# Chapter 6

# Studies on physicochemical properties of crude M35, R31 and J208 Xylanases (Xylan hydrolases)

# 6.1. Introduction:

Crude xylanases and pectinases obtained from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 isolated from camel, cow and buffalo dung respectively exhibited positive synergism when applied as different cocktails (PX, CX, CP, CPX) involving cellulase, xylanase and pectinase enzymes to raw as well as pretreated SCB for saccharification.

Literature on characterization and application of crude xylanase-pectinase enzymes from *B. safensis* and *B. altitudinis* is sparse and those reported are as follows. Rahmani et al., (2014), optimized the production of xylanase activity from marine *B. safensis* P20 through submerged fermentation of sugarcane bagasse and Djohan et al., (2016) used crude xylanase produced by marine *B. safensis* LBF-002 for saccharification of rice straw waste biomass. Alkalophilic xylanase was characterized from *B. safensis* MX47 isolated from decaying material by Chi et al., (2012). Xylanase from *B. altitudinis* DHN-8 was characterized and its application in sorghum straw saccharification, bio-deinking and bio-bleaching were studied by Adhyaru et al., (2014, 2017). Reports regarding the production of pectinase by *B. altitudinis* are lacking while *B. safensis* DSKK5 isolated from earthworm gut was reported for pectinase but the quantification and characterization studies were not done (Singh et al., 2014). Besides these, two of our publications on characterization of xylanase and pectinase activities from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 explained their role in raw agrowaste saccharification (Thite and Nerurkar, 2015; Thite and Nerurkar, 2018).

Hemicellulose or xylan is a major polysaccharide that masks the cellulose and causes hindrance in the action of cellulase. Xylan degrading enzymes therefore have a major impact on cellulose accessibility up to such an extent that they are sometimes considered as core enzymes (Banerjee et al., 2010c). The cell free spernatant (CFS) was characterised for enzyme activities responsible for degradation of xylan and pectin. As xylan hydrolase is an important activity present in M35, R31 and J208 crude xylanase, studies regarding their physicochemical properties were undertaken as they can serve as the suitable process parameters for agrowaste saccharification.

# 6.2. Materials and Methods:

## 6.2.1. Chemicals, crude polysaccharide substrates, enzymes and their producers:

All required chemicals, purchased from HiMedia (Mumbai, India) or Sigma-

Aldrich (Missouri, USA) or SRL Pvt. Ltd. (Mumbai, India), were of assay grade. Citrus Peels (CP) and Wheat Bran (WB) were collected from local market, processed and stored as mentioned in Chapter 2, Section 2.2.1. WB and CP were used as a crude polysaccharide substrate in media for selective production of xylano-pectinolytic enzymes.

Three bacterial cultures *Bacillus safensis* M35, *Bacillus altitudinis* R31 and *Bacillus altitudinis* J208 were used as source of enzymes for the enzyme characterization studies in this chapter. Isolates were maintained by inoculation and incubation on Nutrient Agar (NA) plates at 37 ºC for 24 h followed by storage at 4-6 ºC. Xylanases and pectinases, produced by these three isolates on the production media containing WB and CP respectively were used as crude enzymes for characterization studies and will be stated with the prefix of the bacterial strain they are obtained from. Thus, crude xylanases are referred to as M35 xylanase, R31 xylanase, J208 xylanase and crude pectinases are referred to as M35 pectinase, R31 pectinase and J208 pectinase throughout the studies. Primafast®200 (Genencore, Du-Pont) cellulase was suitably diluted and used as crude source of cellulase for saccharification studies and is referred as commercial cellulase throughout the studies.

### 6.2.2. Media for enzyme production under submerged fermentation:

*B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 cultures were separately inoculated into 10 ml of nutrient broth and incubated at 37 °C at 160 rpm till their optical density ($OD_{600nm}$) reached to 0.2 for inoculum. 0.5% of this inoculum was inoculated into 150 ml of BHM-YEP medium containing 3.27 Bushnell Haas Medium (BHM), 0.25 Yeast extract (YE) and 0.75 Peptone (P) (as g/1000 ml) and crude substrate either WB or CP (as 0.5% w/v) in 500 ml Erlenmeyer flask, incubated at 37 °C for 36 h in shaking condition at 160 rpm for production of xylanase or pectinase respectively. Media was sterilized by autoclaving at 10 lbs for 20 min. Grown cultures were then centrifuged at 10,000 rpm for 20 min, and cell free supernatant (CFS) was further used as crude enzymes to characterize xylanase and pectinase activities.

### 6.2.3. Enzyme activity assays:

The physicochemical characterization studies of xylanase and pectinase activities were performed using the crude cell-free supernatant (CFS). The methods commonly used for enzyme activity estimations were followed as mentioned below with several modifications in substrate preparations or incubation conditions mentioned

further when and where they were applied.

**6.2.3.1. Hydrolase activity:**

50 µl of appropriately diluted enzyme source was added to 250 µl of 50 mM Tris-Cl buffered 0.5% w/v substrate. After incubation in water-bath at 40 °C for 10 min, reaction was stopped by addition of 300 µl dinitro salicylic acid (DNS) reagent, and incubated in boiling water-bath for 10 min. Once the system was cooled down to room temperature, volume was made up to 1.5 ml by adding distilled water and absorbance was measured at 540nm (adapted and modified, Miller, 1959; Ghose and Bisaria, 1987).

**6.2.3.1a. Xylanase activity:**

Xylanase assay was performed using 50 mM Tris-Cl pH 8.0 buffered 0.5% w/v xylan as a substrate as mentioned above. The amount of enzyme required to release an end product equivalent to one µmol of D-xylose in reaction mixture per unit time in optimum incubation conditions was considered as one unit of xylanase activity.

**6.2.3.1b. Polygalacturonase (Polygalacturonic acid hydrolase or PGase) activity:**

PGase assay was performed using 50 mM Tris-Cl pH 8.0 buffered 0.5% w/v polygalacturonic acid (PGA) as a substrate as mentioned above. The amount of enzyme required to release an end product equivalent to one µmol of D-galacturonic acid in reaction mixture per unit time in optimum incubation conditions was considered as one unit of polygalacturonase activity.

**6.2.3.1c. Cellulase (Filter paper hydrolase or FPase) activity:**

FPase assay was performed using 50 mM Tris-Cl pH 7.0 buffered 0.5% w/v filter paper as a substrate as mentioned above. The amount of enzyme required to release an end product equivalent to one µmol of D-glucose in reaction mixture per unit time in optimum incubation conditions was considered as one unit of cellulase (FPase) activity.

**6.2.3.2. Lyase activity:**

50 µl of appropriately diluted enzyme source was added to 250 µl of 50 mM Tris-Cl pH 8.0 buffered 0.5% w/v substrate i.e., pectin for pectin lyase and PGA for pectate lyase. After incubation in water-bath at 40 °C for 30 min, enzyme action was terminated by addition of 500 µl 1N NaOH solution followed by incubation at 76 °C for 10 min. To this 600 µl of 1N HCl followed by 500 µl of 0.04 M 2-thiobarbituric acid was added and incubated at 76 °C for 10 min. Developed pink color was estimated

at 550 nm (adapted and modified, Nedjma et al., 2001). The amount of enzyme required to increase the $A_{550nm}$ of the reaction mixture by 0.01 value per unit time in optimum incubation condition was considered as one unit of pectin or pectate lyase.

#### 6.2.3.3. Protein estimation by Bradford method:

CFS was diluted appropriately with distilled water to 1.0 ml and 1.0 ml of Bradford reagent was added. After incubation in dark for 10 min, absorbance of the reaction mixture was measured at 595 nm. Bovine serum albumin (BSA) was used as standard based on which the amount of protein equivalent to BSA was calculated (adapted and modified, Walker, 1996).

### 6.2.4. Substrate specificity for crude xylanases and pectinases:

To study the substrate specificity, CFS from individual culture grown on BHM-YEP media containing either WB or CP was used as crude enzyme source for xylanase and pectinase respectively.

#### 6.2.4.1. Substrate specificity of xylanases:

Two different xylan substrates were used to study the substrate specificity of xylanases. Individual crude xylanase enzyme was incubated with 50 mM Tris-Cl pH 8.0 buffered 0.5% beechwood or birchwood xylan substrate individually. Xylanase assay was performed as mentioned in Section 6.2.3. Activity on the birchwood xylan was considered as 100% and based on that substrate specificity was measured in terms of % relative activity (RA) for other substrates (Li et al., 2006).

$$\%\ Relative\ activity\ on\ xylan = \frac{Activity\ on\ xylan\ substrate}{Activity\ on\ birchwood\ xylan} \times 100 \qquad \text{(Eq. 6.1)}$$

#### 6.2.4.2. Substrate specificity of pectinases:

Four different pectic substrates were used to study the substrate specificity of pectinases. Individual crude pectinase enzyme was incubated with 50 mM Tris-Cl pH 8.0 buffered 0.5% pectic substrate individually. Four pectic substrates having different Degrees of Esterification (DE) were used as: PGA (0% DE), low- methoxylated pectin (35% DE), high-methoxylated pectin (70% DE) and pectin (65-70% DE), further referred as DE0, DE35, DE65 and DE70 respectively. Lyase (pectate or pectin lyase, PL or PNL) and hydrolase (polygalacturonase, PG) assays were performed on all four substrates as mentioned in Section 6.2.3. Hydrolase and lyase activities on PGA (DE0) was considered as 100% and based on that substrate specificity was measured in terms of % relative activity (RA) for other substrates having DE35, DE65 and DE70 in

hydrolase and lyase assays. (Maisuria et al., 2010; Maisuria and Nerurkar, 2012).

$$\%\ Relative\ activity\ on\ pectin = \frac{Activity\ on\ pectic\ substrate}{Activity\ on\ PGA} \times 100 \quad \ldots(\text{Eq. } 6.2)$$

## 6.2.5. SDS-PAGE and activity staining of xylanases and pectinases:

SDS PAGE (adapted and modified, Laemmli, 1970) was performed using a 10% polyacrylamide gel incorporated with substrate for activity staining (zymogram analysis). Protein molecular weight markers (PMWM) (Range: 29-205 kDa: Carbonic anhydrase 29, Ovalbumin, 43, Bovine Serum Albumin 66, Phosphorylase b 97.4 and Myosin from rabbit muscle 205) were run alongside the crude protein samples. After electrophoresis at 80V for 180 min, each gel was cut in two parts. One part of the gel containing protein molecular weight marker was stained with silver salts (Sambrook et al., 2001). The second part of gel containing the substrate was renatured and activity staining was performed. Renaturing steps involved three times washing with 2% Triton X-100 in 50mM Tris-Cl pH 8.0 for 1 h each, followed by incubation of 24 h in 50 mM Tris-Cl pH 8.0 and then zymogram was developed. The activity gel was compared with the silver stained counterpart. Protein bands responsible for activities were measured for their molecular weight according to PMWM using Alpha Ease software V2.0.

### 6.2.5.1. Zymogram of xylanases:

Xylanase zymogram was developed using an acrylamide gel amended with 0.2% birchwood xylan. Renatured and incubated portion of the gel was stained with 0.1% Congo-red prepared in DW for 2-3 min and then washed with saturated NaCl solution till the destaining ceased. 1 N HCl was added to further develop the colour contrast and blue activity band was in purple background was observed for xylanase activity (Teather and Wood, 1982; Ruijssenaars and Hartmans, 2001).

### 6.2.5.2. Zymogram of pectinases:

Pectinase zymogram was developed using an acrylamide gel amended with 0.1% pectin or PGA. Renatured and incubated portion of the gel was stained with 0.1% toluidine blue-O in 50 mM Tris-Cl pH 8.0 for 2-3 min and then washed with deionized distilled water till the destaining ceased (Maisuria and Nerurkar, 2012). A colourless band was observed in purple-blue backgrounds for both pectin and PGA substrates.

## 6.2.6. Kinetic studies for xylan hydrolysis by xylanases:

Enzyme kinetic studies for crude xylanases from all three isolates, were performed based on the Michaelis-Menten model equation. The kinetic parameters $K_m$

and $V_{max}$ were determined by measuring the enzyme activity of xylanase as mentioned in Section 6.2.3.1., except the substrate concentration was taken in range from 0.05 to 3.0% w/v (or 5 to 30 mg/ml) instead of 0.5%, with a fixed amount of CFS enzyme. The nonlinear form of Michaelis-Menten equation was transformed to several linearized forms such as Lineweaver-Burk plot, Edie-Scatchard, Hanes Wolf & Edie Hofstee plots. The $K_m$ and $V_{max}$ values were calculated from the slope and intercept on Y axis based on the above linearized forms and further catalytic efficiency from the ratio of $V_{max}/K_m$ was calculated.

