Original Article

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Biochemical characterization and biolog based identification of efficient jute retting bacterial isolates from retting water

Syandan Sinha Ray[1], Suparna Das[2], Md. Nasim Ali[1,3]*, Lucina Yeasmin[1], Bijan Majumdar[2]

[1]Department of Agricultural Biotechnology, IRDM Faculty Centre, Ramakrishna Mission Vivekananda University, Ramakrishna Mission Ashrama, Narendrapur, Kolkata, West Bengal, India, [2]Division of Crop Production, Central Research Institute for Jute and Allied Fibe, ICAR, Barrackpore, Kolkata, West Bengal, India, [3]Department of Agricultural Biotechnology, Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India

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***Address for correspondence:**
Md. Nasim Ali, IRDM Faculty Centre, Ramakrishna Mission Vivekananda University, Ramakrishna Mission Ashrama, Narendrapur, Kolkata, West Bengal, India.
E-mail: nasimali2007@gmail.com

ABSTRACT

Jute is considered as one of the cheapest natural fibers after cotton in terms of its production and uses. Retting is the most important post-harvest operation to yield high-quality jute fiber and is solely carried out by various types of retting microorganisms. The present study was undertaken to screen and characterize the efficient retting microbes isolated from retting water based on their enzymatic activity followed by biolog based identification of those efficient microbes. These isolates were characterized on the basis of qualitative and quantitative estimation of Pectinolytic, Xylanase, and Cellulase activity. Out of 40 isolated strains only 3 were finally identified as efficient jute retting microorganism having high Pectinolytic and Xylanase activity coupled with less Cellulase activity. These identified three microorganisms may provide a suitable means to develop a new retting technique, especially under water stress condition.

KEY WORDS: Biochemical characterization, biolog, jute retting, microorganisms

INTRODUCTION

Jute is considered as one of the cheapest sources of natural fiber after cotton in terms of its production and its uses (Ahmed and Nijam, 2008). Among all natural industrial fibers nearly 15% of total outputs are supplied from jute which indicates its economic importance (Haque *et al.*, 2001a). Retting, the most important post-harvest operation carried out by various types of retting microorganism, ultimately improves the quality of jute fiber and its economic value thereafter (Islam and Rahaman, 2013). It may be defined as the process of separation and extraction of fiber from non-fibrous tissues and woody part of the stem through dissolution and decomposition of pectins, gums and other mucilaginous substances (Dasgupta *et al.*, 1977; Majumdar and Dey, 1977). This is because a jute retting microbe is supposed to be highly efficient which is having high Pectinolytic and Xylanase (EC 3.2.1.8) (Collins *et al.*, 2005) activity with no or less Cellulase (EC 3.2.1.4) (Sarrouh *et al.*, 2012) activity. During retting, degradation of pectin carried out by Pectinase mainly Polygalacturonase (PG) and pectin lyase (PLN) (EC 4.2.2.10) (Duvetter *et al.*, 2009) activity of retting microbes while partial degradation of Xylan (major component of hemicellulose) done by Xylanase to make the fiber soft in nature (Ahmed and Akhter, 2001; Das *et al.*, 2012a). Application of mixed enzyme (Cellulase, Xylanase and Pectinase) on jute shown significant positive changes on physical characteristics of jute fibers in terms of whiteness index, brightness index (Vigneswaran and Jayapriya, 2009). Until now there were several attempts that has been taken to test the pectinolytic activity of microorganisms like fungi (Haque *et al.,* 2001b), bacteria (Das *et al.*, 2012a) for retting and pilling of jute (Banik and Ghosh, 2008) and identified through technique like 16S rDNA analysis (Das *et al.*, 2012a; Das *et al.*, 2011; Munshi and Chattoo, 2008) or through Bergey's manual (Banik and Ghosh, 2008; Ali, 1958; Jalaluddin, 1965) and assessment of bacterial community done through biolog ecoplates under different stages of jute retting at different location (Das *et al.*, 2013). Until now there is no report available on the identification of retting microorganisms through using BIOLOG Microplate system from retting water.

