# Chapter 7

# Selection and optimization of inducer substrate(s) in medium for concurrent production of xylanase-pectinase enzymes

## 7.1. Introduction:

Studies from earlier chapters revealed several characteristics of cellulase free xylano-pectinolytic enzymes obtained from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 which imparted industrial importance to these enzymes. Since xylanase and pectinase both are equally important for the biomass saccharification experiments, it would be economical and convenient to obtain both the enzymes from a single optimized production media. This production medium should contain one or several components which can induce the xylanase as well as pectinase enzyme production simultaneously. Though commercially obtained pure polysaccharide substrates induced production of the respective enzymes in higher amount, their cost is a major parameter that affects the enzyme production process economics. In the present case, birchwood or beechwood xylan induced higher xylanase but not the pectinase, while citrus pectin induced higher pectinase but not the xylanase from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208. Such expensive substrates incur higher production cost. Therefore, the replacement of these pure polysaccharide substrates with their inexpensive counterparts would make the enzyme production process cost effective. Agrowaste biomass is easily available in bulk and at low cost and can be a good replacement in the production media. Further, selection of the suitable fermentation substrate and optimization of physical parameters such as temperature, pH, agitation etc., can determine the production rate and cost of the process.

There are several reports available on optimization of media components and physical parameters of growth conditions for production of either xylanase or pectinase enzymes. Kaur et al., (2016) optimized media for hyper production of xylanase from *B. pumilus* 3GAH. Kallel et al., (2016) optimized xylanase production from *B. mojavensis* UEB-FK. Optimized pectinase production from *B. licheniformis* has been reported from orange peel (Bibi et al., 2016). The reports on optimized production of both xylanase-pectinase enzymes from a same medium are very few. *B. pumilus* AJK was reported for concurrent production of cellulase free xylanase and pectinase enzymes from submerged fermentation of wheat bran and citrus peel containing medium (Agrawal et al., 2016; Kaur et al., 2017). While *B. firmus* SDB9 was reported for concomitant production of xylanase, pectinase and cellulase enzymes from submerged fermentation of pectin salt medium (Bhagat et al., 2016). Besides these, there are no reports regarding optimization of inducer substrate concentrations for concurrent production of cellulase

free xylano-pectinolytic enzymes from *B. safensis* and *B. altitudinis* strains.

Hence, the present studies were taken up with use of pure and crude fermentation substrates individually to ascertain their role as inducer of enzymes acting on xylan and pectin. Xylan or hemicellulose forms a substantial part of plant cell wall as compared to pectin. Hence xylanase or xylan hydrolase has an important role in enhancing cellulose accessibility. Pectin even though a minor component also hinders the accessibility to cellulose. Among the pectinases even though PGase is produced by the selected cultures here, pectin lyase and not the hydrolase acts directly on pectin substrate as shown in Chapter 6. Therefore, concurrent production of xylanase in terms of xylan hydrolase and pectinase in terms of pectin lyase was studied. The inducer concentration in production medium was optimized by using design expert software for concurrent production of xylanase-pectinase enzymes from each of the isolates.

## 7.2. Materials and Methods:

### 7.2.1. Chemicals, bacterial strains and crude polysaccharide substrates:

All required chemicals were purchased from HiMedia (Mumbai, India) or Sigma-Aldrich (Missouri, USA) or SRL Pvt. Ltd. (Mumbai, India) and were of analytical grade. Three bacterial isolates *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 obtained from camel, bull and buffalo dung respectively were used to optimize the inducer component for enhanced concurrent production of xylanase and pectinase enzymes in media under this chapter. Isolates were maintained by inoculation and incubation on Nutrient Agar (NA) plates at 37ºC for 24 h followed by storage at 4-6 ºC.

