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Assessment of Changes in Community Level Physiological Profile and Molecular Diversity of Bacterial Communities in Different Stages of Jute Retting

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Abstract: Retting of jute is essentially microbiological and biochemical in nature. Community Level Physiological Profiles (CLPP) as well as genomic diversity of bacterial communities were assessed in water samples collected during pre-retting, after 1st and 2nd charges of retting. The water samples were collected from two widely cultivated jute growing locations, Sonatikari (22°41'27"N; 88°35'44"E) and Baduria (22°44'24"N; 88°47'24"E), West Bengal, India. The CLPP, expressed as net area under substrate utilization curve, was studied by carbon source utilization patterns in BIOLOG Ecoplates. Molecular diversity was studied by polymerase chain reaction followed by denaturing gradient gel electrophoresis (PCR-DGGE) of total DNA from water samples. Both between locations and stages of retting, substrate utilizations pattern were carbohydrates> carboxylic acids>polymers>amino acids>amines/amides>phenolic compounds. Differential substrate utilization pattern as well as variation in banding pattern in DGGE profiles was observed between the two locations and at different stages of retting. The variations in CLPP in different stages of retting were due to the change in bacterial communities.

Key words: Jute retting, BIOLOG, community level physiological profiles, denaturing gradient gel electrophoresis, bacterial diversity

INTRODUCTION

The quality of jute fibres is largely determined by the retting process (Candilo *et al*., 2010) and water-retting is in vogue in India and elsewhere. Jute bundles are steeped in water and subjected to naturally controlled decomposition of biopolymers like pectins, celluloses and hemicelluloses that hold the bast cells to the rest of the stem (Haque *et al*., 2001a). The decomposition is essentially a microbiological and biochemical process (Haque *et al*., 2001b) and is controlled by bacterial communities present in retting water. Knowledge concerning the bacterial diversity together with their metabolic profiles during retting process is thus important (Tamburini *et al*., 2003).

Bacterial communities associated with retting processes of different fibre crops have been reported earlier. Tamburini *et al*. (2003) characterized both aerobic and anaerobic pectinolytic bacterial communities present in the retting water of hemp or flax by amplified ribosomal DNA restriction analysis. Munshi and Chattoo (2008), studied bacterial communities associated with water retting of jute. The bacterial community structure of flax retting in China has been recorded by using denaturing gradient gel electrophoresis (DGGE) and culture-dependent method (Hongzhi *et al*., 2009).

Jute is an important cash crop in many Asian countries including India. Much attention is now paid to improve jute fibre quality to cope with the challenge from synthetic fibres. In India, water retting is usually undertaken for obtaining jute fibres. Retting water varies from place to place with respect to its physico-chemical, microbial and biochemical properties, which affect jute fibre quality (Das *et al*., 2010). However, scarcity of water

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compels the farmers to use same water bodies repeatedly for retting, leading to poor quality jute fibre in the long run (Das *et al*., 2010).

Information concerning changes in CLPP of bacterial community and their diversity during jute retting, more particularly when it is done in the same water bodies repeatedly is lacking. Present communication deals with the CLPP of bacteria and their diversity in repeatedly used jute retting water.

MATERIALS AND METHODS

Sample collection: Water samples were collected from four and three ponds of the jute growing villages of Sonatikari (22°41'27"N; 88°35'44"E) and Baduria (22°44'24"N; 88°47'24"E), respectively, located in the district of (N) 24-Parganas, West Bengal, India. It is a general practice in the selected areas that after harvest, jute is charged in retting ponds in a phased manner. The jute crop is harvested from some parts of the cultivated area and charged in the designated ponds of the respective farmers. After the retting is completed from the first charge, another charge is given in the same pond with the jute crop harvested from the rest of the cultivated land. The water samples were collected during different stages of retting, such as pre-retting, after 1st (July, 2011) and after 2nd charges of retting (August, 2011). For each retting pond, five replicated samples were collected in sterile glass vials (Borosil®) from areas in close proximity to the submerged mat of jute bundles, at a depth of 1-3 ft, to get maximum coverage of the retting consortia. The subsamples were then pooled to create a single composite sample (Munshi and Chattoo, 2008). The samples were then transported to the laboratory in portable cooling boxes and on reaching the laboratory were preserved at 4°C.