Beside enzymatic activity, water is a crucial factor for retting of jute. At water stress condition improper retting leads to low-quality jute which found recently in major jute growing areas of Bangladesh (Islam and Rahaman, 2013). Under this situation, the identification of efficient retting microorganisms can provide a suitable way for the development of new retting technique, especially under water scarcity condition. The present study was undertaken to characterize and identify the efficient retting microbes present in the jute retting water on the basis of their enzymatic activity, i.e., Pectinolytic, Xylanse, and Cellulase activity.

MATERIALS AND METHODS

Isolation and Selection

Retting water samples were collected from different retting tank. The enumeration of retting microbes was done following serial dilution technique and pour plate method (Parmer and Sclunidt, 1965). The retting water was serially diluted up to 10^{-6} times. Each dilution was plated in Pectin agar media (Pectin - 1%, Yeast extract - 1%, Sodium chloride - 0.5%, and Agar -2%) and was incubated for three days at 34°C. The pure cultures of 40 morphologically distinct isolates on Pectin agar plate were developed for further analysis.

Enzymatic Study of Isolated Microorganism

For the qualitative study of 40 isolates with respect to formation of halozone (Plate 1a-c), spot inoculations of the isolates were done on Pectin agar plate, Xylan agar plate following the modified method of Kaur *et al*. (2011) and in case of carboxymethyl cellulase (CMC) agar plate it was according to modified method of Baharuddin *et al*. (2010). Then the plates were incubated for 72 h in Orbital Incubator Shaker (Model CIS-24 BL, Make REMI). After that media surface of Pectin and Xylan, agar plate was kept flooded with 2% CTAB (Cetyl Trimethyl Ammonium Bromide) for 30 min. The flooding was done with 0.03% Congo red in case of CMC agar plate. Then the plate surfaces were washed using distilled water to get the halozone on media surface. Potency index (zone diameter/colony diameter) of each strain measured with three replication (Phutela *et al*., 2005).

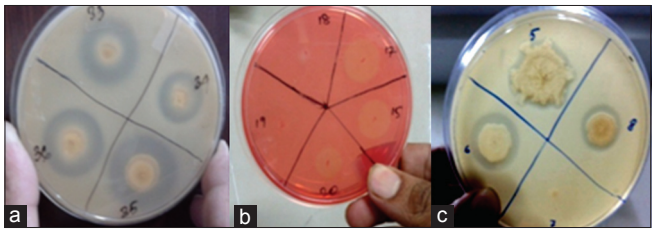

Plate 1: (a) Qualitative study of Pectinolytic enzyme using Pectin as substrate (Halozone formation around the colony in Pectin agar plate). (b) Qualitative study of Cellulase enzyme using carboxymethyl cellulase (CMC) as substrate (Halozone formation around the colony in CMC agar plate). (c) Qualitative study of Xylanase enzyme using Xylan (Halozone formation around the colony in Pectin Xylan agar plate)

All 40 strains then were used for quantitative analysis of Pectinolytic activity in terms of PG and PLN at 48 and 72 h of incubation following modified enzymatic assay of Phutela *et al*. (2005) and Nedja *et al*. (2001) respectively. But out of 40 only 8 strains showing higher potency index for Xylanase and lower potency index for cellulase were selected for the enzymatic assay of Xylanase and Cellulase following the method of Monisha *et al*. (2009) and Miller *et al*. (1960) respectively at 48 and 72 h after inoculation in Xylan broth and CMC broth.

Statistical analyses were performed using SPSS v16.0 for Windows (SPSS Inc., USA) with 5% probability level of statistical significance.

Identification of Isolates

After analyzing data, microbial strains having significantly high Pectinolytic and Xylanase activity with low Cellulase activity were identified up to species level by using Biolog (Model: BioLoG Microstation™) system based on the metabolic fingerprinting pattern of isolates using the software ML_51_01_ml3.

RESULTS AND DISCUSSION

Qualitative Analysis

Among 40 strains, most strains exhibited high Pectin and Xylan degrading capacity defined by high potency index and maximum of them were shown high potency index in CMC agar plate. All data of qualitative analysis were not given (Table 1). All microbial strains were formed halozone in Pectin agar plate indicating all had considerable jute retting capacity. For this reason,

Table 1: Qualitative study - Potency index (zone diameter/colony diameter) of finally selected microbial strains for quantitative assay (S11, S33 and S37 were selected for identification)

Strain number	Potency index in pectin agar media	Potency index in CMC agar media	Potency index in Xylan agar media
S10	2.09	1.65	2
S11	2.5	1.08	2.33
S15	2.04	1.5	2.11
S16	2.29	2.5	1.9
S29	3.33	3.2	2.9
S30	3.2	3	2.4
S33	1.67	1.50	3
S37	1.52	1.25	1.72

CMC: Carboxymethyl cellulose

quantitative assay for Pectinolytic activity was done in all 40 isolates.