Citrus peels (CP), wheat bran (WB), cottonseed cake (CSC), rice bran (RB) were collected from local market, processed and stored as mentioned in Chapter 2, section 2.2.1. Molasses (M) was collected from local market and stored at -20°C to avoid microbial growth. Individually all of these were used as crude complex polysaccharide substrates for enzyme production. Xylan (Xn) and Pectin (Pn) were used as commercially extracted pure complex plant polysaccharide substrates and Glucose was used as pure substrate for enzyme production. Henceforth, these polysaccharides will be referred as pure and crude substrates throughout this chapter. The crude xylanases are referred to as M35 xylanase, R31 xylanase and J208 xylanase as well as crude pectinases are referred to as M35 pectinase, R31 pectinase and J208 pectinase throughout the studies.

### 7.2.2. Influence of different substrates amended in BHM-YEP medium on production of xylanase and pectinase enzymes:

*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 0.2. 0.5% of this was inoculated into 250 ml Erlenmeyer flask containing 100 ml of sterilized BHM-YEP composed of (g/1000ml) Bushnell Haas Medium (BHM) 3.27, Yeast extract (YE) 0.25 and Peptone (P) 0.75. To this 0.5% (v/v or w/v) of pure or crude fermentation substrates i.e., citrus peel (CP), cotton seed cake (CSC), glucose (G), molasses (M), pectin (Pn), rice bran (RB), wheat bran (WB) and xylan (Xn) were added individually. Medium was sterilized by autoclaving at 10 lbs for 20 min and incubated at 37 °C up to 72 h at 160 rpm. At every 12 or 24 h aliquots were withdrawn from flasks, and further analyzed for various parameters as mentioned below.

#### 7.2.2.1. Measurement of growth:

Growth as absorbance in terms of $OD_{600nm}$ was estimated in aliquots withdrawn from the flasks in 24 well microtiter plate on Tecan infinite M200 Pro using i-Control software based on normalization with uninoculated media control.

#### 7.2.2.2. Xylanase (Xylan hydrolase) activity:

50 µl of appropriately diluted crude CFS from individual *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 was added to 250 µl of 50 mM Tris-Cl pH 8.0 buffered 0.5% w/v xylan and incubated in water-bath at 40 °C for 10 min. The 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 540 nm on Tecan infinite M200 Pro using i-Control software (adapted and modified, Miller, 1959; Ghose and Bisaria, 1987). The amount of xylanase 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.

#### 7.2.2.3. Pectinase (Pectin lyase) activity:

50 µl of appropriately diluted crude CFS from individual *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 was added to 250 µl of 50 mM Tris-Cl pH 8.0 buffered 0.5% (w/v) pectin and incubated in water-bath at 50 °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 were added and incubated at 76 °C for 10 min. Developed pink coloration was estimated at 550 nm on Tecan infinite M200 Pro using i-Control software (adapted and modified, Nedjma et al., 2001). The amount of enzyme required to increase the absorbance at 550 nm of the reaction mixture by 0.01 value per unit time in optimum incubation condition was considered as one unit of pectin lyase activity.

#### 7.2.2.4. Protein estimation:

Amount of secretory protein in CFS was estimated by Bradford reagent (Kruger, 1996). CFS were properly diluted using DW to 1.0 ml volume. 1.0 ml of Bradford reagent was added and reaction mixture was incubated in dark. After incubation of 10 min, absorbance was measured at 595 nm on Tecan infinite M200 Pro using i-Control software. Bovine serum albumin (BSA) was used as standard and amount of protein in equivalent to BSA was calculated.

### 7.2.3. Screening for enzyme inducer substrates:

The growth ratio, xylanase activity ratio, pectinase activity ratio for each isolate was calculated using the values of growth, xylanase and pectinase activities obtained on individual pure or crude complex fermentation substrate (CP, CSC, M, Pn, RB, WB and Xn) to the simple fermentation substrate (G) as per Section 7.2.2. These values were compared to discriminate between the enzyme inducing and growth enhancing nature of various complex substrates.

$$Xylanase\ activity\ ratio\ = \frac{Xylanase\ units\ obtained\ on\ polysaccharide}{Xylanase\ units\ obtained\ on\ glucose} \quad \text{(Eq.7.1)}$$

$$Pectinase\ activity\ ratio\ = \frac{Pectinase\ units\ obtained\ on\ polysaccharide}{Pectinase\ units\ obtained\ on\ glucose} \quad \text{(Eq.7.2)}$$

$$Growth(OD_{600})\ ratio\ = \frac{Growth\ (OD_{600})\ obtained\ on\ substrate}{Growth\ (OD_{600})\ obtained\ on\ glucose} \quad \text{(Eq.7.3)}$$

### 7.2.4. Determination of effective range of individual inducer substrates:

To study the effect of the inducer concentration on three *Bacillus* isolates for production of xylanase and pectinase, a broad range of concentration (0.5 -10.0% w/v) of individual inducer substrates was amended in BHM-YEP media and production of xylanase and pectinase enzymes was monitored as above.