### 6.2.7. Effect of temperature and pH on activities of xylanases:

Simultaneous effect of various pH at a broad range of temperature on xylanase, activities were assayed as described in Section 6.2.3.1 Crude CFS obtained as mentioned in Section 6.2.2 was appropriately diluted and used as crude xylanase source. 0.5% w/v Xylan was prepared in 50 mM buffers of pH 4.0, 5.0 and 6.0 (Citrate-Phosphate buffer); pH 7.0, 8.0 and 9.0 (Tris-Cl buffer); pH 10.0 and 11.0 (Carbonate-Bicarbonate buffer). The buffered substrates were incubated with individual xylanase enzyme up to 10 min at different temperatures of 30, 40, 50, 60, 70 and 80°C each. Further xylanase activities were measured as mentioned in Section 6.2.3.1. A matrix of individual enzyme activities at various temperature and pH was generated in Office 2013 software, highest activity for each enzyme was considered as 100% and based on that relative activities at other incubation conditions for that enzyme were calculated. Contour plot for relative units for each enzyme, with respect to pH and temperature conditions, was prepared using Akima's polynomial method for interpolation with MINITAB17 software.

- ***Calculations of activation energy ($E_a$) and Temperature quotient ($Q_{10}$):***

Based on the effect of temperature and pH on the enzyme activities, optimum incubation conditions were identified and activation energy ($E_a$) and temperature quotient ($Q_{10}$) were calculated for the optimum and suboptimum conditions using Arrhenius plot as mentioned by Riaz et al. (2007).

Temperature is one of the important parameters which majorly influences the rate of any chemical or biological reactions and Arrhenius equation is the formula which relates the reaction rate constant with the temperature.

$$k = A.e^{\frac{-Ea}{RT}} \quad \text{(Eq. 6.3)}$$

Where, $k$ is the rate constant, T is the absolute temperature (in Kelvin), A is the pre-exponential factor, a constant for each chemical reaction that defines the rate due to frequency of collisions in the correct orientation, $E_a$ is the activation energy for the reaction, $R$ is the universal gas constant (8.314 $JK^{-1}mol^{-1}$).

Solving the equation,

$$\ln k = \ln A - \frac{Ea}{RT} \times \ln e \quad \text{(Eq. 6.4)}$$

Since, $\ln e = 1$, rearrangement of the Arrhenius equation gives a homologue to first order straight line equation, $y = mx + c$

$$\ln k = \left[\frac{-Ea}{R} \times \frac{1}{T}\right] + \ln A \quad \text{(Eq. 6.5)}$$

Where, $y = \ln k, x = 1/T, slope = m = (-Ea/R)$ and $c = intercept\ on\ y = \ln A$. For any enzyme reaction, rate constant for product formation is dependent on concentration of product formed and available substrates for reaction.

$$k = [P]/[S] \quad \text{(Eq. 6.6)}$$

Enzyme units are defined as dependent on concentration of products released or substrates consumed, so enzyme units can be used to calculate reaction rate, and activation energy ($E_a$) can be calculated from the linear plot of ln k or ln (Enzyme activity) versus 1000/T as follow.

$$Ea = -slope \times R \quad \text{(Eq. 6.7)}$$

Increase in the temperature can change the rate of a biological or chemical reaction. Temperate quotient ($Q_{10}$), is a measure of the ratio of the rate as a consequence of increasing temperature by 10 °C. If $k_{T1}$ is the rate constant at $T_1$ and after increase of 10 °C, $k_{T2}$ is the rate constant at $T_2$, Eq. 6.4 can be rewritten as follow,

$$\ln k_{T_1} = \left[\frac{-Ea}{R} * \frac{1}{T_1}\right] + \ln A \quad \text{(Eq. 6.8)}$$

$$\ln k_{T_2} = \left[\frac{-Ea}{R} * \frac{1}{T_2}\right] + \ln A \quad \text{(Eq. 6.9)}$$

Subtracting Eq. 6.9 from Eq. 6.8,

$$\ln k_{T_1} - \ln k_{T_2} = \left[-\frac{Ea}{R \times T_1} + \ln A\right] - \left[-\frac{Ea}{R \times T_2} + \ln A\right] \quad \text{(Eq. 6.10)}$$

Solving the above equation,

$$Q_{10} = \ln \frac{k_{T_1}}{k_{T_2}} = \frac{-Ea}{R} \times \left[\frac{T_1 - T_2}{T_1 T_2}\right] \quad \text{(Eq. 6.11)}$$

$Q_{10}$ is the ratio of two reaction rate constant at difference of 10 °C, which was calculated from Eq. 6.11.

### 6.2.8. Effect of temperature on stability of xylanases:

Stability of crude xylanase and pectinase enzymes at 40-70 °C temperatures were determined by preincubating them at these temperatures for specific time followed by performing the xylanase assay as mentioned in Section 6.2.3.1. CFS from individual isolate was used as crude source of xylanase and pectinase enzymes. CFS for each culture was preincubated at 50 mM pH 8.0 buffer at 40, 50, 60 and 70 ºC up to 60, 30, 15 and 10 min respectively in case of xylanase. Xylanase assays were performed using 50 µl enzyme aliquots withdrawn at different time intervals during preincubation. Enzyme at t=0 min incubation was considered as a control.

- ***Estimation of deactivation rate constant and $t_{1/2}$:***

Xylanase controls were considered as 100% and based on that relative residual activity for other incubation conditions were calculated as mentioned below.

$$\%\, residual\ activity = \frac{E_t}{E_0} \times 100 \qquad \text{(Eq. 6.12)}$$

Where, $E_t$= enzyme activity at incubation of time t, $E_0$ = enzyme activity at 0 min.

Enzyme deactivation is one of the major limitations in their industrial applications hence understanding the deactivation of enzymes becomes necessary. The deactivation of xylanase enzyme is assumed to follow the first order kinetics, which is also known as single step two-stage theory(Naidu and Panda, 2003). The two-state mechanism is as follows:

$$E \rightarrow E_d \qquad \text{(Eq. 6.13)}$$

The assumption in the mechanism is that the active enzyme state *E* directly converts to inactive state $E_d$ without providing any significant number of intermediates. So, the deactivation rate of these enzymes can be calculated by first order expression as,

$$\frac{dE}{dt} = -K_d E \qquad \text{(Eq. 6.14)}$$

Integration of the above equation leads to,

$$\frac{E_t}{E} = exp^{(-K_d t)} \qquad \text{(Eq. 6.15)}$$

Solving the above equation, gives a homologue to first order straight line equation, $y = mx + c$

$$\ln \frac{E_t}{E_0} = -K_d t \qquad \text{(Eq. 6.16)}$$

Where, $K_d$ = deactivation rate constant, $E_t$= enzyme activity at incubation of time t, $E_0$ = enzyme activity at 0 min. The $K_d$ (deactivation rate constant or first order rate constant) values were calculated from a plot of $\ln(E_t/E_0)$ versus time (t) at a particular

temperature. Half-life of an enzyme is defined as the time required by the enzyme to reduce half of its initial activity, and once the value of $K_d$is calculated, the half-life of an enzyme can be calculated as

$$T_{\frac{1}{2}} = \ln\frac{2}{K_d} \quad \text{(Eq. 6.17)}$$

### 6.2.9. Effect of pH on stability of xylanases:

Stability of crude xylanase enzymes at pH 4.0-10.0 were determined by preincubating them at these pH conditions for specific time followed by performing the xylanase assay as mentioned in 6.2.3.1. CFS from individual isolate was used as crude source of xylanase enzymes. CFS for each culture was preincubated at 40 ºC with 50 mM buffer of pH 4.0, 5.0 and 6.0 (Citrate phosphate buffer); pH 7.0, 8.0 and 9.0 (Tris-Cl buffer); pH10.0 and 11.0 (Carbonate-bicarbonate buffer) for 24 h. Xylanase assays were performed using 50 µl enzyme aliquots withdrawn at time interval of every 6 h during preincubation. Activity of a freshly buffered enzyme for each pH value at t=0 was used as a control and considered as 100% and based on that the relative residual activity for other incubation conditions were calculated as mentioned below.

$$\%\, residual\ activity = \frac{E_t}{E_0} \times 100 \quad \text{(Eq. 6.18)}$$

Where, $E_t$ and $E_0$ are residual enzyme activities at preincubation of t and 0 min.

### 6.2.10. Mode of action of xylanases:

Determination of the mode of action for these xylanases for degradation of polysaccharides was done by viscosity and endproducts analysis.

#### 6.2.10.1. Relative flow rate and viscosity analysis:

To study the mode of action of an enzyme, i.e. whether it is of an endo- or exo-acting nature, change in relative flow rate (or relative viscosity) of polymer substrate along with accumulation of released end-product were analyzed simultaneously at various time periods during incubation. The enzyme reaction was performed with 20 ml of 50 mM Tris-Cl buffer (pH 8.0) containing 3.0% beechwood xylan with suitably diluted enzyme individually in Ostwald capillary viscometer for flow rate (viscosity) measurement and in test tube for end-product estimations. Assay mixtures were incubated at 40 °C up to 30 min and accumulated end-products were assayed using the method as mentioned in Section 6.2.3.1 for xylanase. Flow time of only solvent (50 mM Tris-Cl pH 8.0 buffer, without any substrate) was taken as control.

Relative flow time of enzyme-substrate system was calculated as,

$$\text{Relative flow time or relative viscosity} = \frac{T_t}{T_s} \quad \text{(Eq. 6.19)}$$

Rate of viscosity decrease was calculated as,

$$\text{Rate of viscosity decrease } (r_{vd}) = \frac{T_{t1} - T_{t2}}{t_2 - t_1} \quad \text{(Eq. 6.20)}$$

Rate of increase in end-product ($r_{epa}$) was calculated as, flowing formula,

$$\text{Rate of end-product increase } (r_{epa}) = \frac{A_{t2} - A_{t1}}{t_2 - t_1} \quad \text{(Eq. 6.21)}$$

Where, $T_t$ = flow time at t = t, $t_1$ or $t_2$ min for enzyme-substrate reaction system; $T_s$ = flowtime for solvent system; $A_t$ = absorbance ($A_{540}$ for xylanase) due to released end-products at t = t, $t_1$ or $t_2$ min. One unit of endo-activity was defined as an amount of the enzyme required to achieve ~30-50% decrease in relative viscosity of pectin by substrates after a definite time under optimum incubation conditions (Angelova, 2008).

**6.2.10.2. End-product analysis through chromatography:**

To analyze the end products, individual system was prepared for each xylanase using 0.5% xylan substrates prepared in 50 mM Tris-Cl, pH 8.0 buffer. Reaction system was allowed to incubate for various time periods. 20 µl of the sample was loaded at several incubation periods from 5 min up to 120 min for Thin Layer Chromatography and from 5 min up to 10 h for High Performance Liquid Chromatography. TLC and HPLC analyses was carried out as mentioned below.

- ***End-product analysis by TLC:***

5 mM D-xylose (monomer control of for xylanase activity) along with 20 µl of aliquots withdrawn from samples were individually loaded on TLC plates coated with Silica Gel 60G and fluorescent indicator F254. Water-butanol-methanol in 1:4:5 ratio was used as mobile phase. Carbohydrates spots were detected on the chromatogram by spraying with alcoholic p-Anisidine phthalate solution (1.23 g p-Anisidine and 1.66 g phthalic acid in 100ml 95% ethanol) and thereafter gently heating the plate for 15-20 mins at 60 °C (Randerath, 1963).

- ***End-product analysis by HPLC:***

20 µl aliquot withdrawn from samples was loaded in Hi-Plex-H column specific for carbohydrates and alcohols on Shimadzu HPLC system equipped with LC-10AT pump, CTO-10ASVP oven column cabinet, and detected using RID-10A detector. Individually, D-xylose monomers (control for xylanase), buffered substrates without

enzyme treatment and buffered enzyme CFS were also loaded as control. The stationary phase in Agilent Hi-Plex-H column has strong cation-exchange resin consisting of sulfonated, crosslinked styrene-divinylbenzene copolymer in hydrogen form with diameter of 7 to 11 µm and deionized water was used as mobile phase for separation of hydrolysate products under isocratic conditions as recommended by Agilent. Chromatographs for individual sample were compared with the respective controls with respect to retention time and peak area.

### 6.2.11. Effect of various chemical modulators on xylanase activities:

Effects of modulators of various groups like monovalent, divalent and trivalent cations, cationic chelators, oxidizing and reducing agents, detergents were studied by incubating enzymes with the system containing 0.5% xylan prepared in the individual solution containing 1 mM concentration of the most of the chemical modulators except a few which were used in % w/v or % v/v as mentioned in Table 6.1. Xylanase assay was performed as mentioned in Section 6.2.3.1. Assay system without any modulator was used as control and activity on native substrates was considered as 100% and based on that relative activity for modulator supplemented systems were calculated.

$$\%\, relative\ activity = \frac{E_M}{E_0} \times 100 \qquad \text{(Eq. 6.22)}$$

Where, $E_M$= enzyme activity in presence of modulator, $E_0$ = enzyme activity in absence of modulator.