In CMC agar plate halozone formed surrounding area of the colonies (Plate 1b) represented that the isolates (S15, S17, S18, S20) having the cellulose degrading capacity, so they were not considered for retting. Xylanse activity of microbial strain is act as a bleaching agent which increase the brightness of fiber. For this reason, out of 40 strains only 8 strains were selected for quantitative enzymatic analysis of Cellulase and Xylanse activity on the basis of low (<1.5) and high potency index (>1.5) in CMC and Xylan agar plate respectively (Table 1).

Quantitative Analysis

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A quantitative study of Pectinolytic enzyme involved estimation of PG and PLN activity at 48 and 72 h interval. It was found that most of the microbial strains have high PG activity at 72 than 48 h (Figures 1 and 2). At 48 h, PG activity ranges from 2.08 ± 0.07 to 6.8 ± 0.42 µmol/ml/min while at 72 h it ranges from 2.30 ± 0.023 to 7.71 ± 0.1 µmol/ml/min. Highest and lowest PG activity found in S37 and S24 respectively at 48 h. At 72 h, PG activity found highest in S20 and lowest in S1 (Table 2). During PLN estimation result was opposite (Figures 3 and 4). Most of the microbial strains shown high PLN activity at 48 h than 72 h. PLN activity of strains at 48 h ranged from 11.70 ± 0.12 to 184.83 ± 0.2 U/ml and at 72 h it ranged from 20.63 ± 1.18 to 161.20 ± 1.38 U/ml of culture filtrate (Table 2). In 48 h, PLN activity found highest in S3 while it was lowest in S18, S19, S21, S22, S23, S24 and S27. From this view, we can found that S24 was worst performer among 40 strains on the basis of Pectinase activity. The result clearly indicates that during the early stage of retting water complex Pectinolytic compound breaks down to simple form as PG acid through several enzymes like PLN which later breaks down by PG. Another thing which may conclude from this view (Table 2) that in jute retting microorganisms PG activity and PLN activity both are independent of each other because we have not get any microbial strains having both of high PLN and high PG activity at 48 and 72 h. Similar results obtained by Das *et al.* (2012a). where microbial strains having higher PG activity failed to show higher PLN activity.

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Since the strains having low potency index were selected for this purpose all shown low Carboxymethyl cellulase (CMCase) activity at 48 and 72 h interval. At 48 h, the Cellulase activity ranges from 2.4 ± 0.03 to 3.23 ±.07 µmol/ml/min while at 72 h it was from 1.29 ± 0.12 to 2.56 ± 0.02 µmol/ml/min (Table 3). It was found that all these strains have high Cellulase activity maximum in 48 than 72 h (Figures 5 and 6). At both 48 and 72 h minimum CMCase activity found in S1. Since Pectinase activity was low in S1 in comparison to other strains made it unsuitable for jute retting. The strains S33 and S37 were found to be efficient retting microbes because both Xylanase and Pectinase were found comparatively high (Tables 1 and 2).

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The response of the selected strains to Xylanase activity was very erratic. The strains (S1, S19 and S23) were shown higher activity in 72 than 48 h (Figures 7 and 8).

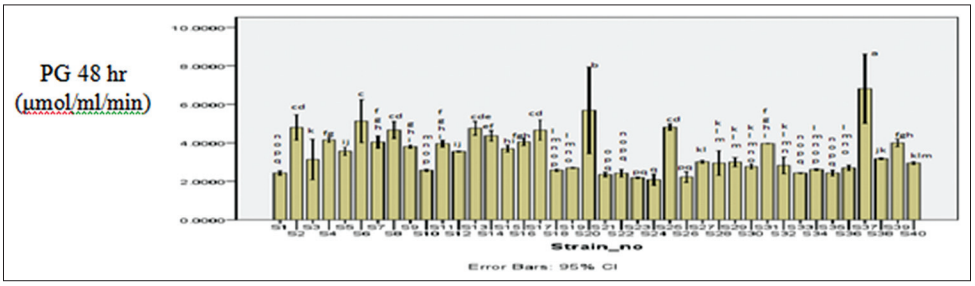

Figure 1: Polygalacturonase (PG) activity at 48 h of microbial strains.