### 7.2.5. Cumulative effect of inducer substrates on enxyme production:

To study the combined effect of selected inducers substrates on three *Bacillus*

isolates for production of both xylanases and pectinases, 0.5 %w/v of each were amended together in BHM-YEP media and their production was monitored by activity measurement as in Section 7.2.2.

### 7.2.6. Use of Response Surface Method (RSM) for optimization of crude inducer substrate(s) concentration for xylanases and pectinases production:

Based on the observations obtained in above mentioned experiments, effects of selected two inducer substrates on induction of xylanase and pectinase production from selected *Bacillus* spp. were analyzed through central composite design (CCD) using response surface methodology (RSM) which allows to develop up to a cubic model. In present study, Design Expert Software (version 7.1.6, State-Ease, Minneapolis, USA) was used to design and analyse the experiments. Since, initially, the relationship between the independent variable(s) and response(s) is unknown and the preliminary step in RSM is to identify a suitable approximation for the true functional relationship between the response and the independent variables. In present studies using Central Composite Design (CCD) method, a narrow range of concentration (0.05-3.0% w/v) for each of inducer substrate was used as two independent variables (F1) and (F2) for further optimization of their concentration in order to obtain the maximum response of xylanase ($R_1$) and pectinase ($R_2$) productions from each of M35, R31, J208 xylanase or pectinase separately. The Central Composite Design of 11 runs with actual values of two selected variables (inducer substrates $S_1$ and $S_2$) at various levels is shown in Table 7.1. Enzyme units were estimated as mentioned in Section 7.2.2.

**Table 7.1. $2^2$-CCD of 11 runs with actual values of selected variables:**

| Standerd Order | Run Order | Point type | $F_1$: $S_1$ (g) | $F_2$: $S_2$ (g) |
|---|---|---|---|---|
| 1 | 6 | Factorial | 0.482 | 0.482 |
| 2 | 8 | Factorial | 2.568 | 0.482 |
| 3 | 11 | Factorial | 0.482 | 2.568 |
| 4 | 5 | Factorial | 2.568 | 2.568 |
| 5 | 9 | Axial | 0.050 | 1.525 |
| 6 | 4 | Axial | 3.000 | 1.525 |
| 7 | 7 | Axial | 1.525 | 0.050 |
| 8 | 2 | Axial | 1.525 | 3.000 |
| 9 | 10 | Centre | 1.525 | 1.525 |
| 10 | 1 | Centre | 1.525 | 1.525 |
| 11 | 3 | Centre | 1.525 | 1.525 |

### 7.2.7. Validation of optimized parameters for concurrent production of xylanase-pectinase enzymes:

All three *Bacillus* cultures were inoculated into 10 ml NB and incubated at 37˚C at 160 rpm till optical density (OD) reached to 0.2. 0.5% inoculum from this culture was inoculated into sterilized 100 ml modified production medium (MPM) containing (g/1000 ml of) Bushnell Haas Medium (BHM) 3.27, Yeast extract (YE) 0.25 and Peptone (P) 0.75, and optimized concentrations of inducer substrates in 250 ml Erlenmeyer flask. Media was sterilized by autoclaving at 10 lbs for 20 min and incubated at 37 °C up to 96 h at 160 rpm. Aliquots were drawn from grown cultures at every 24 h to measure $OD_{600}$ and then centrifuged at 10,000 rpm for 10 min. CFS obtained thereby was further used for enzyme activity assay of xylanase and pectinase. Further enzyme activities and growth ratios in optimised medium and glucose containing medium as mentioned in Section 7.2.2. were compared.