**Table 6.1. List of modulators studied for their effects on xylanase activities:**

| Category | Modulator |
|---|---|
| Monovalent cations | LiCl, NaCl, KCl, $NH_4Cl$ and $NaNO_3$ (1mM each) |
| | NaCl (1%, 2% and 3% w/v) |
| Divalent and Trivalent cations | $CaCl_2.2H_2O$, $CdCl_2$, $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, $HgCl_2$, $MnSO_4.H_2O$, $MgSO_4.7H_2O$, $NiCl_2.6H_2O$, $Pb(NO_3)_2$ and $FeCl_3$ (1mM each) |
| Cation metal complex | $K_2Cr_2O_7$, $K_3[Fe(CN)_6]$, $Na_2B_4O_7$ and $Na_2MoO_4.2H_2O$ (1mM each) |
| Cation chelators | EDTA and EGTA (1mM each) |
| Reducing agents | Sodium azide ($Na\text{-}N_3$), Dithiothreitol (DTT), 2-Mercaptoethanol (2-MCEt-OH) (1 mM each) |
| | 2-Mercaptoethanol (2-MCEt-OH) (1% v/v) |
| Detergents | Tween 20, Tween 80, Triton X-100 and Sodium Dodecyl Sulphate (SDS) (1% v/v or 1% w/v, each) |
| | SDS (1mM) |

### 6.2.12. Data Analysis:

- All the quantitative estimation experiments were performed in triplicates (n=3) and data and error values are represented as Mean ± Standard Error of Mean (SEM)

either in GraphPad Prism 6.0 or Origin 8.0 software.

- Statistical analysis was carried out using Two-way ANOVA method in GraphPad Prism 6.0.

## 6.3. Results and Discussion:

*B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 produced cellulase free xylanases and pectinases that exhibited positive synergism with cellulase. Biotechnological applications of such industrially important enzymes depend on several physicochemical parameters (Basu et al., 2008). The characterization helps in improving the effective usage of enzymes as well as in maintaining desired level of enzyme activity over a longer period of time and improve its stability during their industrial applications (Tari et al., 2008).

### 6.3.1. Analysis of enzyme activities in crude CFS:

Table 6.2 represents the measured protein concentrations as well as cellulase, xylanase and pectinase activities of WB or CP induced crude enzymes on different substrates as mentioned in Section 6.2.3. CP induced enzyme from the three *Bacillus* M35, R31 and J208 exhibited highest pectinase activities with negligible xylanase activities. This included polygalacturonate hydrolase activities as well as pectin and pectate lyase activities. Thus, the saccharification activity studied earlier on raw agrowastes as well as pretreated SCB biomass were contributed by PGase, PNL and PL all three enzymes wherever the crude pectinase from the three *Bacillus* cultures was used. WB induced enzymes from the same isolates exhibited highest xylan hydrolase activity, moderate pectin and pectate lyase activities were observed from them, which were several folds lower than the CP induced enzyme. WB induced enzyme possessed xylan hydrolase and polygalacturonase activities in comparatively equal amounts. Remarkably both crude xylanases and pectinases from individual cultures exhibited no cellulolytic activity on filter paper. However, on the other hand in addition to 5.0 units of cellulolytic activity, commercial cellulase Primafast®200 possessed 4.8 units of xylanase activity equivalent to the xylanolytic enzymes of the *Bacillus* isolates. It also possessed 4.6 units of PGase activity and negligible PL and PNL activities. Hu et al., (2011) reported 438 units of xylanase activities and 475 units of CMCase activity from a commercial preparation of Celluclast 1.5L. While Commercial Multifect ® CL and Accellerase®1500 were reported with 313.17 and 97.0 units of xylanase activities with 208.32 and 178.37units of endoglucanase activities (Maitan-Alfenas et al., 2015b).

**Table 6.2. Enzyme activities of commercial cellulase, crude xylanase and pectinase enzymes from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208:**

| | Enzyme and Preparation | Protein (µg/ml) | FPase (FPU/ml) | Xylanase (U/ml) | PGase (U/ml) | PNL (U/ml) | PL (U/ml) |
|---|---|---|---|---|---|---|---|
| Pectinase (CP) | *B. safensis* M35 | 52.1 ± 2.7 | **0.0** | 0.5 ± 0.1 | **11.3 ± 0.7** | **9461.3 ± 106.2** | **10425.6 ± 160.4** |
| | *B. altitudinis* R31 | 67.4 ± 5.6 | **0.0** | 0.3 ± 0.0 | **14.6 ± 0.5** | **9625.0 ± 399.2** | **12996.8 ± 98.3** |
| | *B. altitudinis* J208 | 65.5 ± 3.9 | **0.0** | 0.7 ± 0.1 | **14.8 ± 0.2** | **14077.3 ± 124.1** | **14221.2 ± 84.5** |
| Xylanase (WB) | *B. safensis* M35 | 66.3 ± 5.2 | **0.0** | **4.8 ± 0.0** | 4.8 ± 0.1 | 578.9 ± 35.2 | 701.8 ± 54.9 |
| | *B. altitudinis* R31 | 36.7 ± 3.3 | **0.0** | **5.0 ± 0.1** | 4.1 ± 0.0 | 1872.5 ± 120.3 | 2677.3 ± 178.32 |
| | *B. altitudinis* J208 | 39.4 ± 2.2 | **0.0** | **5.5 ± 0.1** | 4.9 ± 0.1 | 2261.3 ± 280.5 | 2848.0 ± 90.2 |
| | Primafast®200 | 529.2 ± 14.1 | **5.0 ± 0.2** | 4.8 ± 0.2 | 4.6 ± 0.1 | 122.6 ± 19.2 | 170.1 ± 12.2 |

Values presented are Mean ± Standard Errors of the Mean (SEM), for n=3.

## 6.3.2. Substrate specificity studies:

Since, the xylanases and pectinases were suitable as accessory enzymes with cellulase in cocktail mediated plant biomass saccharification process, it was necessary to study their substrate specificity i.e., their ability to act on plant cell wall polymers like xylan and pectin.

### 6.3.2.1. Activity of crude xylanases on xylan:

Xylan from beechwood and birchwood were used for the substrate specificity studies. As these substrates are prepared from two different plant sources, their general composition varies as depicted in Table 6.3. Presence of more glucuronic acid in beechwood xylan attributes to higher amount of glucurono-xylan heteropolymer which makes it more complex than birchwood xylan. Hence, for substrate specificity studies, xylanase activity on birchwood xylan was defined as 100% and relative xylanase activities on beechwood xylan were calculated. Results for substrate specificities of xylanase enzymes from three isolates M35, R31 and J208 are depicted as Figure 6.1.

**Table 6.3. General composition of birchwood and beechwood xylan (Source: Megazyme):**

| Properties | Constituents in % (w/w) | | | | | |
|---|---|---|---|---|---|---|
| | Xylose | Glucuronic acid | Other sugars | Protein | Ash | Moisture |
| Birchwood xylan | 85.6 | 8.7 | 5.7 | 0.1 | 7.6 | 5.1 |
| Beechwood xylan | 81.3 | 13.0 | 5.7 | 0.2 | 4.7 | 4.1 |

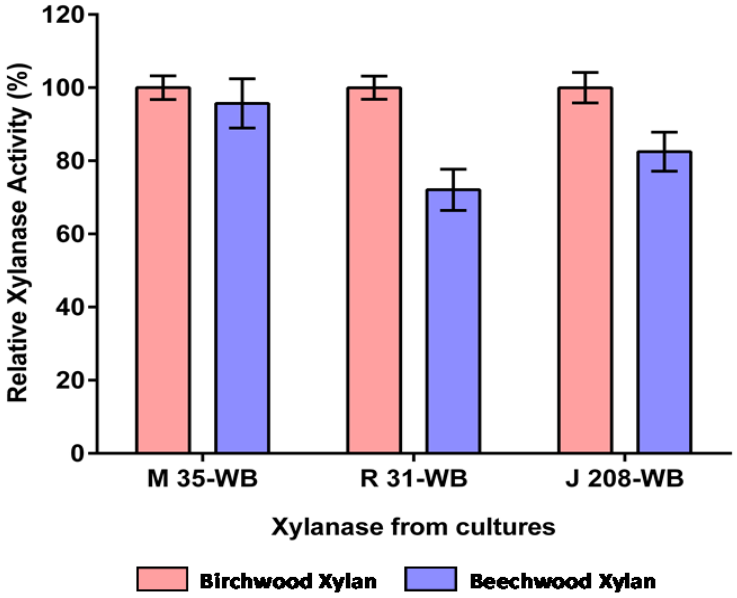


**Figure 6.1. Activity of crude xylanases from three *Bacillus* isolates on xylan:**
Xylanase activities from WB induced crude xylanases; Errors represented as Standard Errors of the Mean (SEM), for n=3.

M35 xylanase demonstrated ~95% activity whereas R31 and J208 xylanases respectively demonstrated 72% and 82% activity on beechwood xylan relative to 100% activity on birchwood xylan. Structural complexities for xylan substrates has been observed in an increased order for oat-spelt xylan, birchwood xylan and beechwood xylan (Gray et al., 2007; Shi et al., 2013). Studies regarding the xylanase from *Paecilomyces themophila* supports the results observed here with ~20% decrease in xylanase activity on beechwood xylan when compared to birchwood xylan (Li et al., 2006). Shi et al., (2013) reported 98.4% and 87% conversion of xylan polymers respectively from birchwood and beechwood to mono and oligomers by xylanase from *Thermotoga thermarum* within 5 h of incubation. Thus, as the complexities of xylan substrates increase, the specificities of xylanases tend to decrease. In our studies, with increase in the complexities of xylan substrate also the xylanase exhibited only 5% to 28% decrease in activities on complex substrate. This suggests that these xylanases possess the ability to work on diverse β-1,4-linked backbone complex xylan substrates.

### 6.3.2.2. Activity of crude pectinases on pectic substrates:

Pectic polymers are linear homogalacturonan (HG) consisting a backbone of α (1→4) linked D-galacturonic acid residues, which after esterification is converted to methyl esters and free acids. The ratio of esterified galacturonic acid groups to total galacturonic acid groups is termed the degree of methyl esterification (DE). Pectin is

made up of HG with high DE whereas pectic acid or polygalacturonic acid is HG with no esterification. Four pectic substrates with diverse values of DE, i.e., DE0, DE35, DE65, DE70 were used to study the substrate specificity of crude pectinases from all three *Bacillus* cultures based on hydrolase and lyase assays. Since, pectic polysaccharides with higher DE values are structurally more complex, and PGA (DE0) being the simple polymer the pectinase activity on PGA (DE0) was defined as 100% and relative pectinase activities on various pectic substrates with DE35, DE65, DE70 were calculated.