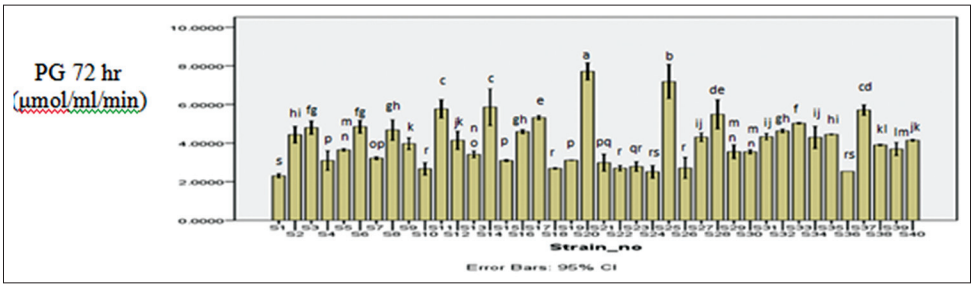

Figure 2: Polygalacturonase (PG) activity at 72 h of microbial strains.

Table 2: Xylanase and Cellulase activity at 48 and 72 h of finally selected microbial strains.

Strain number	CMCase 48 h (μmol/ml/min)	CMCase 72 h (μmol/ml/min)	Xylanase 48 h (μmol/ml/min)	Xylanse 72 h (μmol/ml/min)
S1	2.4±0.03^{f}	1.29±0.12^{d}	3775.48±72.11^{e}	4466±113.26^{e}
S11	2.64±0.02^{e}	1.70±0.04^{c}	12928.0±242.89^{a}	12836.8±376.05^{a}
S15	2.81±0.035^{d}	2.13±0.02^{b}	11894.26±428.26^{b}	11835.3±129.63^{b}
S18	2.99±0.07^{c}	2.20±0.04^{b}	10068.2±190.68^{c}	9228.35±441.09^{c}
S19	3.04±0.05^{bc}	2.38±0.04^{ab}	3857.0±135.82^{e}	11949.43±315.0^{b}
S23	3.15±0.02^{ab}	2.56±0.02^{a}	3172.41±419.41^{e}	5792.08±44.31^{d}
S33	3.1±0^{abc}	2.25±0.21^{b}	12291.2±221.21^{ab}	11662.07±440.2^{b}
S37	3.23±0.07^{a}	2.35±0.03^{ab}	4715.71±108.83^{d}	2988.51±57.51^{f}

Sig value for all significant value 0.00.

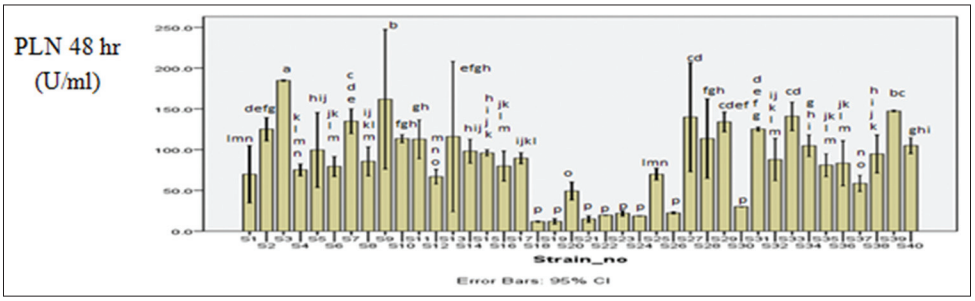

Figure 3: Pectin lyase (PLN) activity at 48 h of microbial strains.

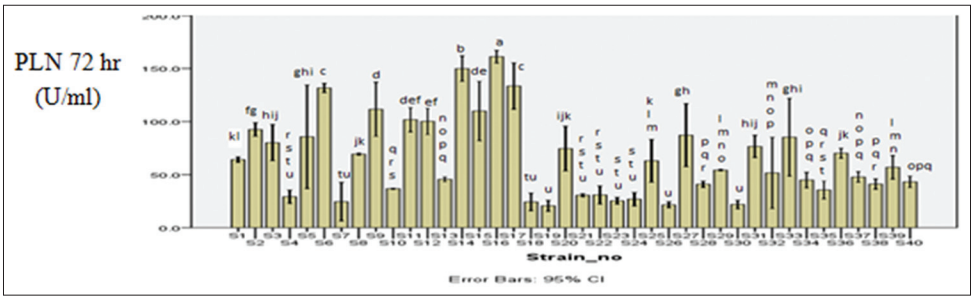

Figure 4: Pectin lyase (PLN) activity at 72 h of microbial strains.