### 7.2.8. Kinetic characteristics for enzyme production:

Kinetic characteristics for enzyme production process were estimated in terms of volumetric rate of enzyme production as well as specific rate of enzyme production or economic coefficient. These parameters for each substrate were compared to find the best substrate for production of enzymes (Sikyta, 1995; Sohail et al., 2009).

- **Volumetric rate of enzyme production process ($Q_p$):** It is defined as total units of enzyme produced in one hour when organism was grown in 1L medium ($IUlit^{-1}h^{-1}$).
- **Specific rate of enzyme production** or **Economic coefficient ($Y_{p/s}$):** It is defined as total units of enzyme produced per gram of introduced substrate ($IU/g^{-1}$)

### 7.2.9. Data Analysis:

- All quantitative estimation experiments were performed in triplicates (n=3) and result data along with error values are represented as Mean ± Standard Error of Mean (SEM) for each experiment either in GraphPad Prism 6.0 or Origin 2017 software.
- Statistical analysis was carried out using Two-way ANOVA method in GraphPad Prism 6.0.
- Design Expert Software version 7.0 was used to perform Response Surface Studies using Central Composite Design for enzyme inducer optimization.

## 7.3 Results and Discussion:

Potential enzyme inducing fermentation substrate was selected based on the

studies of growth and xylanopectinolytic enzyme production by three isolates.

### 7.3.1. Growth and production of xylanases and pectinases on different substrates by *Bacillus* isolates:

Growth of microorganisms and production of xylanase and pectinase activities were estimated on different crude and pure polysaccharide substrates and glucose are as mentioned below.

#### 7.3.1.1. Growth on glucose and different polysaccharide substrates:

Figure 7.1 A, B and C represents the growth of *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 respectively in terms of $OD_{600}$. Increase in growth of each isolate was observed after supplementation of BHM-YEP medium with the selected eight fermentation substrates. Least increase in growth on glucose supplementation and highest growth on xylan supplementation within 24 h of incubation for each isolate was observed. The decrease in growth was in this order: pectin (Pn), wheat bran (WB), molasses (M), rice bran (RB), cotton seed cake (CSC), and citrus peel (CP). In case of CSC supplementation, all three isolates achieved stationary phase at ~36h of incubation exhibiting extended lag and log phase. Thus, the luxuriant growth of isolates on these fermentation substrates indicated that they secreted enzymes degrading the polysaccharides down to their oligo or monomers which were used for growth.

#### 7.3.1.2. Production of xylanase on glucose and polysaccharide substrates:

Figure 7.2 A, B and C represents xylanase activity exhibited by *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 respectively on BHM-YEP medium with and without supplementations. For all three isolates, highest xylanase units of 6-8 units were observed in commercially extracted beechwood xylan supplemented media followed by WB supplemented media within 12 h of incubation. Moderate xylanase activities were observed in CSC and RB supplemented media after 36 h of incubation. CP, P, M and glucose exhibited very less or negligible xylanase activities.

#### 7.3.1.3. Production of pectinase on glucose and polysaccharide substrates:

Figure 7.3 A, B and C represents pectinase activity units exhibited by *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 respectively on BHM-YEP medium with and without supplementations. Pectinase activities were detected within 12 h of incubation from WB and CP were supplemented media for all three isolates, later on after 48 h citrus pectin supplemented media exhibited the highest pectinase activities in range of 220-280 units for all isolates. Moderate pectinase activities were observed in

CSC and RB supplemented media after 24 h of incubation. X, M and glucose exhibited very less or negligible pectinase activities. Chaudhri and Suneetha, (2012) also have reported similar kind of observations for pectinase production.