Average pH, Ec and COD of the water samples from the studied locations in the pre-retting stage were 6.9, 0.26 and 52 mg L^{-1}, respectively. The same values for the 1st and 2nd charges of retting were 6.2, 1.6 dS m^{-1} and 199 mg L^{-1} and 6.3, 1.2 dS m^{-1} and 180 mg L^{-1}, respectively.

CLPP analysis: The patterns of potential carbon source utilization by the jute retting bacterial communities were assessed by BIOLOG Ecoplate system containing triplicates of 31 different environmentally relevant carbon sources and control well (Biolog Inc., Hayward, CA, USA) (Choi and Dobbs, 1999). Aliquots of 150 μL corresponding to 10^3 cfu (determined by dilution plate on Luria agar) for each sample were inoculated to each well of BIOLOG Ecoplates and were incubated in the dark at 28°C. The absorbance values were read at 590 nm with a BIOLOG microplate reader at 12 h intervals until 72 h.

Ecoplate data analysis: A quantitative analysis was performed by plotting the ratio of absorbance of one well to the total sum absorbance of all the wells for that sample against time for the 31 response wells. Absorbance values used for such calculation were obtained after subtraction of each value with the absorbance of the control well containing water. All the positive absorbance values for the response wells were taken into account. The negative absorbance values were considered as zero. CLPP was expressed as the net area under curve (Guckert *et al*., 1996; Chakraborty *et al.,* 2011) for each well of the 31 response wells over a period of 72 h of incubation.

DNA isolation from water samples: Genomic DNA from water samples was isolated by modified extraction method of Tsai and Olson (1991). To 5 mL of water samples in sterile Oakridge tubes 13.5 mL of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM Na_2-EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl and 1% CTAB) and 100 μL of lysozyme (10 mg mL^{-1}) was added and were incubated at 37°C in water bath for 45 min with gentle shaking. Then 1.5 mL of 20% SDS was added and the samples were incubated in a 65°C water bath for 2 h with gentle end-over-end inversions every 15-20 min. After incubation equal volume of chloroform-isoamyl alcohol (24:1) was added and centrifuged at 10,000 g for 15 min. The aqueous layer was taken in another centrifuge tube and to it equal volume of phenol-chloform-isoamyl alcohol (25:24:1) was added. The sample was centrifuged at 10,000 g for 15 min and the aqueous phase was collected. To it 0.6 volume of isopropanol was added and the DNA was precipitated at room temperature for 1 h. The sample was then centrifuged at 16,000 g for 20 min, the supernatant discarded and the pellet was washed with 70% alcohol. After centrifugation at 16,000 g for 15 min the pellet was air dried and resuspended in 50 μL sterile deionized water.

PCR amplification of 16S rDNA for DGGE analysis: The PCR of the isolated DNA samples was conducted in a final volume of 50 μL. The primers used for DGGE-PCR were 357F (5'-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATT-C(A/C)TTTGAGTTT-3'). Prior to the 357F primer a GC clamp (5'-CGCCCGCCGCGC-GCGGCGGGCGGGGCGGGGCACGGGGGG-3') was attached.

The reaction mixture included 20-50 ng of isolated genomic DNA, 2U Taq polymerase (Promega, USA), 1×PCR buffer with 1.5 mM $MgCl_2$, 200 μM each dNTP and 10 pmol of each primer (IDT, USA). Before amplification cycle, DNA was denatured at 94°C for 5 min. The touchdown PCR was performed as follows: The denaturing temperature of each cycle was carried out at 94°C for 1 min, annealing temperature was initially set at 65°C for 1 min and was then decreased by 1°C every cycle until it reached 55°C. Twenty-four additional cycles were carried out at 55°C. Primer annealing was performed for 1 min and primer extension was carried out at 72°C for 2 min. The final extension step was at 72°C for 30 min. The samples were held at 4°C until analysis.