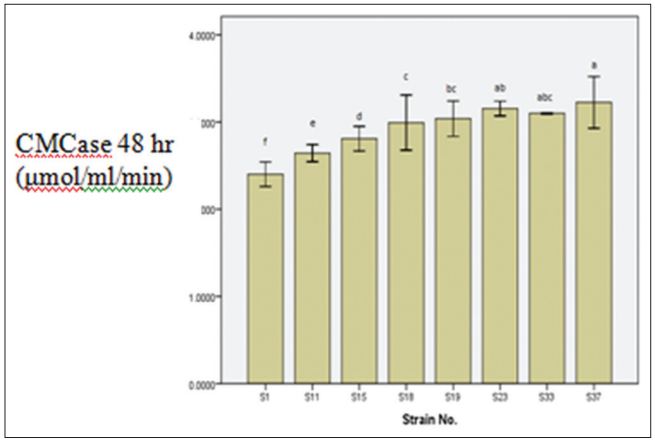

Figure 5: CMCase activity at 48 h of selected microbial strains.

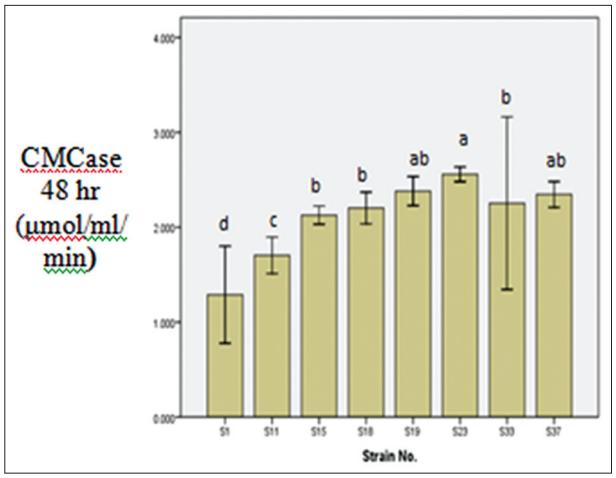

Figure 6: CMCase activity at 72 h of selected microbial strains.

At 48 h Xylanase activity ranges from 3775.48 ± 72.11 to 12928.0 ± 242.89 μmol/ml/min while at 72 h it was from 12836.8 ± 376.0 to 2988.51 ± 57.51 μmol/ml/min (Table 2). At both 48 and 72 h S11 and S33 recorded highest Xylanse activity.

Overall potency index for Pentinase and Xylanse activity were high coupled with low potency index for Cellulase activity were recorded for three strains No. 11, 33, and 37. Besides, the quantitative assay showed that all these strains having high Pectinolytic activity as well Xylanse activity