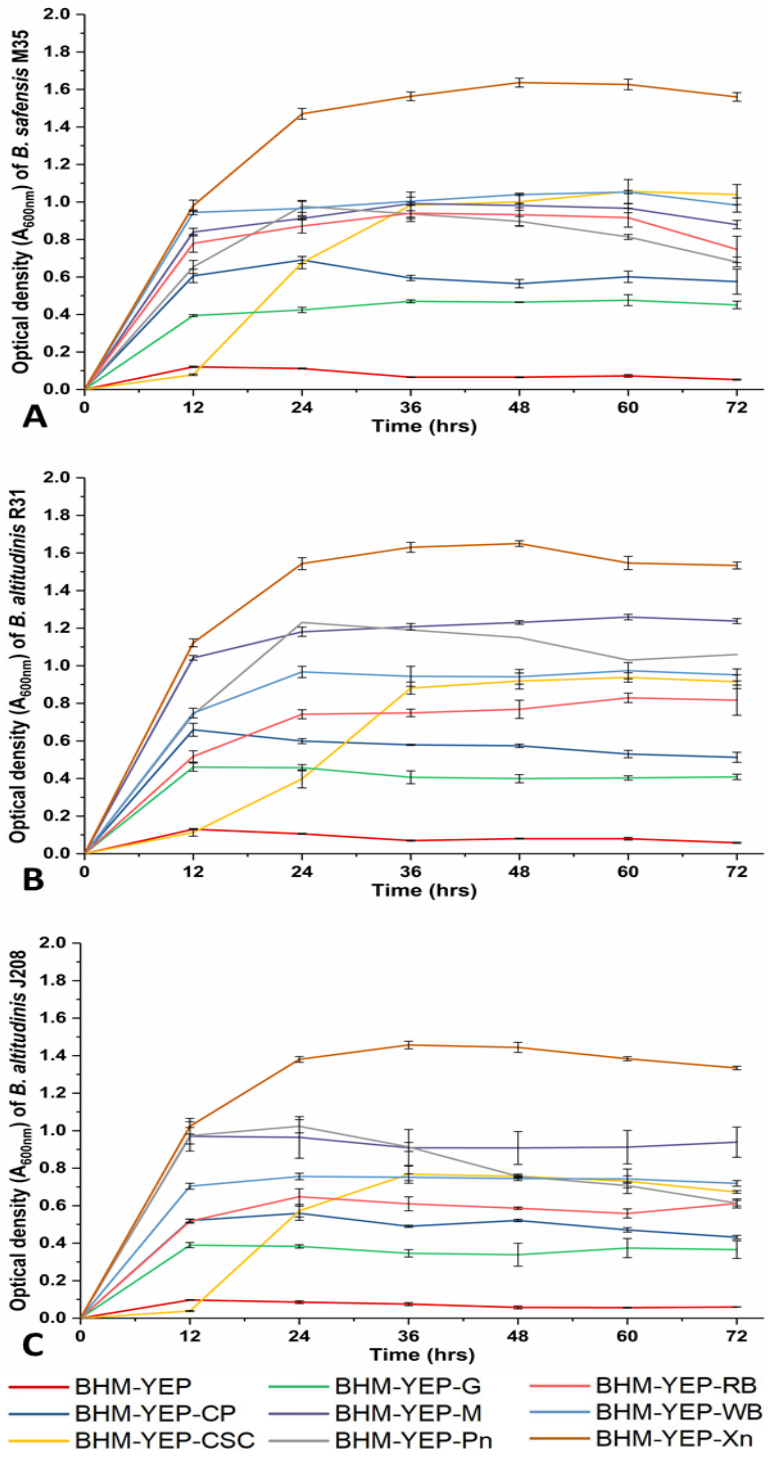



**Figure 7.1. Growth of *Bacillus* cultures on BHM-YEP medium supplemented with and without individual substrates:**
**(A)** *B. safensis* M35 **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208 up to 96 h on BHM-YEP media supplemented individually with citrus peel (CP), cotton seed cake (CSC), glucose (G), molasses (M), pectin (Pn), rice bran (RB), wheat bran (WB) and xylan (Xn). Values plotted are Mean ± Standard Error of Mean (SEM) for n=3.

