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Studies on the effect of pH and carbon sources on enzyme activities of some pectinolytic bacteria isolated from jute retting water

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Abstract: The effect of pH of the assay media and different carbon sources in the culture media on the activities of 4 different enzymes, namely polygalacturonase (PG), pectin lyase (PNL), xylanase, and cellulase, of some pectinolytic bacteria were studied. The pectinolytic bacterial strains were isolated from the jute retting water bodies of 2 important jute growing areas of North 24 Parganas, West Bengal, India. The highest PG (12.45 to 41.0 IU/g cell wet weight) and PNL (22.55 to 75.7 U/mL) activities occurred at the alkaline pH of 8.0 and 8.5, respectively. Maximum xylanase activity (0.01 to 0.735 µmol/mL/min) occurred at pH 7.0. An acidic pH of 5.0 favored the cellulase activity (0 to 0.168 µmol/mL/min). All the enzymes were highly substrate specific and inducible in nature. The highest PG and PNL activities were observed in the presence of pectin, whereas the highest xylanase and cellulase activities were detected in the presence of xylan and cellulose, respectively, as the sole carbon source in the media.

Key words: Pectinolytic bacteria, polygalacturonase, pectin lyase, xylanase, cellulase

Introduction

Jute (*Corchorus* sp.) is one of the most versatile bast fibers, occupying second position among the natural industrial fibers of the world and accounting for nearly 15% of the total output of natural fibers, making it economically significant (1). The Gangetic delta of India and Bangladesh contributes about 80%-85% of the world's jute production (2). Jute fibers are obtained through the process of retting in water bodies. It is essentially a biochemical process decomposing biopolymers such as pectins, hemicelluloses, and other mucilaginous substances that hold the bast fibers to the rest of the stem (3). Biological retting is the cheapest and most universally practiced method for the commercial extraction of jute fibers (4).

Pectin is a heteropolysaccharide, composed of α-1,4 linked galacturonate chains with a high percentage of methyl esterification, and is the main cementing agent holding the fiber to the stalk. During microbiological retting, the depolymerization of pectin is operated by pectinases, primarily comprising a mixture of 4 enzymatic activities: PG, PNL, pectate lyase, and pectin esterase. However, PG (5) and PNL (6) are the primary retting enzymes.

Partial removal of non-fibrous materials like hemicelluloses makes the jute fiber considerably softer for finer spinning (7). For this, the enzyme xylanase is useful, since xylan is a major component of hemicellulose. This enzyme allows the selective removal of hemicellulose without affecting the strength of the cellulosic fiber itself. Pectinolytic microorganisms, having xylanase activity but devoid of cellulase activity, are an additional beneficial aspect for improving the fiber quality of jute (8).

The PG, PNL, xylanase, and cellulase activities are intimately related to the retting process and quality of the jute fiber. These enzymes are produced by a consortium of bacteria in the water. The production, activity, and stability of the enzymes are dependent on different carbon sources and the pH of the media (9). The pH and the availability of different carbon sources undergo changes as the retting progresses (10). The pH and media composition, specifically the carbon source, may affect the production of enzymes. Such information for jute retting bacteria is yet to be studied.

We studied the effect of different pH of the enzyme assay media and different carbon sources in the culture media, under laboratory conditions, on enzyme activities like, PG, PNL, xylanase, and cellulase of some pectinolytic bacterial strains isolated from jute retting water bodies.

Materials and methods

Altogether, 38 pure pectinolytic bacteria were isolated in pectin containing media, following conventional microbiological procedures, from jute retting ponds (static water) occurring in 2 notable jute-growing belts, i.e. Sonatikari (22°41′27″N, 88°35′44″E) and Baduria (22°44′24″N, 88°47′24″E), West Bengal, India. Among the isolates, 8 bacterial strains were used for this study, based on their higher pectinolytic activity and lower/nil cellulase activity. The chemical properties of the retting water samples were: total hardness (142-399 ppm), Ca content (42-162 ppm), Mg content (19-49 ppm), Fe content (0.018-1.977 ppm), HCO_3^- content (4-18 me L^{-1}), and Cl^- content (0.4-4.8 me L^{-1}).

Isolation of genomic DNA

DNA was prepared from all of the 8 isolates using the sodium dodecyl sulfate proteinase K-cetyltrimethylammonium bromide (CTAB) method (11). The DNA preparations were treated with RNase A, and the DNA concentrations were estimated by visual examination of ethidium bromide-stained agarose gels, as well as by spectrophotometric examination.