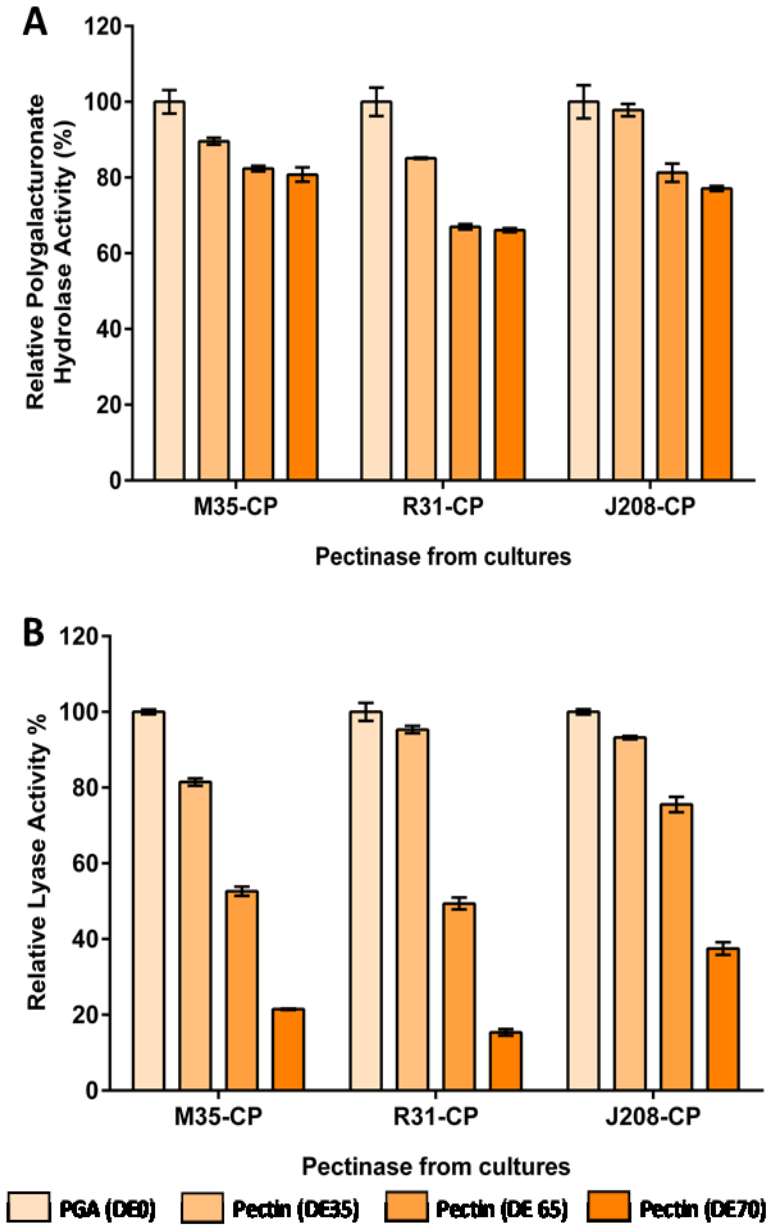


**Figure 6.2. Activity of crude pectinases from three *Bacillus* isolates on pectin:**
**(A)** Polygalacturonate hydrolase activities and **(B)** Pectin and pectate lyase activities from CP induced crude pectinase on different pectic substrates; Errors represented as Standard Errors of the Mean (SEM), for n=3.

Hydrolase activity on DE35 pectin and DE65-DE70 pectin were found to be in range of 85-97% and 66-80% respectively by three pectinases as shown in Figure 3.2(A) against 100% activity on PGA (DE0). Likewise, lyase activity on DE35 pectin and DE65-DE70 pectin were found to be in range of 81-93% and 15-38% respectively by three pectinases as shown in Figure 3.2(B) against 100% activity on PGA (DE0). When both activities of DE65 and DE70 were analyzed, the hydrolase activity didn't have much difference in both the substrates but lyase activity is affected and decreased with increase in DE. In case of *Sclerotinia sclerotiorum,* more specificity of pectinase towards least esterified substrate was exhibited by highest activity on nonmethylated Na-polygalacturonte, 40% activity on moderately esterified citrus pectin and 0% activity on highly esterified apple pectin (Riou et al., 1992). Similarly, pectinase from *Bacillus subtilis* Btk-27 has been exhibited only 34% substrate specificity towards apple pectin, against the100% towards citrus pectin (Oumer and Abate, 2017). Such substrate specificities have also been supported by reports in our laboratory in case of *Erwinia carotovora* subsp. *carotovora* (*Ecc)* BR1 toward moderate and high methyl esterified pectin for hydrolase activity (Maisuria et al., 2010) and for lyase activity (Maisuria and Nerurkar, 2012) respectively.

These reports support our observations that increase in DE gradually decreased the amenability of pectin to the enzyme reaction and yet three pectinases were able to retain 15-38% of the activity on DE70 pectin substrate. This suggests that although these three pectinases had more specificities towards least esterified pectic substrates, they possessed ability to work on diversely esterified complex pectic polymers also. Such pectinases can be of particular interest because they break pectin polymers causing increase in structural porosity and also open up the pockets to expose cellulose for cellulase activity (Yadav et al., 2009).

Thus, substrate specificity studies of xylanase and pectinase enzymes from the three *Bacillus* cultures revealed their ability to act upon simple to complex structural polysaccharide substrates which are present in plant cell wall and hence can be useful for their application in plant biomass saccharification.

### 6.3.3. SDS-PAGE-Zymogram analysis of crude xylanases and pectinases:

Since all of the reported xylanase enzymes from *Bacillus* spp. are of hydrolase type, the complex xylan substrate, i.e., beechwood xylan was used for zymographic studies of xylanases whereas, hydrolase, lyase and esterase activities were reported by

pectinases, two different pectic substrates viz., PGA (DE0) and pectin (DE70) were used for the pectinase zymography studies.

### 6.3.3.1. Zymogram analysis of xylanases:

Activity staining for xylanase from three isolates on beechwood xylan polymer substrate developed a thin blue band in the pale purple background indicating the breakdown of xylan (denoted by red arrow in activity stained gel) as shown in Figure 3.3 (A, B and C). This band was compared with the silver stained counter-part gel and corresponding silver stained protein band (denoted by red arrow in silver stained gel) responsible for the activity on zymogram was identified. A single band from each culture was found responsible for activity on xylan. The approximate molecular weight for this band was determined as ~100kDa for *B. safensis* M35 (Figure 6.3A) and *B. altitudinis* R31(Figure 6.3B) as well as *B. altitudinis* J208 (Figure 6.3C) using PMWMs and software Alphaease (2.0) Alpha imager. Adhyaru et al., (2017) has reported xylanase of ~99kDa from *B. altitudinis* DHN8. There are several diverse reports on xylanase sizes from diverse organism. Kaur et al., (2014) reported two different xylanases from *B. pumilus* AJK with size of ~24.5 and ~13kDa. Chi et al., (2012) reported *Bacillus* sp. MX47 to produce xylanase of ~26.4kDa.

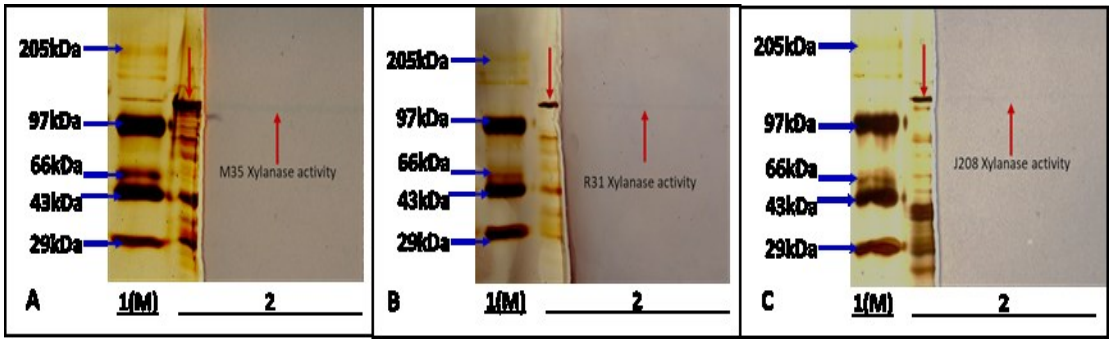


**Figure 6.3. SDS-PAGE and zymogram analysis for crude xylanase enzymes:**
SDS-PAGE and Zymogram analysis on birchwood xylan: Samples loaded: Lane 1(M): Protein molecular weight marker, and 2: CFS of WB induced crude xylanase from **(A)** For *B. safensis* M35 **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208.

When NCBI database of shotgun sequencing genome assemblies for two type strains *B. safensis* Fo36b$^T$ (ASJD01000001.1-ASJD01000038.1) and *B. altitudinis* 41Kf2b$^T$ (ASJC01000001.1-ASJC01000039.1) were analyzed for different xylanases, annotations revealed presence of genes for endo-1,4-β-xylanase which cleaves xylan backbone randomly, β-1,4-xylanase which cleaves terminal xylosidic bond and β-xylosidase which cleaves the xylobiose in xylose. All three of them belonged to the group xylan hydrolases suggesting that the xylanase activity represented here in

zymogram was by mostly either endo-or exo-xylan hydrolases.

### 6.3.3.2. Zymogram analysis of pectinases:

Activity staining for pectinases from three isolates was performed using two different pectic polymer substrates PGA (DE0) and pectin (DE70). Unstained bands in toluidine blue-O stained blue coloured pectic background indicated the breakdown of pectic polysaccharides. Zymogram on PGA and pectin as shown in Figure 3.3(A) and 3.3(B) respectively were compared with their silver stained counter-part gel and corresponding silver stained protein band responsible for the activity on zymogram was determined for *B. safensis* M35, *B. altitudinis* R31 and J208. A single protein band from each culture supernatant was found responsible for activity on both PGA and pectin substrates. The approximate molecular weight for this band was determined as ~32kDa for *B. safensis* M35 and ~34kDa for *B. altitudinis* R31 and J208 using PMWMs and software Alphaease (2.0) Alpha imager. Zhou et al., (2017) has reported pectate lyase with molecular weight of 43.1kDa from the *B. subtilis* PB1 and cited several other bacterial pectin lyases with their molecular weight as follows: *Bacillus pumilus* (37kDa) (Klug-Santner et al., 2006), *Bacillus clausii* (35kDa) (Li et al., 2012), *Bacillus subtilis* (45kDa) (Liu et al., 2012) and several others. Kaur et al., (2014) had reported polygalacturonase hydrolase (of ~40kDa) from *B. pumilus* AJK.

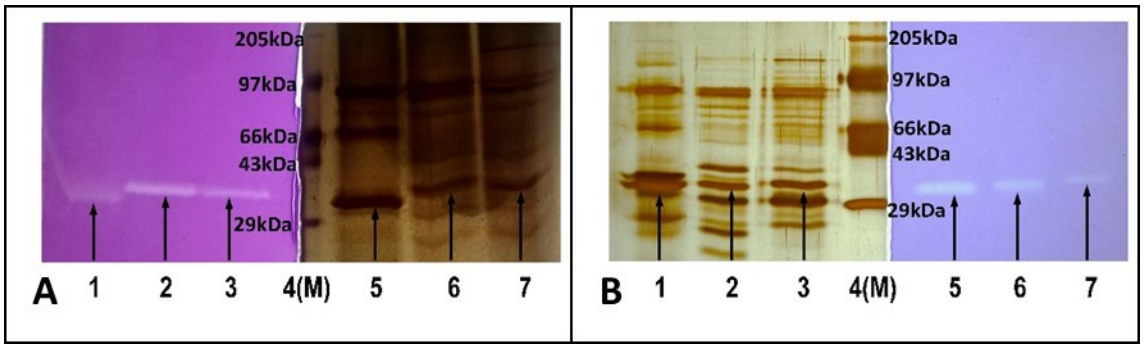


**Figure 6.4. SDS-PAGE and zymogram analysis for crude pectinase enzymes:**
SDS-PAGE and Zymogram analysis on **(A)** PGA (DE0) and **(B)** pectin (DE70) incorporated 10% acrylamide gel for crude pectinase enzymes from three *Bacillus* isolates; Samples loaded: Lane 1 and 5: *B. safensis* M35 pectinase, Lane 2 and 6: *B. altitudinis* R31 pectinase, Lane 3 and 7: *B. altitudinis* J208 pectinase, Lane 4 (M): Protein molecular weight marker**.**

When NCBI database of shotgun sequencing genome assemblies for two type strains *B. safensis* Fo36b$^T$ and *B. altitudinis* 41Kf2b$^T$ were analyzed for different pectinases, annotations revealed presence of genes for endo-polygalacturonase (Endo-PGase) which acts on simple PGA (DE0) but annotations for endo- or exo-methyl galacturonase which works on highly esterified pectin substrates (DE35, DE65 or

DE70) were absent in database. The annotations for pectin methyl esterase and pectate lyase were present in both organisms. Usually, pectin is either hydrolysed by polymethyl galacturonase or pectin lyase or first de-esterified by pectin methyl esterase to pectin and then further acted upon by polygalacturonase or pectate lyase. Hence, PME de-esterifies methyl esterified pectic backbone and releases pectic acid which can act as substrate for PGase, PL or PNL enzymes. While, polygalacturonase alone can not act on pectin, pectin lyase is an important enzyme that can directly act on pectin (Jayani et al., 2005). The zymogram and annotation analyses results prompted that, these *Bacillus* strains M35, R31 and J208 may possess predominantly lyase instead of hydrolase. Notably, PGase activity was extremely low as compared to the pectin lyase activity (Table 6.2). Thus, further characterisation of the PGase activity was not undertaken. However, studies on the physicochemical characterization of M35, R31 and J208 pectin lyase activity in crude pectinase, has been published (Thite and Nerurkar, 2018).

