suspension was mixed well by a vortex mixer and allowed to stand for half an hour for sedimentation of the soil particles. The relatively clear upper portion of the suspension was decanted out in a sterile tube and served as the source of bacteria. The suspension was serially diluted and plated on Burk's nitrogen free agar plates. After 72 h of incubation at 30°C, the biggest colony on the agar plate was picked up and transferred to Burk's Nitrogen free broth twice and subsequently in agar slants. They were purified by streaking and subsequently preserved in the same medium slants. Isolation was done from North 24 parganas on the first year and South 24 Parganas in the second year.

2.2.5 Effect of the addition of nitrogen fixing bacteria individually and along with microbial consortia from Raigunge (MC7) and Samsi (MC6) to the retting water in Kolkata:

The lower middle to bottom portion of jute plants grown in the plot adjoining the college were cut and were allowed to ret in tap water contained in plastic retting tubs. Fifteen such plant stems were retted for each experiment. Jute: water ratio was kept at 1:20. The microbial inocula of the microbial consortia, MC6 and MC7 were obtained from 72 h old cultures in nutrient agar. Nitrogen fixing culture 1, 2 and 3 will be referred to as NFC1, NFC2 and NFC3 respectively. Their inocula were obtained from 72 h cultures in Burk's nitrogen free

agar. The concentration of the bacterial suspensions made from these slants in sterile tap water was adjusted 10^7 per ml and these served as inocula. Each inoculum was subsequently introduced to the retting water singly or in combination of two. Each inoculum : retting water was kept at 1 :100. In the control set up, no inoculum was added. After 18 days of retting, the retted fibers were water washed and air dried. The fineness of the fibers was measured by the gravimetric method at the National Institute of Jute and Allied Fibers. The experiment was repeated in two consecutive years. In the last year, whole plants were retted. The fineness and strength of the retted fibers were measured by the Air Flow method and the Bundle Strength method respectively at the Institute of jute Technology, University of Calcutta.

2.2.6 Determination of the number and nutritional types of bacteria in control and experimental retting waters:

The retting water samples were pipetted out at regular intervals, diluted and plated on nutrient agar, Omeliansky's-pectin agar, Omeliansky's-xylan agar and Burk's N_2 free agar plates. After 72 h incubation at 30°C, colonies were counted and average number of colony forming units (CFU)/ml of original samples were determined.

2.2.7 Identification of the nitrogen fixing bacteria (NFC3): Colony morphology and gram staining character were studied. Identification was done by 16s rRNA sequencing.

III. Results

Table I shows the location and climatic conditions of the regions studied.

Fig. 1 shows the quantification of the retting bacteria of the different regions. The result shows that MC7 comprises the highest number of bacteria growing on nutrient agar followed by MC6. Highest number of bacteria growing on Omeliansky's- Pectin agar was found in the case of Gopalnagar (MC4) followed by MC7 and MC6. Highest number of pin point colonies showing distinct zone of clearance around them was found in MC7 followed by MC6. MC7 and MC 6 comprise the highest total number of bacteria on nutrient agar and highest number of bacteria having significant pectinolytic activity showing distinct zone of clearance. Fig. 2 shows the comparative softening (reduction of rigidity) of the microbial consortia- treated root cuttings against the non-treated sample (control). The results of the extent of softening (inverse of rigidity) arranged in a decreasing order shows the following trend: Raigunge (MC7) – Samsi (MC6) -Gopalnagar (MC4) -Nadia (MC3) -Tarakeswar (MC2) – Control.

3.1 Effect of the addition of microbial consortia MC7 and MC6 in the retting water in a laboratory scale experiment:

It was found that, on the 6^{th} day of retting, partial retting was better in the inocula- amended retting water than in the control. But the bacterial growth was either at par (MC7) or slightly lower (MC6) in the experimental retting water than in the control. On the 11^{th} day, however, growth of the control retting microflora increased at a higher rate; while the experimental microfloras grew at a much slower rate. With the result final retting was better in the control (Fig. 3a and 3b). On comparative analysis of growth vis-a-vis retting, it was found that, jute stems in MC6- and MC7- amended retting water showed higher retting, if similar growth was considered. Control jute stems showed better retting solely due to higher growth, particularly in the later period of retting.