Amplification of 16S rDNA

The PCR of the genomic DNA of the 8 isolates was conducted in a final volume of 50 µL. The reaction mixture included 20-50 ng of isolated genomic DNA, 2U taq polymerase (Promega, USA), 1 × PCR buffer with 1.5 mM $MgCl_2$, and 200 µM each dNTP and 10 pmol of each primer (IDT, USA). The primers were chosen to amplify partial 16S rDNA sequences. The forward primer 515F was (5'-3') GTGCCAGCAGCCGCGGTAA and the reverse primer 1492R was (5'-3') TACGGYTACCTTGTTACGACTT. Before the amplification cycle, the DNA was denatured for 5 min at 94 °C and after amplification an extension step (7 min at 72 °C) was performed. The cycling parameter consisted of 29 cycles, with denaturation at 94 °C for 30 s, primer annealing at 56 °C for 1 min, and extension at 72 °C for 1 min. The samples were held at 4 °C until analysis by agarose gel electrophoresis. All the amplified PCR products were agarose gel eluted using a Promega gel elution kit.

Sequencing of the 16SrDNA fragment and BLAST search

The amplified and gel-eluted PCR fragments of the 16S rDNA were sequenced in an ABI 3100 genetic analyzer with the primer 515F. Sequencing reaction was performed by using the Big Dye Terminator Cycle Sequencing Kit V3.1 (Applied Biosystems, Foster City, USA) following the manufacturer's protocol. The partial 16S rDNA sequences of the isolated strains were compared with those available in the public databases. Identification to the species level was determined as a 16S rDNA sequence similarity of >99% with that of the prototype strain sequence in the GenBank.

Pectinolytic activity of the isolates

The bacterial strains were grown in modified yeast extract-pectate broth (1% pectin, 1% yeast-extract, 0.5% NaCl, pH 7.0) for 2 days at 30 °C, with shaking (250 rpm). The culture was centrifuged (7000 × *g*, 10

min, 4 °C) and the cell-free supernatant was used for the assay of pectinolytic activities.

PG activity

The activity of the organisms was tested by the dinitrosalicylic acid (DNS) method, as described by Kobayashi et al. (12). About 0.1 mL of the cell-free supernatant was added to a reaction mixture composed of 100 mM Tris-HCl buffer (pH 8.0), 0.4 mM $CaCl_2$, and 1% (w/v) polygalacturonic acid (pH adjusted to 6.8 with NaOH), in a total volume of 1.0 mL. The reaction mixture was incubated at 37 °C for 15 min and terminated by adding 1.0 mL of the DNS reagent, followed by heating at 100 °C for 5 min. The reducing sugars formed in the solution were measured at 535 nm. Enzyme activity was determined by a standard curve prepared by different concentrations of galacturonic acid (Sigma) solutions against absorbance. In 1 min, at the assay temperature of 37 °C by 1 mL of supernatant, 1 unit (IU) corresponds to the release of 1 μmol of the reducing groups (final products of the reaction). The IU was corrected by the culture-wet weight (IU/g cells). The analyses were carried out in duplicate.

To study the effect of pH on PG activity, different assay buffers were used, i.e. 0.2 M phosphate buffer at pH 7.0 and 0.1 M acetate buffer at pH 5.0. The effect of carbon sources like glucose and starch on PG activity was determined by growing the organisms in the presence of glucose and starch, as the sole carbon sources, instead of pectin. The cell-free culture filtrate was assayed for PG activity.

PNL activity

The PNL activity of the isolates was assayed by the method of Pitt (13). Briefly, 1 mL of the cell-free supernatant was added to 5.0 mL of the pectin solution (1% w/v). The volume of the test samples was adjusted to 10.0 mL with Tris-HCl buffer (0.01 M, pH 8.5). The samples were incubated at 37 °C for 2 h. This was followed by the addition of zinc sulfate (0.6 mL, 9.0% w/v) and sodium hydroxide (0.6 mL, 0.5 M). The samples were centrifuged (3000 × *g*, 10 min) and 5.0 mL of the clear supernatant was added to a mixture of thiobarbituric acid (3.0 mL, 0.04 M), HCl (2.5 mL, 0.1 M), and distilled water (0.5 mL). The mixture was heated in a boiling water bath for 30 min, cooled to room temperature, and the absorbance of the colored solution was measured at 550 nm. The amount of enzyme that caused a change in absorbance of 0.01 under the condition of the assay was defined as 1 unit (U) of activity. The assays were done in duplicate.