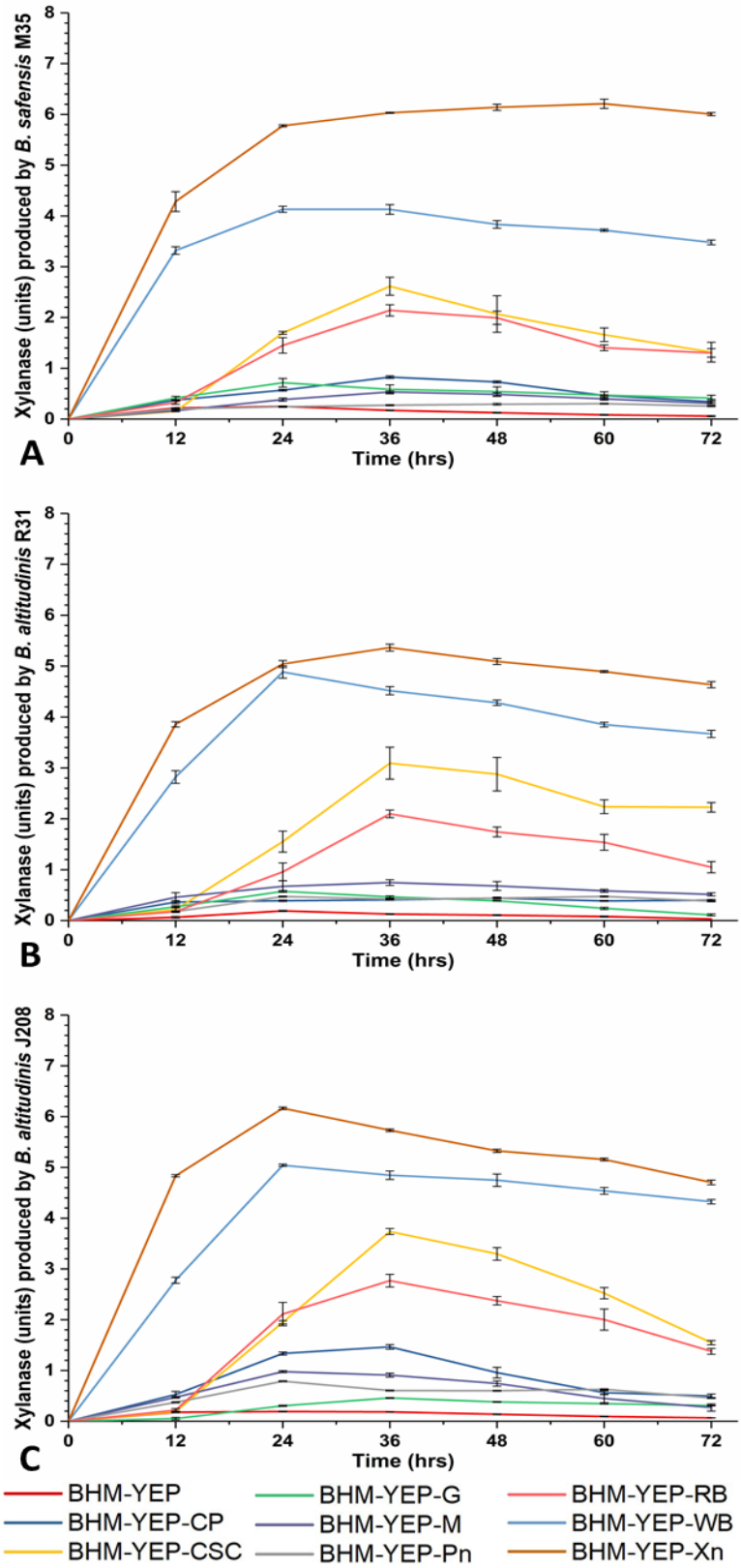



**Figure 7.2. Xylanase production by *Bacillus* isoaltes on BHM-YEP medium with and without individual substrates:**
**(A)** *B. safensis* M35 **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208 up to 96 h on BHM-YEP media supplemented individually with citrus peel (CP), cotton seed cake (CSC), glucose (G), molasses (M), pectin (Pn), rice bran (RB), wheat bran (WB) and xylan (Xn). Values plotted are Mean ± Standard Error of Mean (SEM) for n=3.