3.2 Retting Experiment in the 1^{st} year and the effect of nitrogen fixing bacteria:

It was found that retting performance of microbial consortia Samsi (MC6) and Raigunge (MC7) improved remarkably when used in conjunction with some selected nitrogen fixing bacteria isolated from the soil of North 24 Parganas. Best fiber fineness was achieved when the retting water was amended with microbial consortium MC7 along with nitrogen fixing bacterial culture NFC 2 followed by that with microbial consortium MC6 in combination with another selected nitrogen fixing bacterium NFC 1. Table II depicts the fiber fineness (gravimetric method) of the control samples and the experimental samples.

3.3 Retting experiment in the 2^{nd} year:

Since MC7 along with nitrogen fixing microbial culture NFC 2 as inoculum gave the best result in the first year, it was repeated in the second year. It was found that the microbial consortium (MC7+ NFC2) showed diminished activity in the second year, as reflected in the fiber-fineness data vis-a-vis control (Table II). A nitrogen fixing bacterium NFC- 3 isolated from South 24 Parganas in the second year, however, produced the best result (Table II).

3.4 Retting experiment in the third year with whole stems ribbons:

In the 3^{rd} year, the growth of microbial population in the retting tub was studied as a function of time by the viable count method. Growth of all the nutritional types of bacteria studied was found to be better in the experimental retting waters than in the control. However, best growth was found in retting water amended with

the nitrogen fixing bacteria NFC3 only (Table III). As in the previous years, the fibers of the experimental samples were found to be finer than the control sample but the difference narrowed down further in the third year. The nitrogen fixing bacteria NFC 3- amended retting water produced the finest fibers followed by that of MC 7 + NFC 3 (Table IV). This is commensurate with the growth data (Table III). The control fibers were found to be coarser. The strength of the treated fibers were commensurate with that of the normal ribbon retted fibers

IV. Discussion

Results indicated that difference in microbial community in different agro climatic regions was a great contributor to the quality of jute obtained from different regions (Fig. 1, Fig.2). Diverse climatic and soil conditions in these regions encouraged the growth of different microbial community which contributed to the difference in the retting capability. Assam and Northern regions of West Bengal have higher altitude, heavier rainfall and lower temperature than its Southern counterpart. Raigunge in Uttar Dinajpur and Samsi in Malda situated in between the two regions have somewhat intermediate altitude and climatic features (Table I). Raigunge is situated in the bank of river Kulik and its soil is very fertile. The soil of both Raigunge and Samsi have high potassium and considerable content of phosphorus [9, 10], whereas the soil of Nadia and Tarakeswar have low NPK status[11]. It may be inferred that, the climatic and soil characteristics of Raigunge(MC7) and to some extent Samsi(MC 6) supports the growth of microbial community with better retting capability followed by that of Gopalnagar(MC 4). Fig. 1 shows that though MC4 had maximum number of pectinolytic bacteria, zone of clearance of pin point colonies is better in MC7and MC6. The total number of bacteria on Nutrient Agar plates was highest in MC7 followed by MC6. The total number of bacteria also may have some bearing on retting behavior. It is likely that some of these bacteria have nutritional or supplementary enzymatic functions. Effective degumming of jute fibers by a combination of enzymes have been reported earlier [12, 13, 14].

The markedly higher retting capability of MC7 and MC6 microbial consortia seen in the laboratory encouraged us to use them separately as a supplement in the retting water in Kolkata (south Bengal). It was found that there was antagonism and or competition between the microbial populations of the two different agro climatic regions. With the result the growth of the resultant population slowed down after some time. On comparative analysis of growth vis-a-vis retting, it was found that, jute stems in MC6- and MC7- amended retting water showed higher retting, if similar growth was considered. Control jute stems showed better retting solely due to higher growth, particularly in the later period (Fig. 3a and 3b). To ascertain whether antagonism or limitation of nitrogenous nutrient was the cause of growth retardation of the MC7- amended retting water, a nitrogen fixing bacteria (NFC2) isolated from Naihati (North 24 Parganas) was added to the MC7-amended retting water. A better fiber fineness of jute fibers of the experimental sample as compared to the control clearly showed the beneficial effect of nitrogen fixing bacteria in overcoming the growth block (Table II). However it was not clear to what extent the nitrogen fixing bacteria enhanced the growth of the added microbial community and/or the indigenous microbial population residing on the jute stem surface and retting water.