Analysis of PCR products by DGGE: DGGE was performed with 6% (w/v) polyacrylamide gels in 1×Tris-Acetate-EDTA buffer. The gels contained a linear gradient ranging from 40-70% denaturant (where 100% denaturant contains 7 M urea and 40% formamide). PCR products (10 μL) obtained from total DNA of samples was used for separation in denaturing gradient gels. The electrophoresis was run for 16 h at 60°C, at 100 V. After completion of electrophoresis the gels were stained for 20 min in ethidium bromide solution. The stained gel was immediately photographed on a UV transilluminator.

RESULTS

Community level physiological profile of bacterial community by BIOLOG technique: The color development in the wells was remarkably slow in the first 24 h, which gradually increased with time, recording maximum absorbance values at 72 h. Ecoplate responses by the bacterial communities of different retting stages are expressed as net areas for single carbon source tested.

The bacterial communities in the retting ponds were able to utilize all the 31 carbon substrates. The order of utilization for different groups of carbon sources, both between locations and stages of retting was: carbohydrates>carboxylic acids>polymers>amino acids >amines/amides>phenolic compounds (Fig. 1, 2). The bacterial communities of Sonatikari tended to have greater utilization of carbohydrates but lesser utilization of carboxylic acids than Baduria (Fig. 1). Different stages of retting exhibited differences in the utilization of carbon sources. The utilization pattern of different carbon sources by the bacterial communities irrespective of locations (Fig. 2) revealed that carboxylic acids, polymers and carbohydrates were better utilized after 1st charge than after 2nd charge of retting, whereas amino acids and phenolic compounds were least utilized after 1st charge of retting.

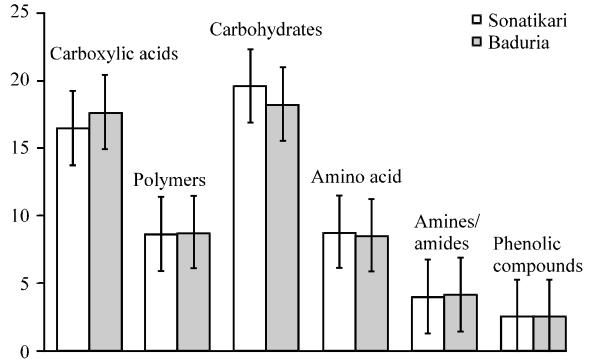

Fig. 1: Irrespective of retting stages, net area under substrate utilization curve of Sonatikari. The error bars indicate the standard error of mean

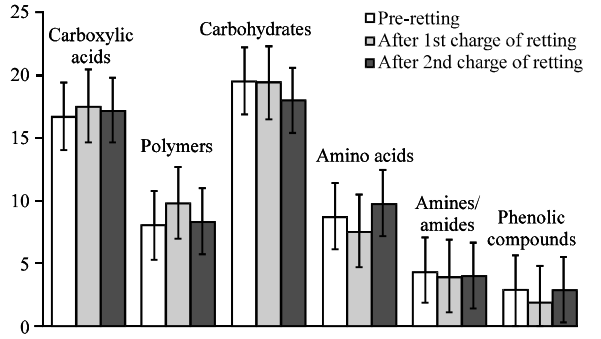

Fig. 2: Irrespective of locations, net area under substrate utilization curve of Baduria. The error bars indicate the standard error of mean

Among the main groups of carbon sources, there were differences in the utilization of individual carbon substrates by the bacterial communities in both the locations irrespective of the stages of retting (Fig. 3). The highest utilized carboxylic acid was D-galacturonic acid in Baduria and D-glucosaminic acid in Sonatikari. Among the carbohydrates, α-D-lactose was best utilized, with the retting microbiota collected from Sonatikari showing a marked preference for this sugar. The highest metabolized carbon substrates among the polymer, amino acid and amine/amide were α-cyclodextrin, L-arginine and putrescein respectively and those were better or equally utilized by the bacterial communities of Baduria than Sonatikari. The phenolic compounds were more or less equally utilized in both the locations.