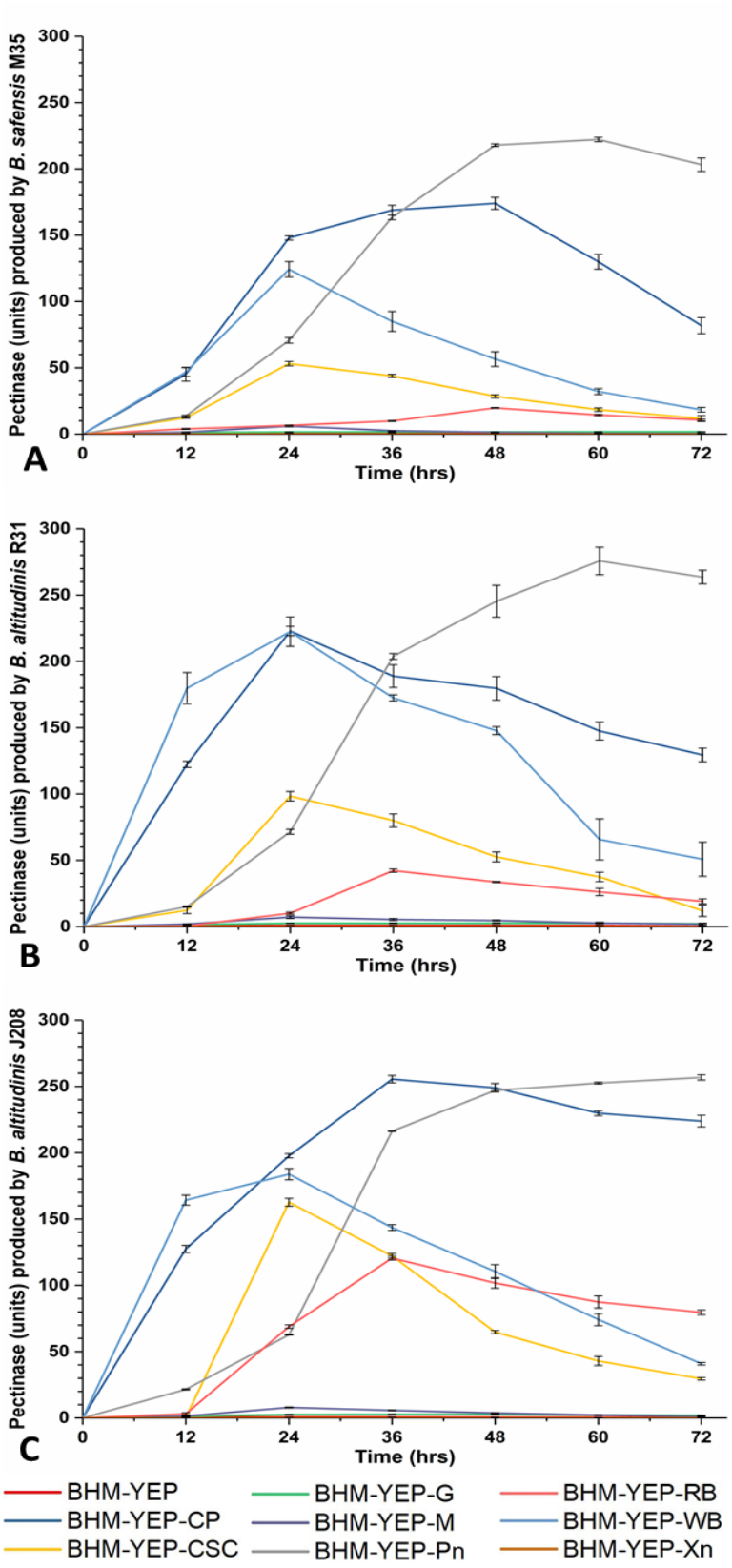
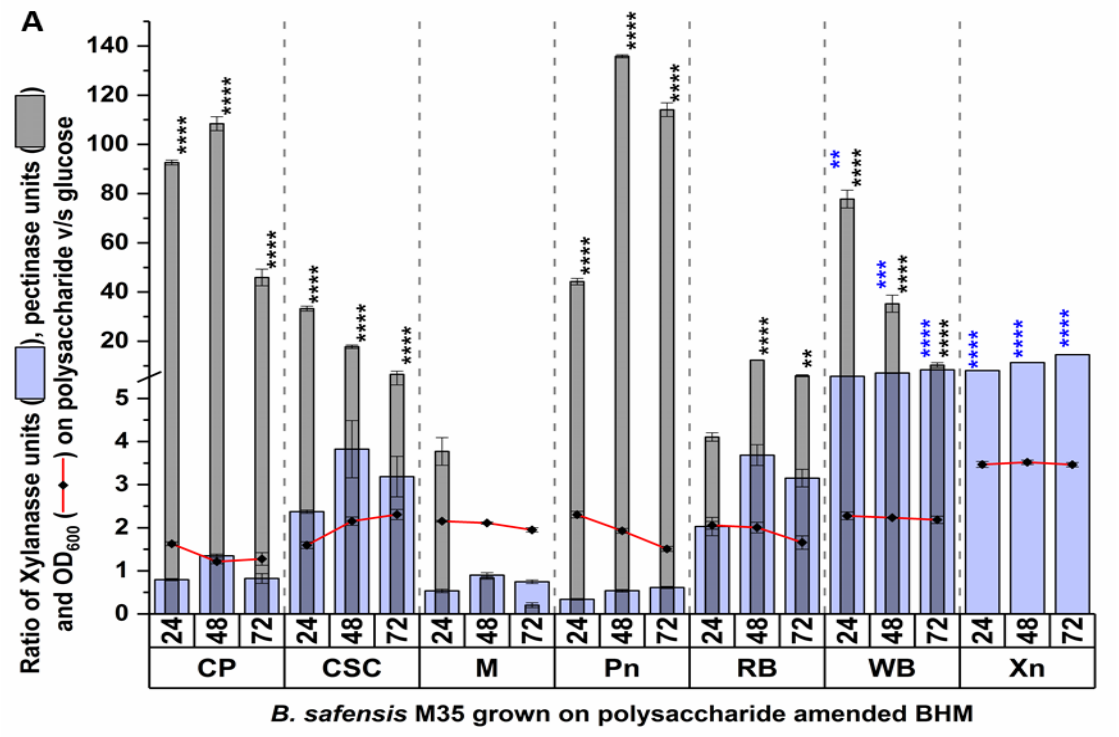