To study the effect of pH on PNL activity, different assay buffers were used, i.e. 100 mM Tris-HCl buffer at pH 7.0 and 0.2 M citrate-phosphate buffer at pH 5.0. The effect of carbon sources like glucose and starch on PNL activity were determined by growing the organisms in the presence of glucose and starch, as the sole carbon sources, instead of pectin. The cell-free culture filtrate was assayed for PNL activity.

Xylanase activity

The xylanase activity of the isolates was assayed by the method of Bailey et al. (14), with some modifications, using 0.5% xylan as the substrate. The organisms were grown in xylan liquid culture media (0.5% xylan, 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4$. $7H_2O$, pH 7.0) for 2 days at 30 °C, with shaking (250 rpm). The culture was centrifuged (7000 × *g*, 10 min, 4 °C) and the cell-free supernatant was used for the assay of xylanase activity.

A suspension of xylan (0.5%) was prepared in a 0.2 M sodium phosphate buffer at pH 7. The reaction mixture containing 1.8 mL of preincubated oat spelts xylan suspension and 0.2 mL of culture supernatant was incubated for 10 min at 50 °C. The reaction was terminated by adding 3.0 mL of DNS reagent and boiling for 5 min. One unit of endo-xylanase activity was defined as μmol of xylose liberated per min per mL of culture supernatant. The assays were done in duplicate.

To study the effect of pH on xylanase activity, different assay buffers were used, i.e. 0.1 M acetate buffer at pH 5.0 and 0.2 M carbonate buffer at pH 9.2. The effect of carbon sources like glucose, starch, and pectin on xylanase activity was measured by growing the organisms in the presence of the sugars, as the sole carbon source, instead of xylan. The cell-free culture filtrate was assayed for xylanase activity.

Cellulase activity

The cellulase (exo-β1,4 glucanase) activity of the isolates was determined according to the method of Miller et al. (15). The organisms were grown in

carboxymethyl cellulose (CMC) broth (1% CMC, 1% yeast extract, 0.5% NaCl, pH 7) for 2 days at 30 °C, with shaking (250 rpm). The culture was centrifuged (7000 × *g*, 10 min, 4 °C) and the cell-free supernatant was used for the assay of cellulase activity.

About 0.05 mL of the cell-free supernatant was added to 0.45 mL of 1% CMC solution (made in 0.1 M sodium citrate buffer, pH 5.0) at 55 °C. The mixture was incubated at 55 °C for 15 min in a water bath. At the end of the incubation, 0.5 mL of DNS reagent was added to the mixture and it was heated in a boiling water bath for 5 min. Then 1 mL of sodium potassium tartarate solution (40%) was added, while the tubes were warm, followed by cooling to room temperature. The absorbance was measured at 540 nm and the concentration of reducing sugar produced was determined against a standard graph with glucose. The enzyme activity was carried out in duplicate and the data were expressed as the μmol glucose released/mL of culture filtrate/min.

To study the effect of pH on cellulase activity, different assay buffers were used, i.e. 0.2 M phosphate buffer at pH 7.0 and 100 mM Tris-HCl buffer at pH 8.0. The effect of carbon sources like glucose, starch, and pectin on cellulase activity was determined by growing the organisms in the presence of glucose and starch, as the sole carbon sources, instead of CMC. The cell-free culture filtrate was assayed for cellulase activity.

Results and discussion

The retting process is the major limitation to efficient and high quality fiber production, thus being the key feature in any future expansion of jute and allied fibers as industrial crops. Bacteria are the main retting agents; therefore, their activity affects the course of the process and quality of the product. The organisms used in this study were isolated for using as jute retting inocula.

The identity of the organisms, as determined by 16S rDNA analysis are as follows: BA1; *Bacillus* sp. L6 (GQ891097), BA15; *Bacillus pumilus* strain EK-17 (GQ891098), BA16; *Bacillus* sp. YACS30 (GQ891099), BA22; *Bacillus pumilus* strain IK-MB13-518F (GQ891100), SO1; *Agrobacterium* sp. BN-2A (GQ891102), SO7; *Bacillus pumilus* strain Geo-03-422 (GQ891103), SO8; *Microbacterium* sp. PVC8 (GQ891104), and SO14; *Bacillus pumilus* strain IK-MB12-518F (GQ891105). The organisms *Agrobacterium* sp. BN-2A and *Microbacterium* sp. PVC8 were not previously reported as jute retting bacteria.