Table 3: PG and PLN activity of all 40 strains at 48 and 72 h

Strain number	PG 48 h (μmol/ml/min)	PG 72 h (μmol/ml/min)	PLN 48 h (U/ml)	PLN 72 h (U/ml)
S1	2.4333±0.02603^{nopq}	2.300±0.02309^{s}	69.9000±8.14064^{lmn}	64.1000±0.57735^{kl}
S2	4.8000±0.15011^{cd}	4.4400±0.09815^{hi}	125.1000±3.2909^{defg}	92.4667±1.47234^{fg}
S3	3.1333±0.24538^{k}	4.8000±0.08083^{fg}	184.8333±0.20276^{a}	80.1000±3.92598^{hij}
S4	4.1500±0.0.02887^{fg}	3.1000±0.11547^{p}	75.2000±1.67432^{klmn}	29.2000±1.44338^{rstu}
S5	3.5700±0.04619^{ij}	3.6467±0.01453^{mn}	99.6000±10.68098^{hij}	85.7000±11.31607^{ghi}
S6	5.1333±0.25693^{c}	4.8500±0.07506^{fg}	79.4333±2.8002^{jklm}	131.7000±0.9815^{c}
S7	4.0433±0.07219^{fgh}	3.2200±0.01732^{op}	134.8667±3.49301^{cde}	24.5000±4.21466^{tu}
S8	4.6667±0.10105^{cd}	4.6800±0.12124^{gh}	85.7000±4.09919 ^{ijklm}	69.3333±0.20276^{jk}
S9	3.8100±0.01732^{ghi}	3.9700±0.06928^{k}	161.9000±19.86085^{b}	111.6333±5.91786^{d}
S10	2.5767±0.00882^{mnop}	2.6700±0.07506^{r}	113.5667±1.12596^{fgh}	36.7667±0.08819^{qrs}
S11	3.9500±0.04041^{fghi}	5.7700±0.1097^{c}	112.9000±5.48483^{gh}	101.7667±2.627^{def}
S12	3.5500±0.00577^{ij}	4.1467±0.10682^{jk}	66.9333±1.99193^{mno}	100.2000±2.88675^{ef}
S13	4.7600±0.08083^{cde}	3.4100±0.04041^{no}	116.0667±21.39083^{efgh}	45.6000±0.51962^{nopq}
S14	4.3667±0.06064^{ef}	5.8633±0.22229^{c}	98.1667±3.37754^{hij}	149.9000±2.77128^{b}
S15	3.6967±0.04333^{hi}	3.0967±0.00882^{p}	96.0333±0.89505^{hijk}	110.0000±6.46632^{de}
S16	4.0500±0.04041^{fgh}	4.5967±0.02028^{gh}	79.9000±4.21466^{jklm}	161.2000±1.38564^{a}
S17	4.6600±0.12124^{cd}	5.3200±0.02309^{e}	89.6333±1.5878^{ijkl}	133.5333±5.05184^{c}
S18	2.5867±0.01453^{lmnop}	2.6900±0.00577^{r}	11.7000±0.11547^{p}	24.3000±1.90526^{tu}
S19	2.7000±0.00577^{lmno}	3.1067±0.00333^{p}	11.9000±0.75056^{p}	20.6333±1.18369^{u}
S20	5.6933±0.5225^{b}	7.7133±0.10105^{a}	49.2000±2.54034^{o}	74.5000±4.84974^{ijk}
S21	2.3600±0.02887^{opq}	2.9867±0.10155^{pq}	15.0000±0.92376^{p}	30.6333±0.31798^{rstu}
S22	2.4167±0.04333 ^{nopq}	2.7000±0.02887^{r}	19.6333±0.03333^{p}	30.8333±0.1.9342^{rstu}
S23	2.1867±0.00882^{pq}	2.7867±0.05487^{qr}	22.0000±0.7506^{p}	25.4333±0.72188^{stu}
S24	2.0833±0.06642^{q}	2.5167±0.07796^{rs}	18.7667±0.08819^{p}	26.8667±1.47234^{stu}
S25	4.8167±0.03756^{cd}	7.1833±0.20497^{b}	69.9000±1.67432^{lmn}	63.0000±4.67654^{klm}
S26	2.2267±0.06064^{pq}	2.7100±0.12702^{r}	22.4333±0.26034^{p}	21.5333±0.66416^{u}
S27	3.0200±0.01732^{kl}	4.3100±0.05196^{ij}	139.8667±15.50186^{cd}	87.1333±6.89936^{gh}
S28	2.9467±0.14723^{klm}	5.4900±0.17321^{de}	113.6000±11.35297^{fgh}	40.9000±0.69282^{pqr}
S29	3.0000±0.05196^{klm}	3.5533±0.08373^{mn}	134.0000±2.77128^{cdef}	54.2667±0.1453^{lmno}
S30	2.7700±0.02887^{klmno}	3.5500±0.02309 ^{mn}	30.1667±0.03333^{p}	21.9000±0.92376^{u}
S31	3.9667±0.00333^{fghi}	4.3267±0.03756	125.3000±0.51962^{defg}	76.6000±2.42487^{hij}
S32	2.8300±0.9815^{klmn}	4.6300±0.02309^{gh}	87.8667±6.03333^{ijklm}	51.6000±7.79423^{mnop}
S33	2.4333±0.00333^{nopq}	5.0267±0.00882^{f}	140.9000±4.04145^{cd}	85.2667±8.4582^{ghi}
S34	2.6133±0.00882^{lmnop}	4.2967±0.12991^{ij}	104.8000±3.11769^{ghi}	44.9000±1.67432^{opq}
S35	2.4300±0.03464 ^{nopq}	4.4500±0^{hi}	81.0000±3.23316 ^{jklm}	35.5667±1.9342^{qrst}
S36	2.6967±0.0318^{lmno}	2.5300±0^{rs}	83.2667±6.37974 ^{jklm}	70.2000±1.03923^{jk}
S37	6.8133±0.41858^{a}	5.7133±0.06064^{cd}	58.7000±2.25167^{no}	47.7333±1.12596^{nopq}
S38	3.1800±0.00577^{jk}	3.9033±0.00882^{kl}	94.8000±5.36936^{hijk}	41.1000±1.09697^{pqr}
S39	4.0000±0.04619^{fgh}	3.6933±0.07796^{lm}	147.3333±0.20276^{bc}	56.9000±2.54034^{lmn}
S40	2.9533±0.01453^{klm}	4.1433±0.00882^{jk}	105.0000±2.25167^{ghi}	43.1333±1.24141^{opq}