In the next year, a better nitrogen fixing bacteria (NFC3) after a laboratory trial was applied in a field trial singly and in combination with the most successful microbial consortium from Raigunge (MC7) to assess its effect on (MC7)- amended retting water and on the un- amended retting water (Control). It was found that, growth was better in the two experimental samples than in the control. Growth was highest in NFC3 amended retting water followed by (MC7 +NFC3) amended retting water, although the latter had higher initial population due to administration of two inocula in equal proportions (Table III). Highest growth was encountered on Nutrient Agar plates which supported the growth of the most diverse bacterial species with various capabilities as mentioned earlier. On Nutrient Agar and Omeliansky-xylan agar plates, stationary phase was attained on the 10^{th} day in case of control retting water sample and on the 5^{th} day in case of both (MC7)- and in (MC7+ NFC3)- amended retting water samples. The number of bacteria on both the plates if arranged in the decreasing order, showed the following trend NFC3→ (MC7+NFC3) →Control. On Omeliansky-pectin agar the same trend was noticed, except that stationary phase was attained late in both control (more than 10 days) and experimental plates (10 days) (Table III).

Since growth was better in (NFC3)- amended retting water than on (MC7 +NFC3)- amended retting water, it seemed there was still antagonism between MC7 and control microbial population. NFC3 increased the growth of the control indigenous population better in absence of MC7. The fiber fineness data also corroborated this (Table IV). Since NFC3 was isolated from the rhizosphere of the jute plants grown in a plot near Kolkata it enhanced the growth of the indigenous retting microbial population very well. Although the control samples showed higher fiber tenacity, the fiber tenacity of the experimental samples was found to be good considering the average fiber tenacity of ribbon retted fibers [11]. Further the coefficient of variance (%) was also found to be low in the experimental samples. Of all the cultures studied, the nitrogen fixing bacteria NFC3 gave the best result (Table IV). Since the nitrogen fixing bacteria NFC3 supported growth and retting most, it was identified by molecular techniques and was found to be *Agrobacterium tumefasciens*.

Agrobacterium species with some pectinolytic activity was reported in retting water in a single instance [7]. Though *Agrobacterium tumefasciens* is known to have pectinolytic activity [15, 16], it is also is known to have nitrogen fixing ability [17, 18].The beneficial effect of nitrogen fixing bacteria *Azotobacter* on retting was reported long back [19]. In this case nitrogen fixation by *Agrobacterium tumefasciens* may be the major cause of growth acceleration of all categories of bacteria of the indigenous population. To test whether this particular strain has also pectinolytic function, the bacteria was grown on Omeliansky's- Pectin agar plates and clear zone of inhibition round the colonies was found. Activation of the retting process by either nitrogen fixing bacteria or by pectinolytic bacteria have been reported, but activation of retting by a single bacteria by both the functions have never been highlighted. This report raises an important possibility that possibly many bacteria accelerate the retting process by more than one function.

V. Conclusion

The study has some important bearing on the selection strategy of retting inoculum and concludes that the inoculum should be from the same agro climatic region and preferably have some nutritional advantage(nitrogen fixing ability) apart from its pectinolytic and other carbohydrase functions.

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Table. I The location and climate of the regions studied.

	Agroclimatic zone	Altitude	Latitude	Longitude	Annual Average Rainfall	Temp-erature
Gopalnagar (Nagaon, Assam)	Agroclimatic zone 1 (Central Brahmaputra Valley Zone)	50.2m	25°99'29.40"N	92°45'81.50"E	2036mm	6°C-38°C
	Agroclimatic zone 2 (Lower Gangetic Plain region of West Bengal)					
Raigunge (North Dinajpur, West Bengal)	New & Old Alluvial Zone	53m	25°36'50.50"N	87°07'38.77"E	1570mm	6°C-38°C

Samsi (Malda, West Bengal)	New & Old Alluvial Zone	25m	25°00'39.03"N	88°08'27.95"E	1545mm	
Nadia (West Bengal)	New & Old Alluvial Zone	15m	23°28'15.48"N	88°33'23.51"E	1385mm	7°C-41°C
Tarakeswar (Hugli, West Bengal)	Old Alluvial Zone	14m	22°54'12.95"N	88°28'38.45"E	1288mm	>10°C-40°C

Ref: [20, 21]

Table II: Fiber fineness of jute fiber samples retted in water amended with microbial consortia and nitrogen fixing bacteria.