PG activities at pH 5.0, 7.0, and 8.0 ranged from 5.25 to 17.96, 1.89 to 22.08, and 12.45 to 41.0 IU/g, respectively (Figure 1). The highest mean activity (27.66 IU/g) was observed at pH 8.0 and the lowest mean activity (9.52 IU/g) was observed at pH 7.0. With the exception of organism SO7, all of the other isolates showed maximum activity at pH 8.0. The SO7 organism recorded the highest activity (17.96 IU/g) at pH 5.0. Regarding the different carbon sources, the PG activities of the isolates in the presence of pectin, glucose, and starch varied from 12.45 to 41.0 IU/g (mean 27.66 IU/g), 5.74 to 14.28 IU/g (mean 10.38 IU/g), and 7.88 to 11.08 IU/g (mean 9.01 IU/g), respectively (Figure 2). The isolates showed the highest PG activity in the presence of the specific carbon source, i.e. pectin. Among the isolates, BA15 showed the highest PG activity (41.0 IU/g) at pH 8.0 in the presence of pectin as the sole carbon source. Tamburini et al. (16) reported that the PG activity of most aerobic bacteria isolated from hemp and flax retting water was between 10 and 39 IU/g,

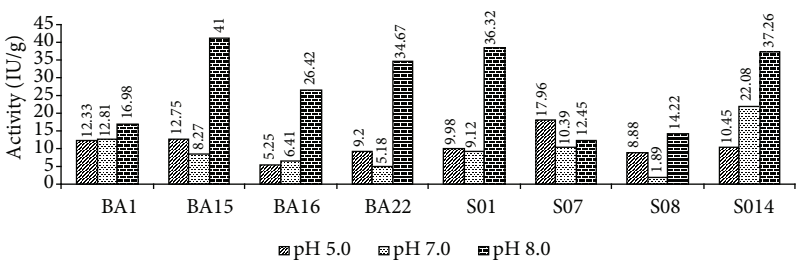

Figure 1. Effect of pH on polygalacturonase activity.

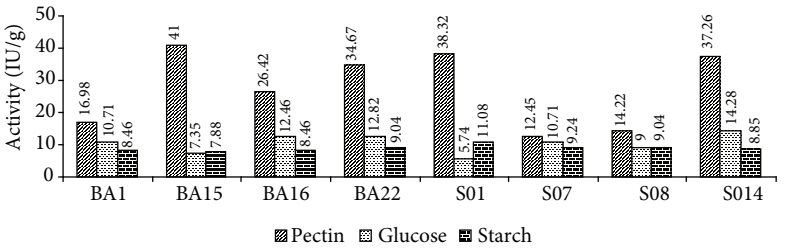

Figure 2. Effect of carbon sources on polygalacturonase activity.

in the presence of pectin at pH 8.0. Under similar conditions, with the exception of organism BA15, all of the isolates showed PG activity between 10 and 39 IU/g. The organism BA15 showed PG activity (41.0 IU/g) higher than the reference strain, *B. subtilis* (39.0 IU/g) (16). However, most of the isolates showed PG activity less than 10 IU/g at pH 5.0 and 7.0. Tamburini et al. (16) reported that the aerobic pectinolytic bacterial strains showed PG activity at pH 8.0. With the exception of organism SO7, all of the isolates showed the highest activity at pH 8.0. Kobayashi et al. (12) also reported that the *Bacillus* sp. strain KSM-P576 had optimum activity at pH 8.0, which is comparable to our finding. Not many reports are available regarding the effect of carbon sources on the PG activity of organisms. Soriano et al. (6) reported that *Paenibacillus* sp. BP-23 showed no PG activity in the presence of glucose, but *Bacillus* sp. BP-7 showed PG activity in the presence of glucose, as the sole carbon source in the medium. Although all of the isolates exhibited PG activity in the presence of both glucose and starch as the sole carbon sources in the media, the highest activity was obtained in pectin containing medium. This indicated that PG is an inducible enzyme (17).