### 6.3.4. Enzyme kinetic studies for xylanases:

Determination of kinetic parameters like $K_m$, $V_{max}$ and *catalytic efficiency* during enzyme kinetic studies compared the affinity between diverse enzymes and their substrates. The constant $K_m$ relates the velocity of an enzyme catalysed reaction to the substrate concentration. $K_m$ being constant value for enzyme-substrate combination, it provides a base for comparative studies of relative suitability of alternate substrates to a particular enzyme from diverse organisms. The lowest $K_m$ value indicates the highest apparent affinity of enzyme for the substrate. $V_{max}$ is the maximum velocity achieved by the enzyme-substrate reaction system. To find out the efficient reaction system, the ratio of $V_{max}/K_m$ known as *catalytic efficiency* was calculated. Highest value of the $V_{max}/K_m$ indicates the maximum velocity achieved by least amount of substrate (Segel, 1976; Price and Stevens, 1999). Based on these, the xylan hydrolases from three isolates *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 were studied for their $K_m$, $V_{max}$ and *catalytic efficiency* values which are presented as mean and standard error of mean calculated from four linear transformations of nonlinear form of Michaelis-Menten equation. and $K_m$, $V_{max}$ and *catalytic efficiency* values for xylanases from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 are listed below in Table 6.4. which suggests that xylanases from the three *Bacillus* spp. exhibited more affinity towards birchwood xylan than beechwood xylan.

**Table 6.4. Kinetic parameters of xylanase enzymes from *Bacillus* isolates on Birchwood and Beechwood xylan:**

| Enzyme and Sources | | Substrates | Kinetic Parameters | | |
|---|---|---|---|---|---|
| | | | $K_m$ (mg/ml) | $V_{max}$ (units) | Catalytic Efficiency |
| Xylanase | *B. safensis* M35 | Birchwood xylan | 4.48 ± 0.28 | 27.65 ± 0.13 | 6.23 ± 0.33 |
| | | Beechwood xylan | 5.15 ± 0.13 | 24.93 ± 0.33 | 4.85 ± 0.10 |
| | *B. altitudinis* R31 | Birchwood xylan | 4.40 ± 0.15 | 22.32 ± 0.27 | 5.07 ± 0.20 |
| | | Beechwood xylan | 6.02 ± 0.23 | 20.30 ± 0.58 | 3.37 ± 0.09 |
| | *B. altitudinis* J208 | Birchwood xylan | 5.76 ± 0.16 | 22.96 ± 0.33 | 3.98 ± 0.10 |
| | | Beechwood xylan | 5.96 ± 0.26 | 22.57 ± 0.71 | 3.80 ± 0.12 |

Values represented are Mean ± Standard Errors of the Mean (SEM), for n=3

M35 xylanase showed least $K_m$ value (5.15 mg/ml) for beechwood xylan while R31 xylanase showed least $K_m$ value (4.40 mg/ml) for birchwood xylan suggesting that M35 xylanase has more affinity for simple xylan substrates while R31 xylanase has more affinity towards complex xylan substrates. $V_{max}$ value observed for M35 xylanase was the highest on both birchwood (27.65 Units) and beechwood (24.93 units) xylan substrate as compared to R31 and J208 xylanases. All three xylanases had higher $V_{max}$ values on birchwood xylan (i.e., 27.65, 22.32 and 22.96 units) compared to beechwood xylan (i.e., 24.93, 20.30 and 22.57 units). The *catalytic efficiency* for all three xylanases was observed to be higher on the birchwood xylan than beechwood xylan, but again these values for J208 xylanase were similar to each-other for both the substrates.

In literature too, varied values of $K_m$ and $V_{max}$ has been reported for different microbial xylanases on diverse substrates. Purified endo-β-1-4-xylanase from *Bacillus halodurans* S7 has been reported for increase in affinity orderly for oat spelt xylan ($K_m$: 4.37 mg/ml, $V_{max}$: 319 units), birchwood xylan ($K_m$: 4.53 mg/ml, $V_{max}$: 230 units) and beechwood xylan ($K_m$: 5.42 mg/ml, $V_{max}$: 252 units) by Mamo et al., (2006), while Lu et al., (2008) has reported a purified xylanase from *Aspergillus ficuum* AF-98 having more affinity for beechwood xylan ($K_m$: 3.267 mg/ml, $V_{max}$: 18.38 units) than birchwood xylan ($K_m$: 3.747 mg/ml, $V_{max}$: 11.1 units). Similarly, crude immobilized xylanase from *Streptomyces olivaceociridis* E-86 was reported for more affinity for beechwood xylan ($K_m$: 1.42 mg/ml, $V_{max}$: 67.6 units) than birchwood xylan ($K_m$: 1.54 mg/ml, $V_{max}$: 90.3 units) by Ai et al., (2005). These and several other reports as referred by Yang et al., (2014) suggesting wide ranges for $K_m$ and $V_{max}$ values irrespective of any correlation between increase or decrease in $K_m$ and $V_{max}$.

### 6.3.5. Effect of temperature and pH on activity of xylanases:

Enzymes that form cocktail for plant biomass saccharification may not have the

same temperature and pH optima. This is a major limiting factor and care must be taken to provide such an incubation condition where all the individual enzyme component of a cocktail should be able to work. In this context the studies regarding effect of temperature and pH on activity of accessory and core enzymes is vital. Therefore, based on the initial screening results as observed in Section 2.3.7 of Chapter 2, a range of temperature from 30-80 °C and a range of pH 4.0-10.0 was used to find out combined effect of temperature and pH on activities of crude xylanase enzymes from *B. safensis* M35, *B. altitudinis* R31 and J208.

When combined effect of pH and temperature on xylanase activity was studied, 8.5, 7.0 and 8.0 units were the highest activities observed for M35 xylanase, R31 xylanase and J208 xylanase respectively at temperature 60 °C and pH 7.0-8.0. Table 6.5 represents the % relative xylanase activities in compatision to highest activities observed at 100%. Xylanases from the three isolates revealed more than 55% activity over a broad range of temperature of 40-60 °C and pH of 5.0-9.0. With gradual decrease in activity above 60 °C, all three xylanases completely lost their activities at 80 °C for all pH conditions. Similarly, these xylanases completely lost their activities at acidic and alkaline conditions of pH 4.0 and 10.0 respectively.

**Table 6.5. Comparison of % relative xylanase activities from three isolates at different temperature and pH incubations:**

| Xylanases | Temperature (°C) | pH 5.0 | pH 8.0 | pH 9.0 |
|---|---|---|---|---|
| ***B. safensis* M35** | 40 | 56.3 ± 0.7 | 59.7 ± 0.1 | 56.1 ± 1.5 |
| | 50 | 79.6 ± 1.4 | **84.5 ± 0.9** | 75.3 ± 0.6 |
| | 70 | 43.2 ± 1.1 | 56.8 ± 0.5 | 40.0 ± 1.4 |
| ***B. altitudinis* R31** | 40 | 65.2± 0.6 | 72.1 ± 1.9 | 65.6 ± 2.4 |
| | 50 | 85.9 ± 3.4 | **95.3 ± 0.1** | 84.6 ± 1.7 |
| | 70 | 31.2 ± 0.1 | 45.5 ± 1.6 | 28.4 ± 0.5 |
| ***B. altitudinis* J208** | 40 | 73.8 ± 2.1 | 77.6 ± 1.7 | 68.8 ± 2.4 |
| | 50 | 87.3 ± 2.3 | **98.5 ± 0.7** | 89.2 ± 0.8 |
| | 70 | 47.5 ± 1.1 | 64.7 ± 1.4 | 40.5 ± 0.3 |

Values presented as Mean ± Standard Errors of the Mean (SEM), for n=3.

Contour plots generated using these relative xylanase activity data exhibits the combine effects of temperature and pH on xylanase activity (Figure 6.5). The pH 7.0 and pH 8.0 were optimum for M35 xylanase and J208 xylanase, whereas a range of pH 7.0 to 8.0 was optimum for R31 xylanase at incubation of 50-60 °C.

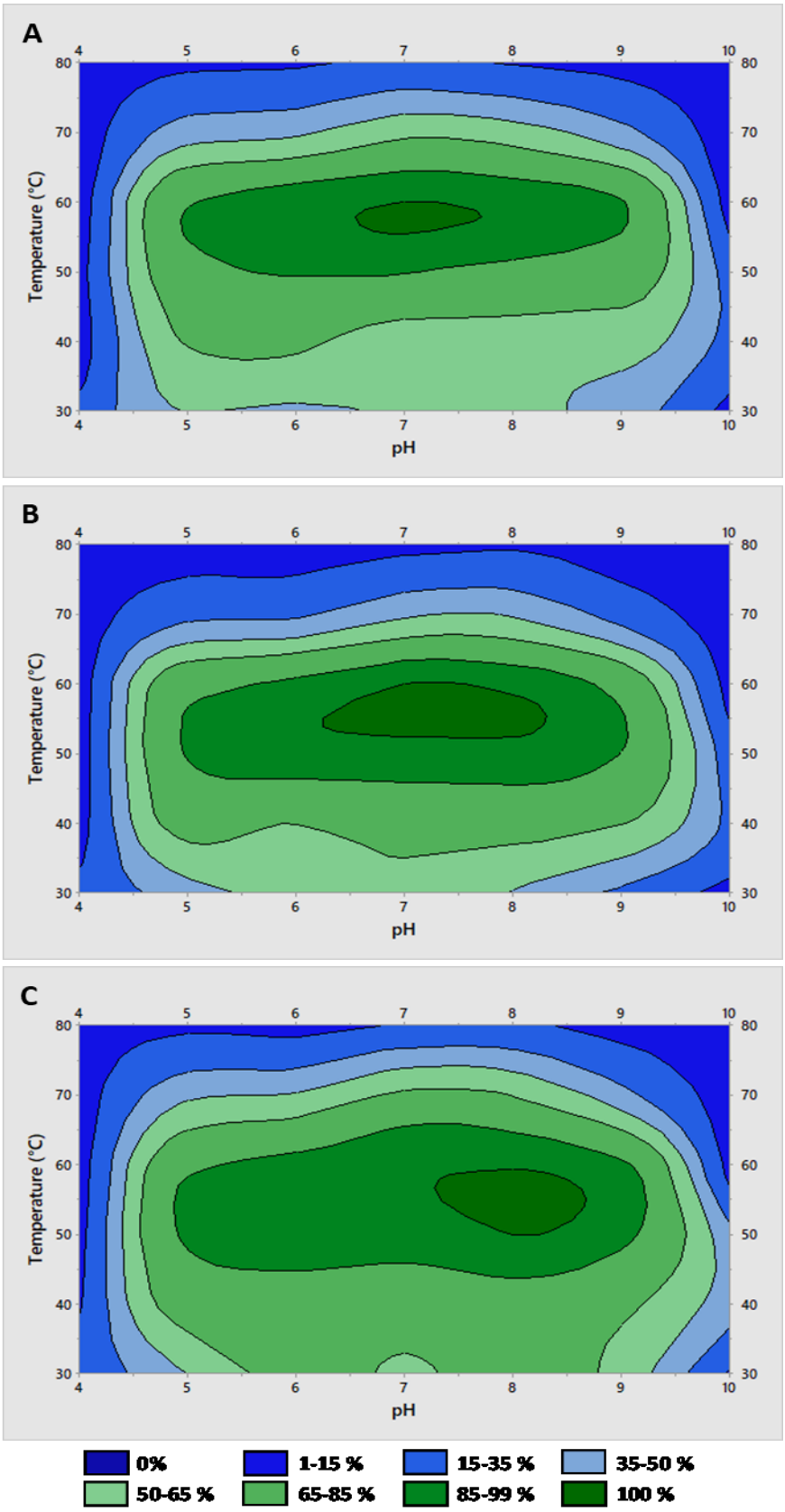


**Figure 6.5. Contour plots of % relative xylanase activity based on the data of Table 6.5:** Effect of temperature and pH on relative activity of crude xylanases of (A) *B. safensis* M35, (B) *B. altitudinis* R31 and (C) *B. altitudinis* J208; X axis (→) represents pH (4.0-10.0), Y axis (↑) represents temperature (30-80 °C) and Z axis represents relative units of xylanase activities as shades of blue and green colours; values plotted are Mean values for n=3.

From the observations it could be predicted that the plot of activity units versus individual pH or activity units versus individual temperature separately for each xylanase is a bell-shaped curve with their peaks in a temperature range of 50-60 °C and pH 7.0-8.0. Crude xylanase from mesophilic *Bacillus* sp. MX47 has demonstrated the pH and temperature optima of 8.0 and 40°C (Chi et al., 2012). Cloned xylanase KRICT PX1 from *Paenibacillus* sp. HPL-001 has shown maximum activity at pH 5.5 and 9.5 at 50 °C and 45 °C, respectively (Hwang *et al*. 2010).

The results suggested that although, the three xylanases have different optimum pH values and broad pH and temperature range for activity, if applied together, their range of pH for higher activity will be expanded to from acidic to alkaline (pH 5.0 to pH 9.0) conditions at temperature of 40-60 °C.