	Sample	Fifer Fineness (tex)	Reduction in coarseness (%)
1st Year	Control	3.1	
	* MC6 +**NFC1	2.5	19.35
	MC7 + NFC2	2.3	25.80
2nd Year	Control	3.3	
	MC7 + NFC2	2.7	18.18
	Control	3.8	
	NFC3	2.7	28.94

*MC – Microbial Consortium, **NFC – Nitrogen Fixing Culture

Reduction in coarseness (%) = Fiber fineness of control sample – Fiber fineness of experimental sample / Fiber fineness of control sample x 100

Table III: Growth (viable count) of microbial population as a function of time in control and experimental retting water sample amended with Microbial Consortium 7 and Nitrogen Fixing Bacteria 3.

Medium of Cultivation	Retting water Sample	Colony Forming Units/ml (log_{10} value) Number of Days of retting			
		0 Days	5 Days	10 Days	15 Days
Nutrient Agar	Control	5.0000	7.4771	7.4954	6.3404
	MC7 + NFC3	5.8260	8.3636	8.0000	7.5797
	NFC3	5.5051	8.3979	8.2041	7.7242
*OMP Agar	Control	3.3617	5.4832	5.6989	5.9030
	MC7 + NFC3	5.3010	5.9138	6.0000	5.9871
	NFC3	4.4771	5.9684	6.2304	5.9242
**OMX Agar	Control	2.9034	5.8808	5.9395	5.3424
	MC7 + NFC3	5.4771	5.9956	6.0581	5.9030
	NFC3	4.3010	6.1760	5.9590	5.4313

*OMP – Omeliansky Pectin, **OMX – Omeliansky Xylan

Table IV: Fiber quality of jute fiber samples retted in water amended with Microbial Consortia and Nitrogen fixing bacteria in the 3rd year.

	Fiber Fineness (tex)	Reduction in coarseness (%)	Fiber Tenacity (g/tex)	(CV %)
Control	3.0		24.8	6.4
MC 6 + NFC1	2.7	10.00	20.1	4.0
MC 7 + NFC3	2.5	16.66	22.3	3.4
NFC 3	2.1	30.00	22.2	3.2

Reduction in coarseness(%) = Fiber fineness of control sample – Fiber fineness of experimental sample / Fiber fineness of control sample x 100

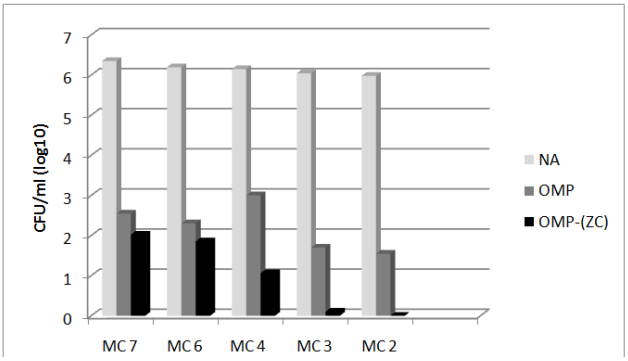

Fig. 1: Number of bacteria present in Microbial Consortia (MC) isolated from different regions on two different media along with their pectinolytic activity on OMP plates.

NA- Nutrient Agar, OMP- Omeliansky-pectin agar, OMP-(ZC) –Number of bacteria with zone of clearance on OMP agar plates.