PNL activities of the isolates at pH 5.0, 7.0, and 8.5 were 0 to 21.47, 0 to 8.09, and 22.55 to 75.7 U/mL, respectively (Figure 3). The isolates showed the highest mean PNL activity at pH 8.5 (41.74 U/mL) and the lowest at pH 7.0 (1.46 U/mL). All of the isolates registered the highest PNL activity at pH 8.5. With the exception of SO7 and SO14, none of the organisms showed detectable PNL activities at pH 7.0. The mean PNL activity of the isolates in the presence of glucose (18.4 U/mL) was higher than of starch (4.85 U/mL) as the sole carbon source (Figure 4). With the exception of SO7 and SO14, none of the organisms showed detectable PNL activity in the presence of starch in the media. In general, the PNL activities of the organisms in the presence of glucose and starch ranged from 13.37 to 24.03 U/mL and 0 to 35.65 U/mL, respectively. The isolate SO7 registered the highest PNL activity (75.7 U/mL) at pH 8.5 in the presence of pectin as the sole carbon source. Published literature suggests that PNL is mainly produced by fungal genera, but a few reports on bacteria and yeast are also available (18). Soriano et al. (6) reported that *Paenibacillus* sp. BP-23 showed PNL activity of 47 U/mL and *Bacillus* sp. BP-7 showed 1.51 U/mL. The optimum pH for PNL activities of the 2 strains was at pH 10.0. *Bacillus* sp. BP-7 showed a second peak of activity at pH 8.0. Kashyap et al. (19) reported that *Bacillus* sp DT7 showed PNL activity of 52 U/mL. The purified enzyme exhibited maximum

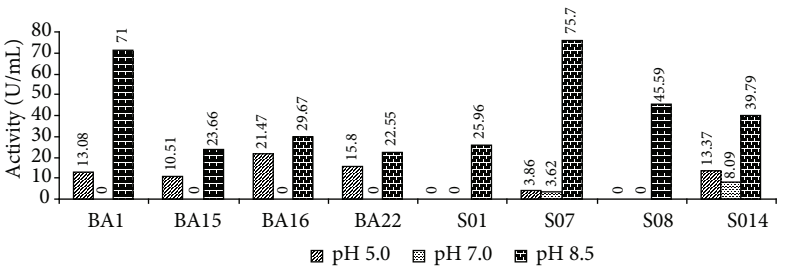

Figure 3. Effect of pH on pectin lyase activity.

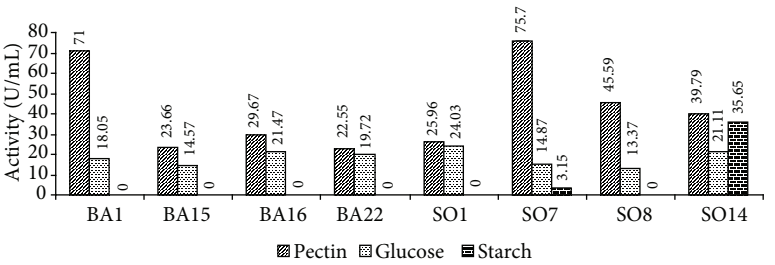

Figure 4. Effect of carbon sources on pectin lyase activity.

activity at pH 8.0. All of the isolates, as found in this study, showed maximum activity at pH 8.5 in the presence of pectin as the sole carbon source in the medium. The organism SO7 exhibited the highest activity at 75.7 U/mL. Soriano et al. (6) found that the addition of glucose to the media abolished the PNL production in *Paenibacillus* sp. BP-23, suggesting that the expression of PNL in this strain is subjected to catabolite repression. In contrast, the expression of PNL activity in *Bacillus* sp. BP-7 was not negatively affected by glucose. The PNL activities of the isolates in the present study were not negatively affected by glucose, but media supplemented with starch repressed the expression of PNL of all of the isolates, except for SO7 and SO14. The results indicated that like PG activity, PNL activity is also inducible and is strain dependent.

The xylanase activities of the isolates at pH 5.0, pH 7.0, and pH 9.2 varied from 0 to 0.333, and 0.01 to 0.735 and 0 to 0.271 µmol/mL/min, respectively (Figure 5). The highest mean xylanase activity took place at pH 7.0 (0.418 µmol/mL/min) and the lowest mean activity was at pH 9.2 (0.078 µmol/mL/min). The isolates showed optimum xylanase activity at pH 7.0. Organisms SO1 and SO8 showed no detectable xylanase activity at pH 5.0 and 9.2. Only the isolate BA16 (0.095 µmol/mL/min) showed xylanase activity in the presence of glucose (Figure 6). The xylanase activity in the presence of starch and pectin varied from 0 to 0.216 µmol/mL/min (mean 0.129 µmol/mL/min) and 0 to 0.147 µmol/mL/min (mean 0.071 µmol/mL/min), respectively. The isolate BA1 showed the highest xylanase activity (0.735 µmol/mL/min) at pH 7.0 in the presence of xylan as the carbon source. The highest xylanase activity of the isolates was found at pH 7.0. Several authors also reported that the pH optima for xylanases isolated from many bacteria are at neutral pH range (20-22). Results indicated that this enzyme was also inducible in nature. The presence of glucose did not induce the production of xylanase, except for the strain BA16. Although xylanase activities of the organisms were detected in the presence of starch and pectin, it was much less than the activity in the presence of xylan as the sole carbon source in the medium.