The variation in individual carbon substrate utilization pattern by the bacterial communities at different stages of retting is evident irrespective of location (Fig. 4). Among the carboxylic acid groups D-galacturonic acid and D-glucosaminic acid substrates were most utilized but the former was best utilized during 2nd charge of retting.

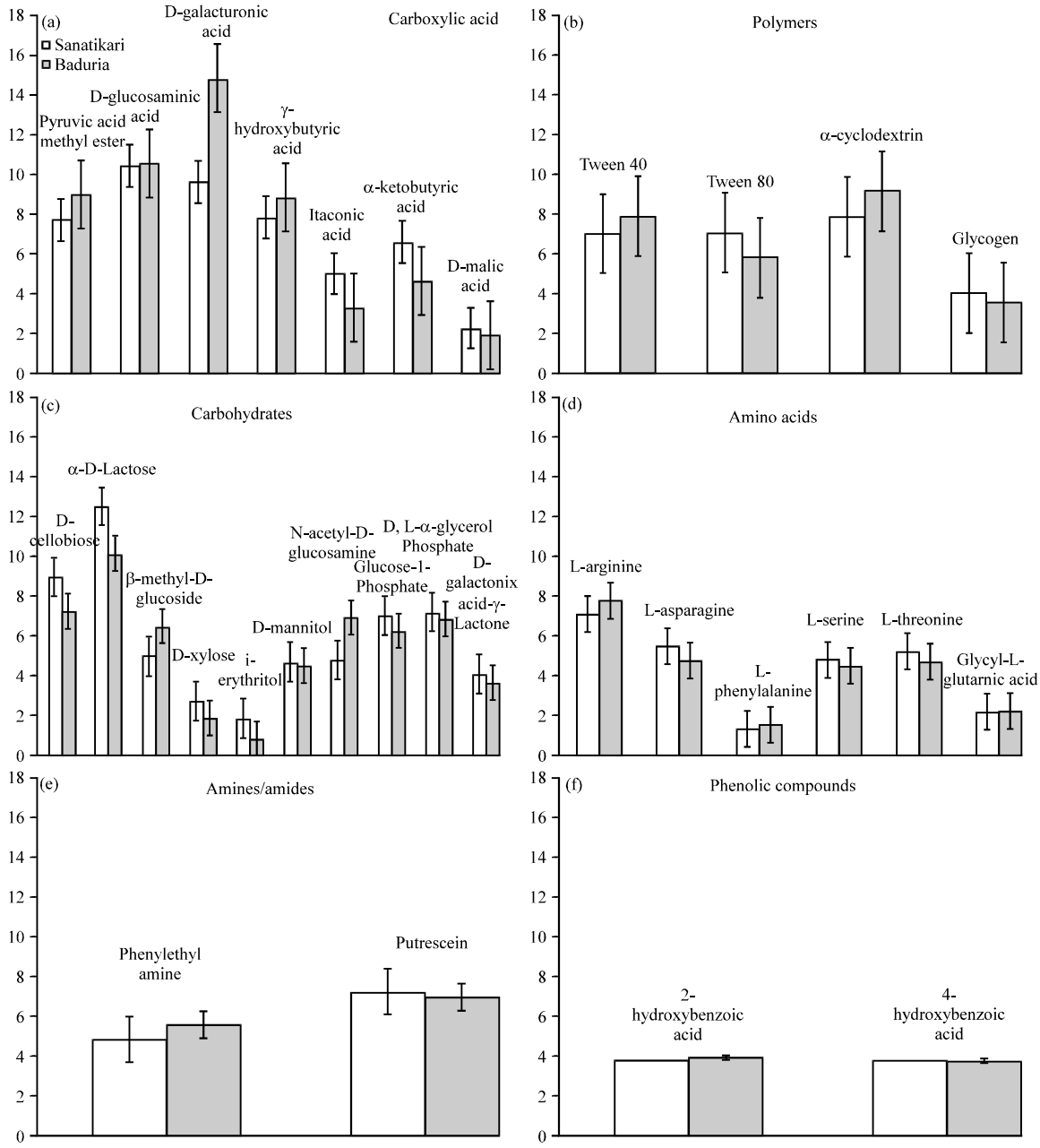

Fig. 3(a-f): Mean utilization of different substrates in BIOLOG plates by the microbial communities of different stages in two locations. Utilization of each substrate is expressed as net area under the substrate utilization curve. The error bars indicate the standard error of mean (a) Carboxylic acid, (b) Polymers, (c) Carbohydrates, (d) Amino acids, (e) Amines/amides and (f) Phenolic compounds

Among the carbohydrates α-D-lactose was most utilized followed by D-cellobiose after 1st charge of retting. L-arginine, among the amino acids, provided the best growth of bacterial community at all stages of retting. The polymers were best utilized after 1st charge of retting with the highest being α-cyclodextrin. At all stages of retting the polymer glycogen was least utilized by the bacterial communities. Putrescein amongst the amine/amide group provided best support for growth. The utilization of phenolic compounds, 2-hydroxybenzoic acid and 4-hydroxybenzoic acid were reduced after 1st charge but increased after 2nd charge of retting.