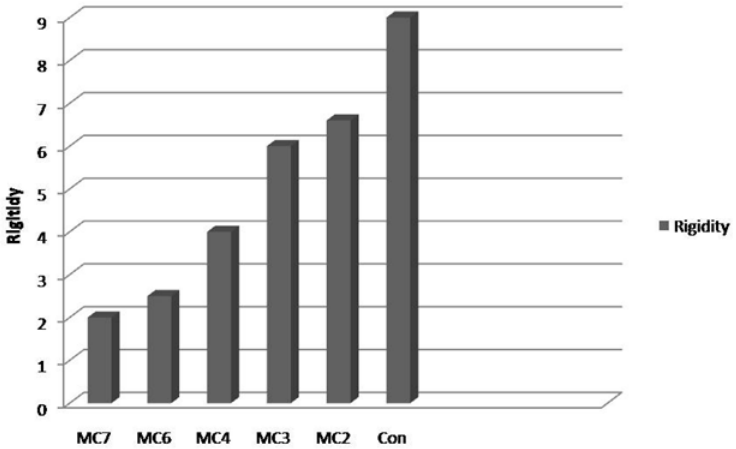

Fig. 2: The softening (reduction of rigidity) of surface-sterilized root cuttings by Microbial Consortia (MC) isolated from different regions

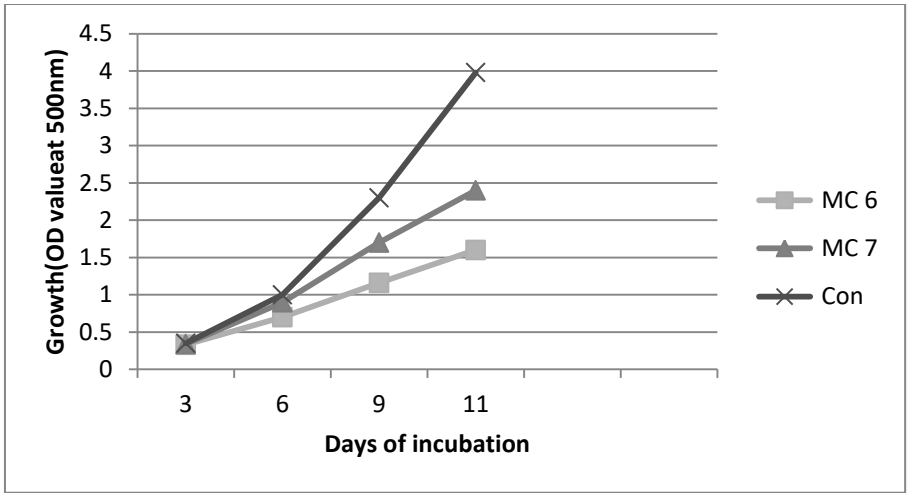

Fig. 3a: Growth of microbial population in retting water amended with Microbial Consortium 6 (MC6) or Microbial Consortium 7 (MC7) as compared with un-amended retting water (Control).

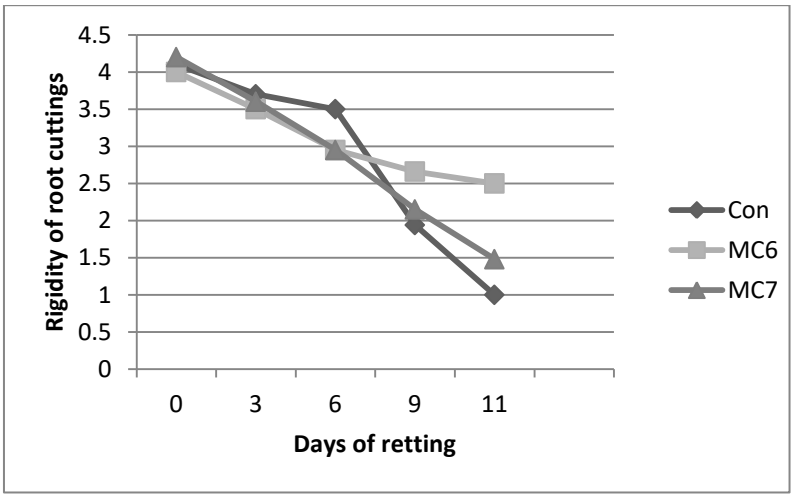

Fig. 3b: Retting (reduction of rigidity) of unsterilized green jute stems in tap water amended with MC6 or MC7