PG: Polygalacturonase, PLN: Pectin lyase Sig value for all significant value 0.00. That means significant at 1% ∞

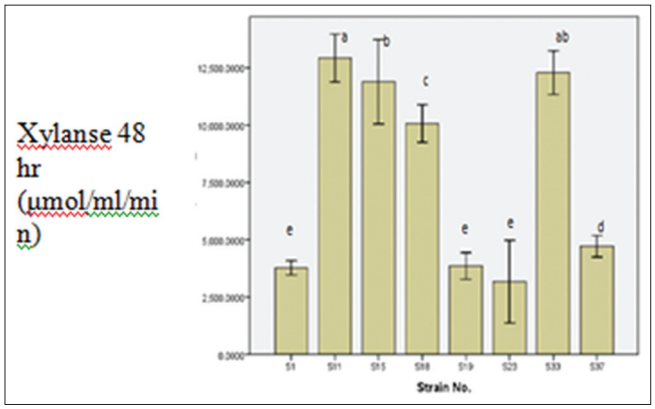

Figure 7: Xylanse activity at 48 h of selected microbial strains

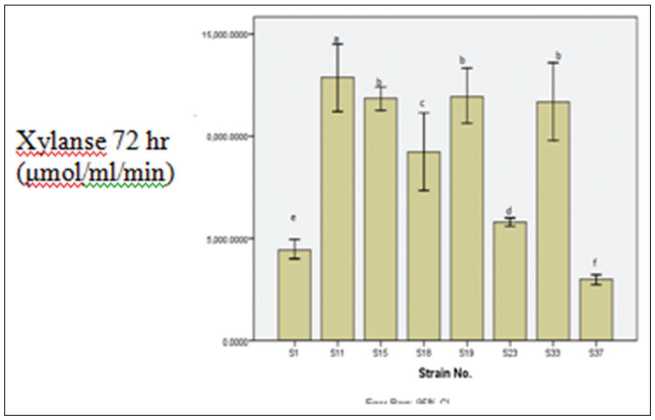

Figure 8: Xylanase activity at 72 h of selected microbial strains

but lower Cellulase activity (Tables 2 and 3). From this result, it was evident that S11, S33 and S37 found most effective strain in terms of their enzymatic activity. S11 having moderate Pectinase coupled with highest Xylanse and lower CMCase value may also be suitably used for retting.

Identification of Selected Retting Microbes

The Biolog Identification System is a new bacterial identification method that establishes an identification based on the exchange of electrons generated during respiration, leading to a subsequent tetrazolium-based color change. Identification through biolog system, it was found 87.3% correct at the genus level and 75.6% correct at the species level at 24 h (Miller and Rhoden, 1991). These three strains (S11, S33 and S37) were then identified by using biolog, an advanced tool for characterization and identification of microorganisms based on the metabolic fingerprinting pattern of the isolates using the software ML_51_01_ml3.

The strains are identified as - *Bacillus pumilus* (Plate 2; strain 11), *B. pumilus* (Plate 3; strain 33) *and Bacillus licheniformis* (Plate 4; strain 37).