- ***Calculations of activation energy ($E_a$) and Temperature quotient ($Q_{10}$):***

During above analysis it was clear that the enzyme activity increased up to 60 °C and decreased thereafter (as can be seen from Figure 6.5). Therefore 60 °C was considered as inflexion temperature and the activation energy ($E_a$) values were calculated for the reaction system at these pH values individually in two phases based on the inflexion temperature as shown by Siddiqui et al., (1996), (i) below inflexion point 30-60 °C and (ii) above inflexion point 60-80 °C.

**Table 6.6. $E_a$ values calculated below and above inflexion points for xylanases:**

| Xylanases | Temp.°C | pH 5.0 | pH 6.0 | pH 7.0 | pH 8.0 | pH 9.0 |
|---|---|---|---|---|---|---|
| M35-xylanase | 30-60 | 14.6 ± 4.4 | 18.6 ± 1.7 | 18.9 ± 0.9 | 19.0 ± 2.3 | 17.1 ± 0.4 |
| | 60-80 | -96.9 ± 2.2 | -96.1 ± 1.5 | -85.1 ± 3.5 | -83.9 ± 3.3 | -109.6 ± 2.4 |
| R31-xylanase | 30-60 | 16.9 ± 1.1 | 17.21 ± 0.3 | 18.2 ± 1.2 | 19.4 ± 1.4 | 17.1 ± 1.2 |
| | 60-80 | -123.7 ± 2.2 | -123.2 ± 0.4 | -110.8 ± 1.3 | -109.2 ± 2.0 | -126.5 ± 2.3 |
| J208-xylanase | 30-60 | 16.8 ± 1.2 | 17.1 ± 1.3 | 18.4 ± 1.2 | 19.1 ± 1.3 | 16.5 ± 2.0 |
| | 60-80 | -67.1 ± 1.1 | -66.8 ± 2.5 | -54.3 ± 1.6 | -53.4 ± 1.6 | -75.1 ± 2.2 |

Values represented are Mean ± Standard Errors of the Mean (SEM), for n=3

Positive $E_a$ values for the temperature below inflexion point and negative $E_a$ values for temperature above inflexion point for each single reaction system was observed in case of all three xylanases. Positive $E_a$ values showed gradual increase in enzymatic rate up to the inflexion whereas the negative values of $E_a$ showed the gradual decrease in enzymatic rate due to thermal denaturation of enzymes after the inflexion point. Similar observations has been reported for activity of polysaccharide free and polysaccharide complexed CMCase enzyme (Siddiqui et al., 1996).

Effect of pH was clearly observed on activities of all enzymes studied, when $E_a$

values below inflexion point and above inflexion points were compared for all pH. The $E_a$ values below 60°C for all three xylanases increased in order as pH 5.0, 6.0, 7.0 and 8.0, but at 9.0 it decreased whereas, above 60 °C the $E_a$ reached to least negative values at pH 8.0 and again increased negatively at pH 9.0. Gradually increasing positive $E_a$ values below inflexion indicates the active range of temperature and pH for enzyme with the highest $E_a$ value being the optimum.

The $Q_{10}$ temperature coefficient is a measure of the rate of change of a biological or chemical system as a consequence of increasing the temperature by 10 °C. When $Q_{10}$ values were calculated for each enzyme at each pH using the both $E_a$ values mentioned above in Table 6.5, it was observed that below 60 °C the $Q_{10}$ values were ≥1.0 while above 60 °C the $Q_{10}$ values were ≤ 0.098. Faulet et al., (2006) has reported $E_a$ values for xylanase A and B obtained from gut of termite *Macrotermes subhyalinus* to be 73.6 and 29.7 KJ/mol and $Q_{10}$ values to be 2.2 (below 60 °C) and 1.4 (below 55 °C) respectively which are in accordance with our results. These $E_a$ and $Q_{10}$ values cumulatively suggested the favourable range of temperature as 30-60 °C and pH 5.0-9.0 for all three xylanases.

### 6.3.6. Effect of temperature on stability of xylanases:

Since, temperature plays an important role in activity as well as stability of an enzyme, effect of temperature on stability of each crude xylanase enzyme was studied in terms of relative residual enzyme activity with increase in preincubation time and temperature.

As shown in Figure 6.6, thermostability studies for crude xylanase activity demonstrated that all three xylanases were highly stable at 40 °C, with more than 90% residual activity for M35 and J208 xylanases and more than 80% residual activity for R31 xylanase up to 150 min. With increase in the preincubation temperature, at 50 and 60°C gradually the enzyme activity decreased with time. As seen from Figure 5.6, at 60 min preincubation M35 xylanase had more than 80% residual activity, while R31 and J208 xylanases had more than 50% residual activities. Further the residual activity decreased with increased temperature and reached zero within 40 min for R31 and J208 xylanases, yet 50% activity was retained by M35 xylanase at 30 min. Notably, similar results have been observed with xylanase from *Bacillus* sp. SV-34S within 15 min of preincubation at different temperatures where the relative residual activity of about 80% was observed up to 60 °C (Mittal et al., 2013). Xylanase from cow rumen metagenome

exhibited short range of thermostability and was stable at 35-40 °C with 70% retained relative activity up to 60 min and lost all of its activity at temperature of 50 °C and 60 °C after 30 min (Gong et al., 2013).

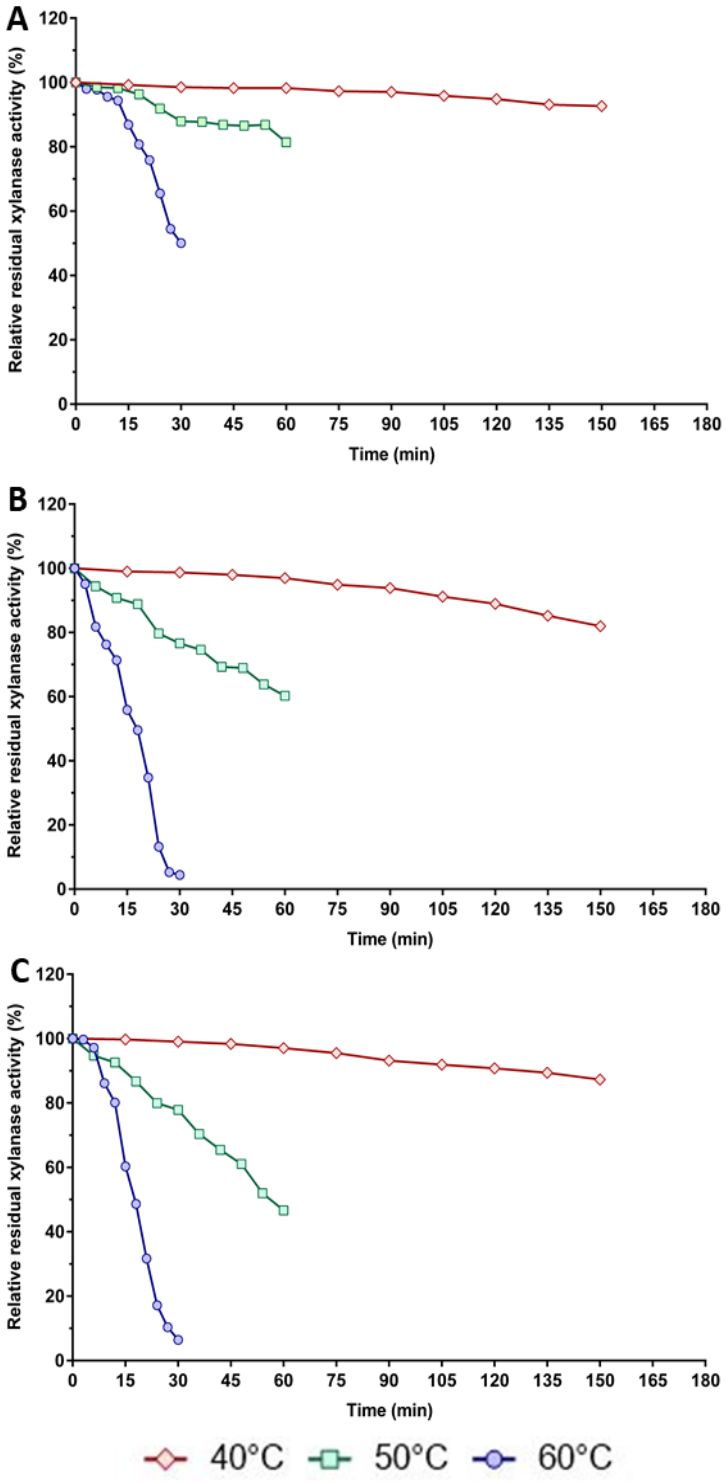


**Figure 6.6. Effect of temperature on stability of xylanases from three *Bacillus* isolates:** Residual activities of crude xylanases obtained from **(A)** *B. safensis* M35, **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208, after preincubation at 40, 50 & 60°C; Values represented are Mean ± Standard Error of Mean (SEM) for n=3.

- ***Half time ($t_{1/2}$) values for crude xylanases:***

Residual activity of xylanase at 40 °C decreased linearly and hence deactivation followed the first order kinetics. While incubation of xylanase at 50 and 60 °C exhibited decrease in residual activity of enzymes and the deactivation didn't follow the first order kinetics. Hence, based on the deactivation rate constant of first order deactivation reaction kinetics, half time values $t_{1/2}$ for all three xylanases at 40°C were calculated and are presented in Table 6.7.

**Table 6.7. $t_{1/2}$ values of xylanase enzymes from the three *Bacillus* isolates:**

| Enzyme and their sources | | $t_{1/2}$ (in h) during preincubation at 40 °C |
|---|---|---|
| Xylanase | *B. safensis* M35 | 24 h |
| | *B. altitudinis* R31 | 10 h |
| | *B. altitudinis* J208 | 13 h |

Although, 45-60 °C was the range of maximum activity as observed in Section 6.3.5., extended exposure of the enzymes to temperatures above 50 °C resulted in decline in stability as well as residual activity. Hence, the temperature of 40 °C can be recommended for applications involving long-term incubation.

## 6.3.7. Effect of pH on stability of xylanases:

Effect of pH on stability of an enzyme is an important attribute as it affects the charges of amino acids on protein molecule which alters the conformation of protein which in turn affects the enzyme stability. Therefore, effect of diverse pH conditions on stability of xylanases from three isolates was studied.

As can be seen from Figure 6.7, M35, R31 and J208 xylanases were comparatively more stable in pH range 6.0 to 9.0 than at pH4.0, 5.0 and 10.0. M35, R31 and J208 xylanases were most stable at pH 8.0 and retained more than 70%, 35% and 50% residual activities. M35 xylanase lost its activity after preincubation of 18h (at pH 4.0) and 24h (at pH 5.0) while other R31 and J208 xylanases lost their activity after preincubation of 18h (at pH 4.0 and 5.0). This suggested that M35 xylanase was more stable and active in broad range of pH 5.0 to 9.0. In another similar finding, recombinant xylanase XynE2 was shown to be stable over a broad pH range, retaining more than 80% of the initial activity after incubation in buffers of pH 4.6–pH12.0 at 37 °C for initial 1 h (Wang et al., 2010).