The cellulase activities of the organisms at pH 5.0, 7.0, and 8.0 varied from 0 to 0.168, 0 to 0.071, and 0 to 0.042 µmol/mL/min, respectively (Figure 7). It was found that for most of the isolates the cellulase activities decreased with the increase in pH values.

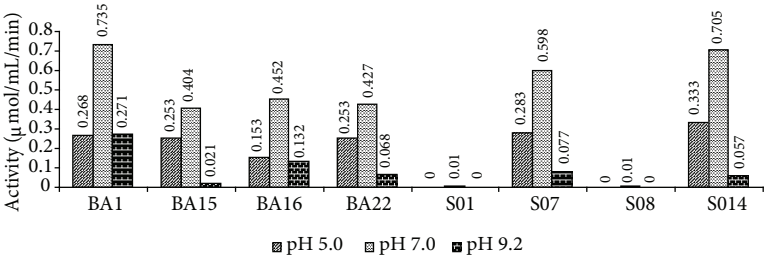

Figure 5. Effect of pH on xylanase activity.

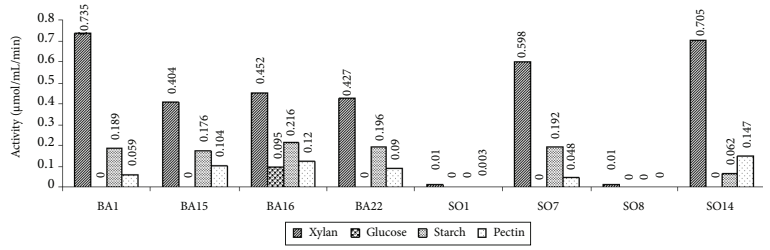

Figure 6. Effect of carbon sources on xylanase activity.

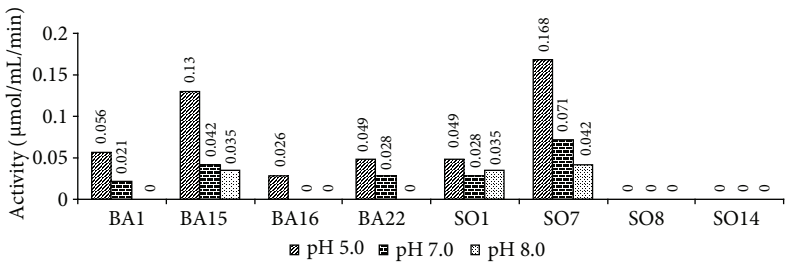

Figure 7. Effect of pH on cellulase activity.

The highest mean activity was found at pH 5.0 (0.06 µmol/mL/min) and the lowest at pH 8.0 (0.014 µmol/mL/min). None of the isolates showed cellulase activity in the presence of glucose, starch, and pectin (Figure 8), which corroborates with previous reports (23), that this enzyme is also inducible in nature.

The isolates showed better pectinolytic and xylanolytic activities at slightly alkaline and neutral pH, respectively, whereas the highest cellulase activity was found at an acidic pH. Therefore, it can be concluded that the pH of the retting water should be kept at neutral to slightly alkaline for proper retting of jute, without affecting the cellulosic fiber. Furthermore, the data indicated that these enzymes are inducible in nature.

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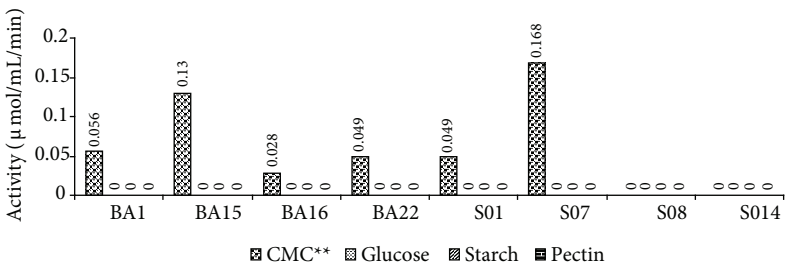

Figure 8. Effect of carbon sources on cellulase activity.
**CMC - Carboxymethyl cellulose

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