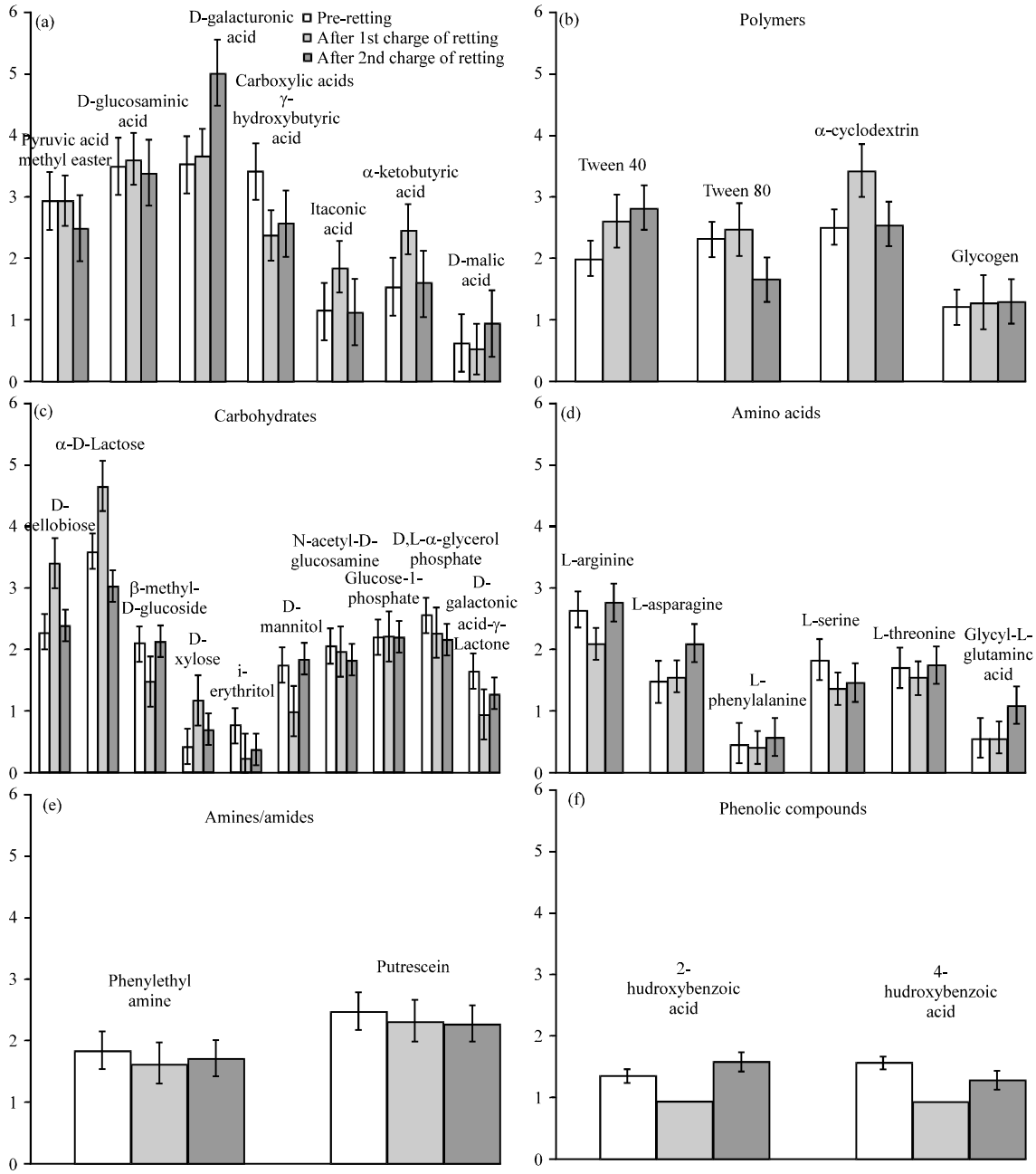

Fig. 4(a-f): Mean utilization of different substrates in BIOLOG plates by the microbial communities of Sonatikari and Baduria at pre, after 1st and 2nd charges of jute retting, Utilization of each substrate is expressed as net area under the substrate utilization curve, The error bars indicate the standard error of mean (a) Carboxylic acid, (b) Polymers, (c) Carbohydrates, (d) Amino acids, (e) Amines/amides and (f) Phenolic compounds

Analysis of the bacterial community by DGGE: The touchdown PCR technique, suggested by Muyzer *et al.* (1993), was used to amplify the V3-V5 region of the 16S rDNA of the isolated DNA samples from retting waters during different charges. This technique was performed to minimize the nonspecific annealing of primers to the non-target DNA and the PCR products were 550 bp in size. Figure 5a and b show the DGGE analysis profile of different ponds located in Sonatikari and Baduria, respectively at different stages of retting. DGGE profile of the ponds within the same locations at different stages of retting was clearly different. The number of bands obtained from the ponds of Sonatikari in the pre-retting, after 1st and 2nd charges of retting were