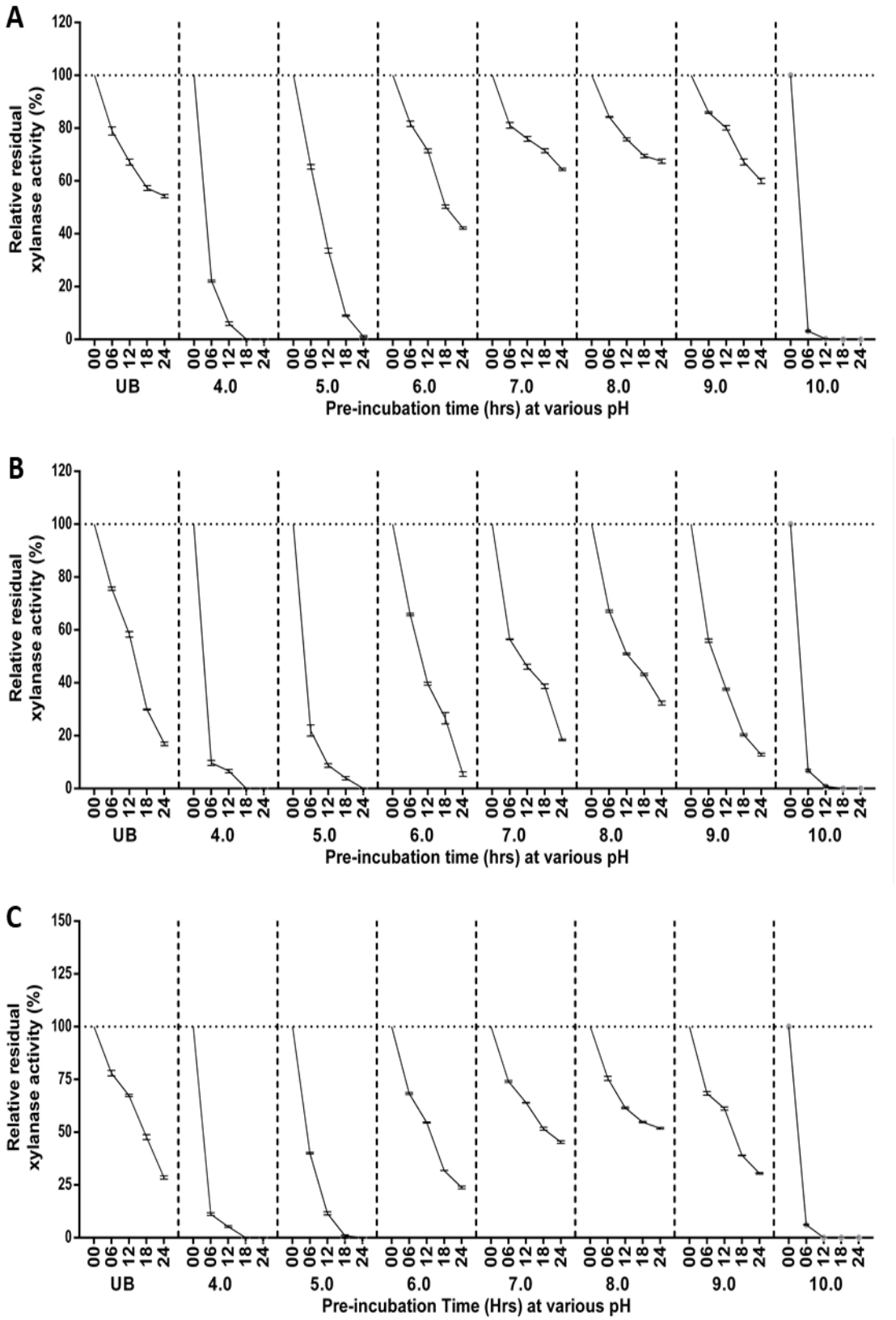


**Figure 6.7. Effect of pH on stability of crude xylanases from three *Bacillus* isolates:** Residual activities of crude xylanases obtained from **(A)** *B. safensis* M35, **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208 after preincubation at 40°C without any buffer (unbuffered, UB) and with various buffers containing pH values 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0; Values represented are Mean ± Standard Error of Mean (SEM) for n=3.

### 6.3.8. Mode of action of crude xylanases:

Viscosity is a characteristic of the fluid that opposes relative motion between two surfaces of the fluid which are moving at different velocities. Since positive correlations between the molecular weight or length of the polymers, or branching pattern of the polymer with viscosity have been proved by Mortimer et al. (1964), it is well known that breakdown of the polymer reduces branching, length and molecular weight of the polymer, which reduces the force required for movement of two fluid layers. This causes increased motion of the layers and reduces the flow time and viscosity. Breakdown of the α-1,4- or β-1,4- linkages from the back-bone or α-1,6- or β-1,6- linkages at branching of polysaccharide polymer chain is responsible for reduction in polymer size and also a decrease in viscosity of polymer solution. Therefore, higher viscosity illustrates less breakdown of polymers while decrease in viscosity portrays more breakdown of polymer (Agrawal et al., 2016). Hence, effect of xylanase activities on the velocity of the xylan substrate was studied to find out the endo- or exo- mode of the all three xylanases enzymes *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 and results are depicted in Figure 6.8.

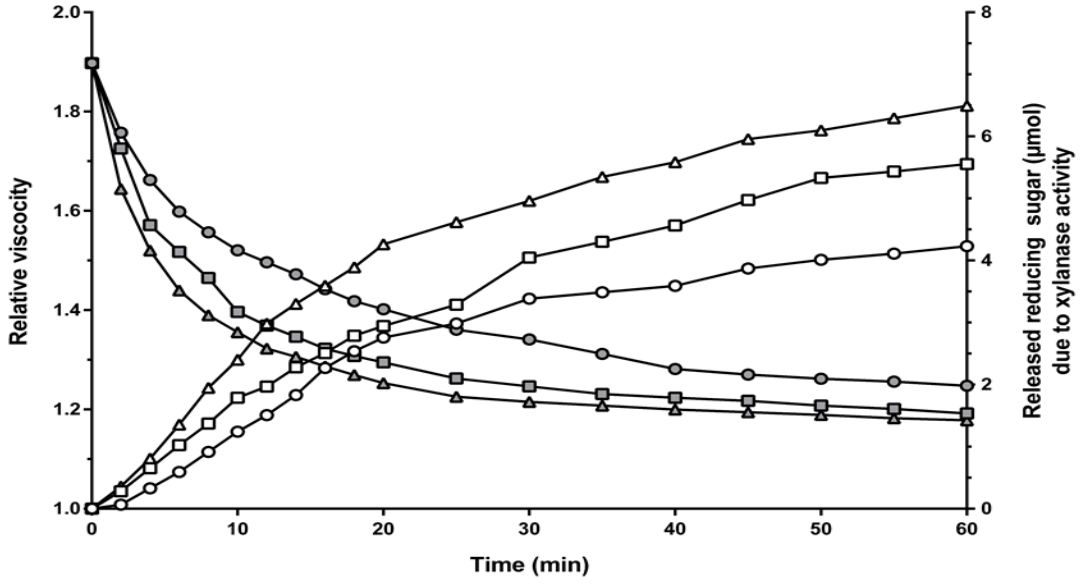


**Figure 6.8. Action of crude xylanases of selected *Bacillus* isolates on viscosity of xylan:** Drop in viscosity (close symbols) and accumulation of released end-products (open symbols) during the breakdown of xylan substrate by xylanases obtained from *B. safensis* M35 (▲,△), *B. altitudinis* R31(■ ,□) and *B. altitudinis* J208 (●,○); Error bars represented as Standard Errors of the Mean (SEM), for n=3.

If an enzyme has purely exo-activity terminal monomers are released from polymer substrate, which cannot bring about a sudden decrease in viscosity within a short time period. Instead the amount of reducing sugar released will go up in short

time. During incubation of individual crude xylanases with xylan at 10th min, the decrease in viscosity was 60%, 55% and 43% by M35, R31 and J208 xylanases respectively while the amount of released end product was 2.4, 1.7 and 1.3 µmol of end-product/ml equivalent to xylose respectively. M35 xylanase decreased viscosity faster as well as released more end products than the other two enzymes followed by J208 and R31 respectively. During the 60th minute of incubation 80, 78 and 72% decrease in viscosity by isolates M35, R31 and J208 respectively was observed with 6.4, 5.5 and 4.4 µmol of released end products/ml equivalent to xylose. Enzyme which can bring more than 40% decrease in viscosity during initial 10 min is considered as endo-acting enzyme as defined by Angelova (2008). Based on the Einstein Equation, which relates molecular weight to viscosity, Khasin et al. (1993), used endo-xylanase from *Bacillus stearothermophilus* T-6 and reported that such drastic reduction in viscosity with slow release of end product can result only from an endo-cleavage of the molecules.

As time increased, the viscosity of xylan polymer approached the value of the solvent suggesting most of the polymer has been broken down to the smaller fragments. The pattern of decrease in viscosity as well as end-product accumulation by the xylanases was further analysed by applying the equations 6.20 and 6.21 where the rate of viscosity decreases ($r_{vd}$) and rate of end-product accumulation ($r_{epa}$) for 10th, 25th, 35th, 50th and 60th min was calculated.

**Table 6.8. Rate of viscosity decrease ($r_{vd}$) and end-product accumulation ($r_{epa}$) by xylanase enzyme from three selected *Bacillus* isolates:**

| Enzyme | Incub. time | *B. safensis* M35 | | *B. altitudinis* R31 | | *B. altitudinis* J208 | |
|---|---|---|---|---|---|---|---|
| | | $r_{vd}$ | $r_{epa}$ | $r_{vd}$ | $r_{epa}$ | $r_{vd}$ | $r_{epa}$ |
| Xylanase | 10 min | 0.2403 | 0.0542 | 0.1787 | 0.0501 | 0.1242 | 0.0377 |
| | 25 min | 0.1142 | 0.0071 | 0.1013 | 0.0076 | 0.0892 | 0.0102 |
| | 35 min | 0.0731 | 0.0018 | 0.0892 | 0.0031 | 0.0769 | 0.0049 |
| | 50 min | 0.0513 | 0.0011 | 0.0767 | 0.0016 | 0.0420 | 0.0020 |
| | 60 min | 0.0396 | 0.0011 | 0.0382 | 0.0015 | 0.0222 | 0.0014 |

Table 6.7 clearly depicts that both parameters i.e. rate of velocity decrease and rate of end-product increase tend to decrease as incubation time increases for xylanases of the three *Bacillus* isolates. Probably the breakdown of polymer length significantly decreased the available endo-action sites on residual polymers for further action of the enzymes and hence the rate of increase in end-product as well as rate of decrease in

viscosity decreased emphasising that the xylanases under study must be of endo-acting in nature. End-product analysis was performed for further confirmation.

### 6.3.9. Chromatographic analysis of end-products of xylanases:

To confirm the endo-acting nature of xylanase from three isolates M35, R31 and J208 end product analysis through TLC and HPLC was performed from individual enzyme substrate system.

#### 6.3.9.1. TLC analysis:

Samples from individual enzyme assay system were run on a TLC plate. Figure 6.9 shows the chromatogram where till 120 min no spots corresponding to D-xylose (Figure 6.9) was obtained. Shi et al., (2013) has reported 85% conversion of xylan polymer to oligosaccharides (containing 2-4 units of monomers) and monosaccharides. The absence monomeric end-products in our results indicated that the xylanases from the selected *Bacillus* spp. must be endo-acting in nature.

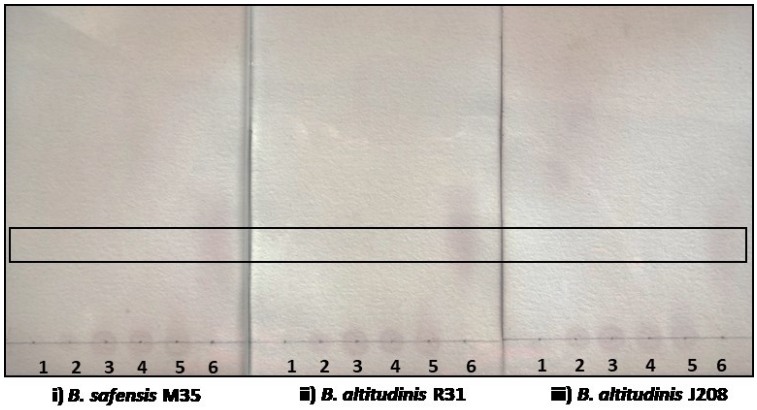


**Figure 6.9. TLC analysis for breakdown of polymers by xylano-pectinolytic enzymes:** Breakdown of beechwood xylan by xylanase obtained from (i) *B. safensis* M35, (ii) *B. altitudinis* R31, (iii) *B. altitudinis* J208. Spots: (1 to 5) Sample after 0, 15, 30, 60, 120 min of incubation respectively and (6) Xylose Control.

#### 6.3.9.2. HPLC analysis:

For additional confirmations, an HPLC analysis was performed from assay system for individual xylanases and pectinases up to 10 h and compared with xylan control at 0 h (Figure 6.10A) as well as D-xylose control (Figure 6.10B). After incubation of 2.5 h and 10 h, the chromatograms for breakdown products for xylanase (Figure 6.10C-6.10H) did not show any monomers as endproducts.

When xylanase chromatograms were compared (Figure 6.10), the broad plateau which appeared at RT of 5-7 min in substrate control, was gradually decreasing in size from the lower retention time and new peaks developed towards higher retention time

(RT: 9.43 and 10.31 min), yet the peak observed for D-xylose control (RT: 12.05 min) was absent even after 10h incubation.