In the present study, all strains identified as *Bacillus spp*. The Pectinolytic strains of *Bacillus spp.* showed cellusolytic activities. These results were in confirmation with the finding of Tamburini *et al.*, (2004) who reported the presence of cellusolytic activities in the Pectinolytic strains that are *Bacillus subtilis*. Zhang *et al.*, (2000) also found low cellulolytic activities (0.04-0.17 U/cm^3) of some strains used in degumming of ramie fibres. Few *Bacillus* strains recorded the Xylanase activity of 11.50 IU/ml and Cellulase activity 1.2-1.5 IU/ml as reported by Subramaniyan and Prema (2002). The presence of *Bacillus* sp. in retting water was also reported by Jalaluddin (1965), and specifically *B. pumilus by* Ali (1958). In comparison to earlier reported microbes belonging to *Bacillus* genus, these strain recorded similar pattern of Xylanase activity with significantly less Cellulase activity which could make essentially more efficient jute retting microbes.

CONCLUSION

Out of 40 Pectinolytic bacterial isolates tested for PG, PLN, Xylanase and Cellulase enzymatic activities, only three isolates were found to have very high Pectinolytic and Xylanase activity with very less Cellulase activity. These identified two microorganisms may provide a suitable means to develop a new retting technique under water scarcity condition to get quality fibre from Jute. If these organisms are to be used as mixed in particular ratio culture, their fibre quality due to retting will be improved, but for this purpose required trial and error method to find out the best ratio to make the mixed culture.

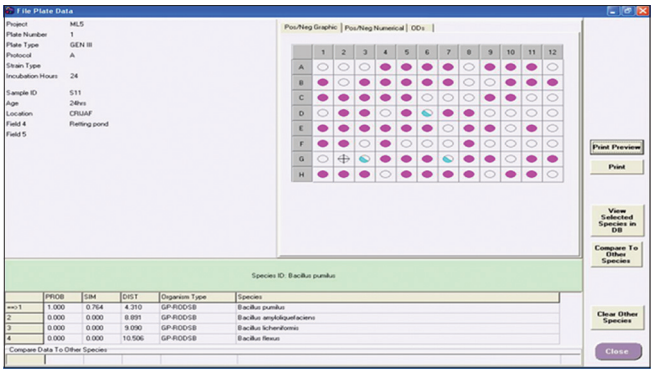

Plate 2: The selected strain (S11) is identified as *Bacillus pumillus*

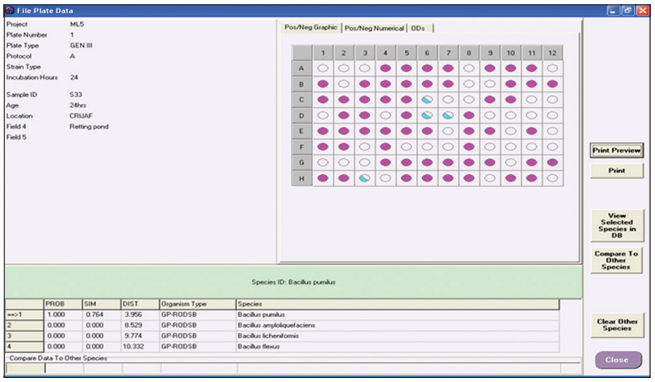

Plate 3: The selected strain (S33) is identified as *Bacillus pumillus*

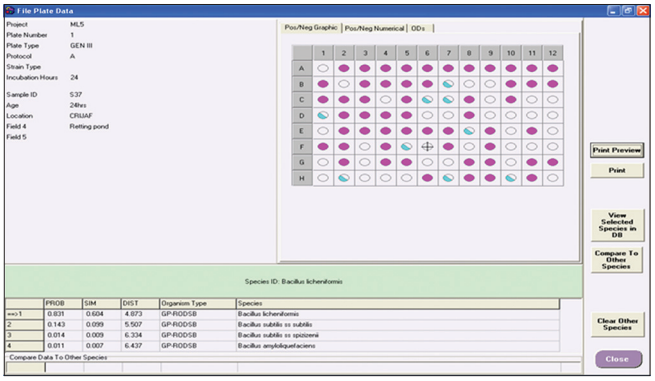

Plate 4: The selected strain (S37) is identified as *Bacillus licheniformis*

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