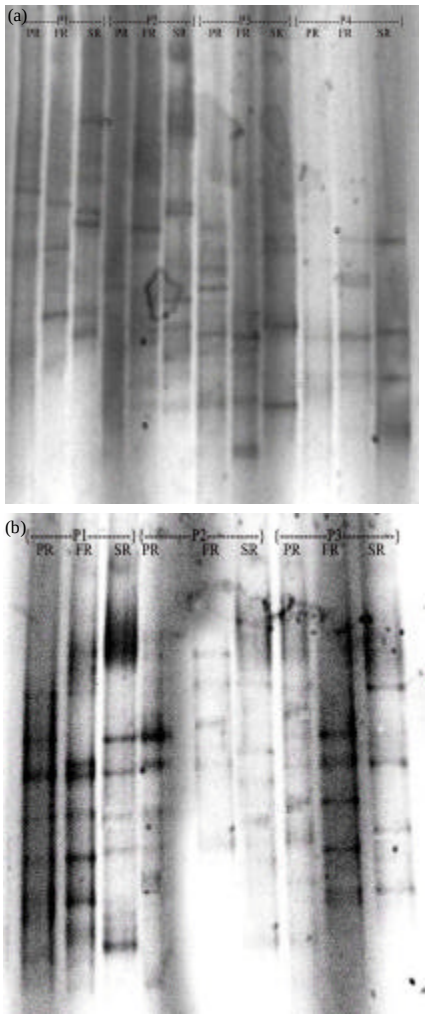

Fig. 5(a,b): DGGE analysis of 16S rDNA fragments obtained after PCR amplification of genomic DNA from bacterial communities of Sonatikari (a) and Baduria (b) ponds (P1, P2, P3, P4) at different stages of retting (PR, pre retting; FR, after 1st charge, SR, after 2nd charge)

2-8 (Mean 4±1), 3-8 (Mean 5±1) and 4-6 (Mean 5), respectively. The same values for Baduria were 4-8 (Mean 6±1), 6-7 (Mean 6±1) and 5-7 (Mean 6) in pre-retting, after 1st and 2nd charges of retting, respectively. Though the number of bands in different stages of retting was more or less similar, but their position varied.

DISCUSSION

Several workers have studied the microbiological aspects of jute retting by traditional microbiological methods (Ali, 1958; Jalaluddin, 1970; Haque *et al*., 2002; Ahmed and Nizam, 2008; Das *et al*., 2012). However, these studies provided little information about the total bacterial communities (Munshi and Chattoo, 2008) and their metabolic profiles. The metabolic profile of the bacterial communities of different environmental samples (Garland and Mills, 1991; Victorio *et al*., 1996; Matsui *et al*., 2001; Tam *et al*., 2003; Merwe *et al*., 2003) have been studied by Ecoplate based BIOLOG technique. PCR-DGGE is a culture independent technique and is useful in assessing bacterial community structure and their phylogenetic diversity Muyzer *et al*. (1993). Such techniques have not yet been used in the microbiology of jute retting water.

Varieties of organic acids, acetone, ethyl alcohol, butyl alcohol and various gases occur in retting water (Debsharma, 1976). Ahmed and Akhter (2001) reported that retting microorganisms utilize a plethora of organic substrates like sugars, pectin, hemicellulose, proteins and others. The bacterial communities of the retting ponds at both the locations as well as in different stages of retting utilized all six groups of carbon sources contained in the BIOLOG Ecoplate. This confirmed metabolic versatility of the bacterial communities. Location wise variation in substrate utilization pattern seems to be related to variation in two groups of water samples. Each pond at a particular location has its own physico-chemical, biochemical and microbiological environment (Das *et al*., 2010) and thus they have their own bacterial communities adapted to that environment.

Irrespective of locations, utilization of D-galacturonic acid was the greatest after 2nd charge of retting. D-galacturonic acid is the end product of pectin degradation by the enzyme polygalacturonase. Increased D-galacturonic acid utilization after 2nd charge of retting might be related to the increase in population of pectinolytic bacteria as earlier observed (Haque *et al*., 2002; Das *et al*., 2011).

Different banding pattern at different stages of retting reflected difference in presence/absence of bacterial groups or variation in species within a group. This phenomenon occurred due to alteration of physico-chemical properties as well as the nutrient status of retting water. Dominant bands in the DGGE pattern of the different retting water samples correspond to bacterial communities that predominate in particular stages of retting. Similar number of bands in different stages of retting but variation in their position indicated change in the type of microorganisms constituting the community. Differential utilization pattern of carbon sources in the BIOLOG Ecoplates resulted in change in bacterial community structure in different stages of retting.

CONCLUSION

BIOLOG system using Ecoplates rapidly screened the metabolic profile of the bacterial communities in ponds during different stages of retting at different locations. PCR-DGGE analysis detected change in bacterial communities during retting process. This study depicted that bacterial communities in retting water changed with the progress of the retting process, with subsequent change in their metabolic profile.

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