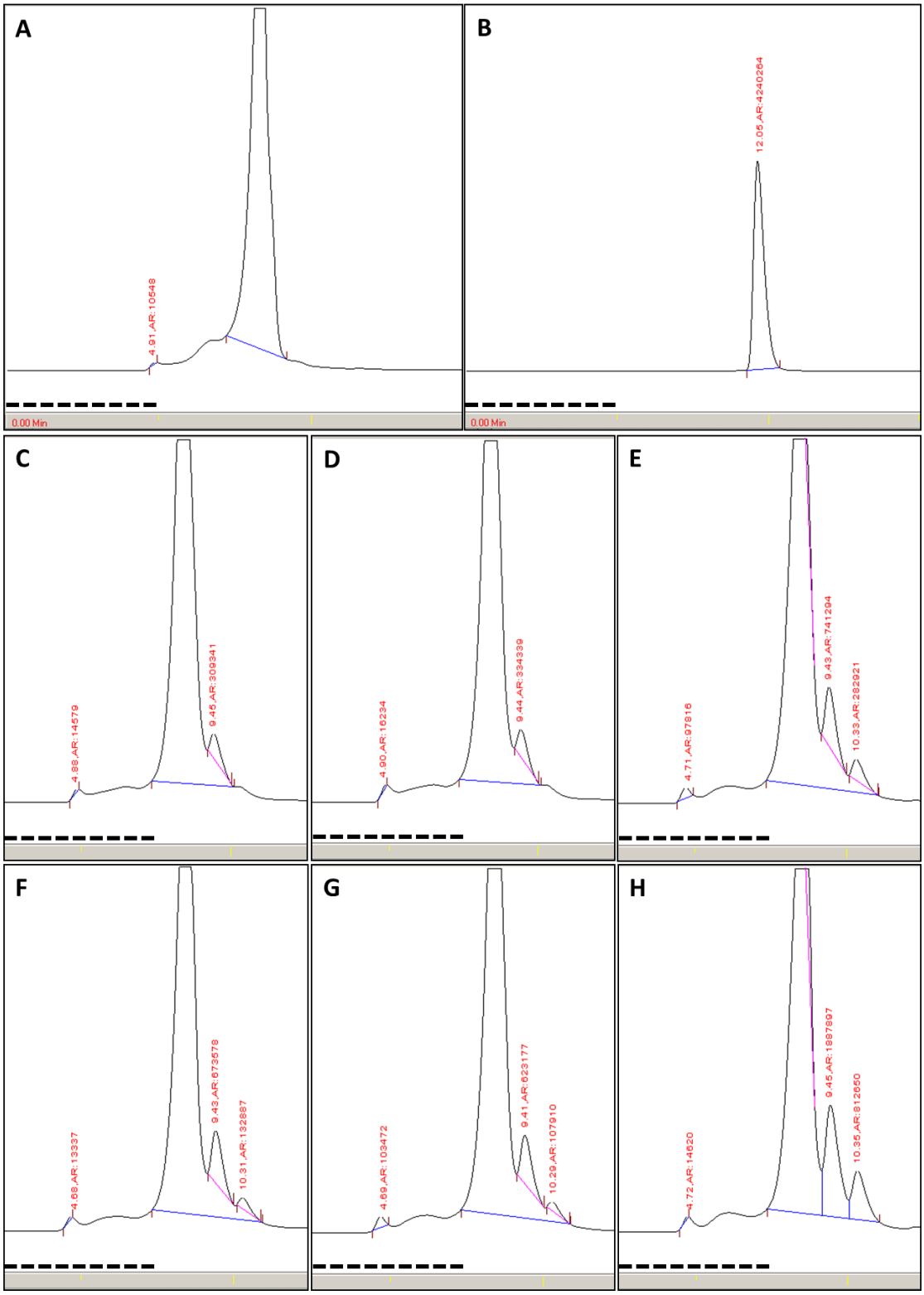


**Figure 6.10. HPLC analysis of xylan hydrolysis by xylanase from individual isolates:** **(A)** Xylan control, **(B)** D-Xylose control; **(C-H)** After incubation of xylan for 2.5 h **(C-E)** and 10 h **(F-H)** with xylanase from *B. safensis* M35 (**C** and **F**), *B. altitudinis* R31 (**D** and **G**) and *B. altitudinis* J208 (**E** and **H**); Peaks are marked by Retention time followed by Area under peak; the dashed line at bottom of each chromatogram represents the Time scale of 5 min.

Summarizing the observations of viscosity, TLC and HPLC analysis suggested that, that break down of xylan polymer structure resulted in release of oligomers (but not monomers) confirming that xylanases from all three selected *Bacillus* strains. are endo acting enzymes and can be termed endo-xylanase. Such broad spectrum bacterial enzymes with endo acting nature are the required as accessory enzymes in plant biomass saccharification.

### 6.3.10. Effect of modulators on xylanase activity:

Pereira et al. (2017) explained that the several modulators like metal ions, metal ion complexes, detergents, solvents etc., diversely affect the activities of lignocellulolytic enzymes secreted by different microorganisms. Ionic charge and ion radius size has a great influence on the activity and stability of the enzymes. Tejirian and Xu (2010) reported that ions with small radius can more strongly attract charged amino acids than the ions with large radius as they affect the enzyme's overall conformation sometimes damaging the catalytic site. Hence, when it comes to the application of modulators with enzyme cocktail to enhance activity, care must be taken that a modulator should not supress any activity of the components. So, xylanase enzymes from three selected *Bacillus* isolates have been characterized for effect of modulators.

As shown in Table 6.9, when beechwood xylan was supplemented with monovalent cations like, $Li^{+}$, $Na^{+}$, $K^{+}$ and $NH^{4+}$, enhanced the xylanase activities in case of all three isolates *B. safensis* M35, *B. altitudinis* R31 and J208. Increased concentration of NaCl lead up to 9%, 22% and 28% decrease in xylanase activity of M35 xylanases, R31 and J208 xylanase respectively. Similarly, cations like $Ca^{2+}$, $Cu^{2+}$ mildly enhanced xylanase activity up to 9%, while $Mn^{2+}$ and $Pb^{2+}$ enhanced activities of all three xylanases by 16 to 30%. On the other hand, $Cd^{2+}$, $Co^{2+}$, $Hg^{2+}$ and $Ni^{2+}$ depleted xylanase activities up to 20-22%, supplementation of $Fe^{3+}$ depleted 65-80% of xylanase activity in all the three cases. Metal ionic complexes mildly enhanced xylanase activities for all three xylanases. Though $Ca^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Ag^{+}$, $Hg^{2+}$, $Fe^{3+}$ have been reported as an enzyme inhibitors for xylanase from *Geobacillus thermoleovorance* (Verma and Satyanarayana, 2012), xylanase from rumen derived metagenomic library (Gong et al., 2013) and for multifunctional glucanase-xylanase isolated from cow rumen (Loaces et al., 2016), in the present study no or mild effect of $Cu^{2+}$ and $Ca^{2+}$was observed, while $Hg^{2+}$and $Fe^{3+}$ acted as inhibitors for the three xylanases. $Mn^{2+}$, $Ca^{2+}$

and $Co^{2+}$ acted as activators for endo β-1,4-xylanase from insect symbiont bacterium *Streptomyces maxicanus* HY-14 (Kim et al., 2014, 2016).

**Table 6.9. Effect of modulators on activity of xylanases from selected *Bacillus* strains:**

| Modulator | | Relative xylanase activity (%) | | |
|---|---|---|---|---|
| **Type** | **Compound** | ***B. safensis* M35** | ***B. altitudinis* R31** | ***B. altitudinis* J208** |
| **Control** | **--** | 100.00 ± 1.56 | 100.00 ± 2.61 | 100.00 ± 1.53 |
| Monovalent Cations | **LiCl** | **109.34 ± 1.84** | **114.17 ± 1.74** | **106.82 ± 1.52** |
| | **NaCl** | **103.93 ± 1.68** | **103.11 ± 1.30** | **101.34 ± 0.70** |
| | **KCl** | **107.75 ± 2.66** | **112.94 ± 2.55** | **122.57 ± 3.01** |
| | **$NH_4Cl$** | **112.85 ± 3.90** | **126.10 ± 4.19** | **126.70 ± 3.81** |
| | **$NaNO_3$** | **111.36 ± 1.52** | **123.68 ± 1.99** | **116.41 ± 0.40** |
| | NaCl (1% w/v) | 91.78 ± 1.72 | 91.56 ± 0.76 | 94.92 ± 2.00 |
| | NaCl (2% w/v) | 91.27 ± 1.63 | 86.38 ± 2.22 | 79.25 ± 1.31 |
| | NaCl (5% w/v) | 90.78 ± 1.46 | 79.64 ± 1.68 | 72.00 ± 1.13 |
| Divalent and Trivalent Cations | **$CaCl_2.2H_2O$** | **105.14 ± 1.20** | **109.76 ± 1.34** | **103.52 ± 1.18** |
| | $CdCl_2$ | 91.81 ± 2.41 | 95.09 ± 1.36 | 79.41 ± 2.53 |
| | $CoCl_2.6H_2O$ | 90.66 ± 1.40 | 81.59 ± 1.29 | 88.08 ± 1.92 |
| | **$CuSO_4.5H_2O$** | **108.09 ± 1.44** | **105.83 ± 1.27** | **105.46 ± 2.41** |
| | $HgCl_2$ | 86.02 ± 0.72 | 93.68 ± 1.52 | 83.65 ± 2.65 |
| | **$MnSO_4.H_2O$** | **116.02 ± 1.40** | **120.25 ± 1.44** | **130.07 ± 1.30** |
| | $MgSO_4.7H_2O$ | 98.43 ± 1.87 | 91.11 ± 1.17 | 94.31 ± 0.94 |
| | $NiCl_2.6H_2O$ | 71.32 ± 0.63 | 73.91 ± 1.50 | 61.48 ± 0.49 |
| | **$Pb(NO_3)_2$** | **116.29 ± 1.80** | **127.46 ± 1.78** | **120.95 ± 1.44** |
| | $FeCl_3$ | 21.30 ± 0.81 | 27.37 ± 1.75 | 35.00 ± 0.89 |
| Cation Metal Complex | **$K_2Cr_2O_7$** | **108.36 ± 1.54** | **112.00 ± 0.66** | **123.95 ± 1.81** |
| | **$K_3[Fe(CN)_6]$** | **112.99 ± 1.27** | **126.26 ± 1.13** | **103.62 ± 1.32** |
| | **$Na_2B_4O_7$** | **103.38 ± 1.91** | **110.87 ± 1.12** | **101.55 ± 2.35** |
| | **$Na_2MoO_4.2H_2O$** | **110.53 ± 2.49** | **116.57 ± 3.46** | **123.78 ± 1.31** |
| Cation Chelators | **EDTA** | **103.11 ± 3.11** | **115.38 ± 1.29** | **119.31 ± 1.37** |
| | **EGTA** | **102.77 ± 3.35** | **114.57 ± 1.80** | **109.66 ± 1.10** |
| Reducing Agents | **Na-$N_3$** | **106.86 ± 1.18** | **117.85 ± 2.90** | **119.65 ± 1.52** |
| | **Dithiothreitol** | **104.14 ± 1.93** | **117.69 ± 0.66** | **122.73 ± 1.73** |
| | **2-MCT-et-OH** | **106.49 ± 2.48** | **142.93 ± 2.49** | **135.50 ± 3.03** |
| | **2-MCT-et-OH (1% v/v)** | **212.91 ± 7.25** | **254.73 ± 9.82** | **272.16 ± 0.80** |
| Detergents | **Tween 20 (1% v/v)** | **102.95 ± 1.51** | **112.22 ± 3.00** | **128.22 ± 2.81** |
| | **Tween 80 (1% v/v)** | **110.28 ± 0.41** | **135.00 ± 1.81** | **121.54 ± 0.99** |
| | **Triton X100 (1% v/v)** | **104.11 ± 1.45** | **130.86 ± 2.16** | **114.17 ± 2.42** |
| | **SDS (1% v/v)** | **103.07 ± 3.32** | **135.67 ± 6.94** | **122.39 ± 2.48** |
| | **SDS** | **106.45 ± 0.75** | **121.73 ± 1.65** | **109.63 ± 0.47** |

2-MCT-et-OH: 2-Mercaptoethanol; Values represented here are Mean ± SEM, for n=3. Values in bold indicate enhanced activity.

When cationic chelators were added no drastic change in xylanase activities from all three isolates were observed. Presence of reducing agents like 2-mercaptoethanol, Na-$N_3$ and DTT as well as ionic and non-ionic detergents had enhanced activity of all three xylanases. Increase in concentration of 2-mercaptoethanol from 1 mM to 1% v/v dramatically enhanced xylanase activities. Inconsistent to this 2-mercaptoethanol and anionic detergent SDS has been reported to reduce the xylanase activities while non-ionic detergents acted as neutral compounds in case of various xylanases (Verma and Satyanarayana, 2012; Gong et al., 2013; Loaces et al., 2016).

Physicochemical characterization studies for three xylan hydrolases from *B. safensis* M35, *B. altitudinis* R31 *and B. altitudinis* J208, indicated that they have broad range of specificities and affinities for substrates, endolytic and mesophilic (40-55 °C) nature with stability and activity at pH 6.0 to 9.0 with certain common modulators like, $Ca^{2+}$, $Cu^{2+}$ and 2-mercaptoethanol acting as enzyme activators. In other words, they possess the attributes that are necessary in enzymes used for biomass saccharification.