**Figure 7.3. Pectinase production by *Bacillus* isolates on BHM-YEP medium with and without individual substrates:**
**(A)** *B. safensis* M35 **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208 up to 96 h on BHM-YEP media supplemented individually with citrus peel (CP), cotton seed cake (CSC), glucose (G), molasses (M), pectin (P), rice bran (RB), wheat bran (WB) and xylan (X). Values plotted are Mean ± Standard Error of Mean (SEM) for n=3.

Thus, from above observations it was clear that, all three cultures grew on all substrates but produced either xylanase and/or pectinase enzymes on a few of them. WB, CSC and RB could concurrently produce both the xylanases and pectinases. In literature also several bacteria and fungi have been reported for production of xylanase and pectinase enzymes individually from such crude polysaccharide substrates (Kashyap et al., 2000; Nandini and Salimath, 2001; Reginatto et al., 2017; Yegin et al., 2017). Hence, the next step was to select better inducer substrate(s) for concurrent production of xylanase and pectinase enzymes.

### 7.3.2. Screening of better enzyme inducer substrate(s):

If the organisms are luxuriantly growing on a complex substrate and a simple substrate individually, microbial growth ($OD_{600}$), xylanase and pectinase activities can be compared by calculating the ratio of growth or enzyme activity on complex substrate v/s simple substrate (Section 7.2.3). This comparison would help to screen a better inducer substrate. Figure 7.4 (A, B and C) represents such comparison of growth, xylanase and pectinase ratio for *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 respectively. Two-way ANOVA analysis was carried out at 99.99% confidence interval ($p < 0.0001$) and significance difference between enzyme activity ratio and growth ratio is presented in figures for xylanase (blue asterisks) and pectinase (black asterisks).



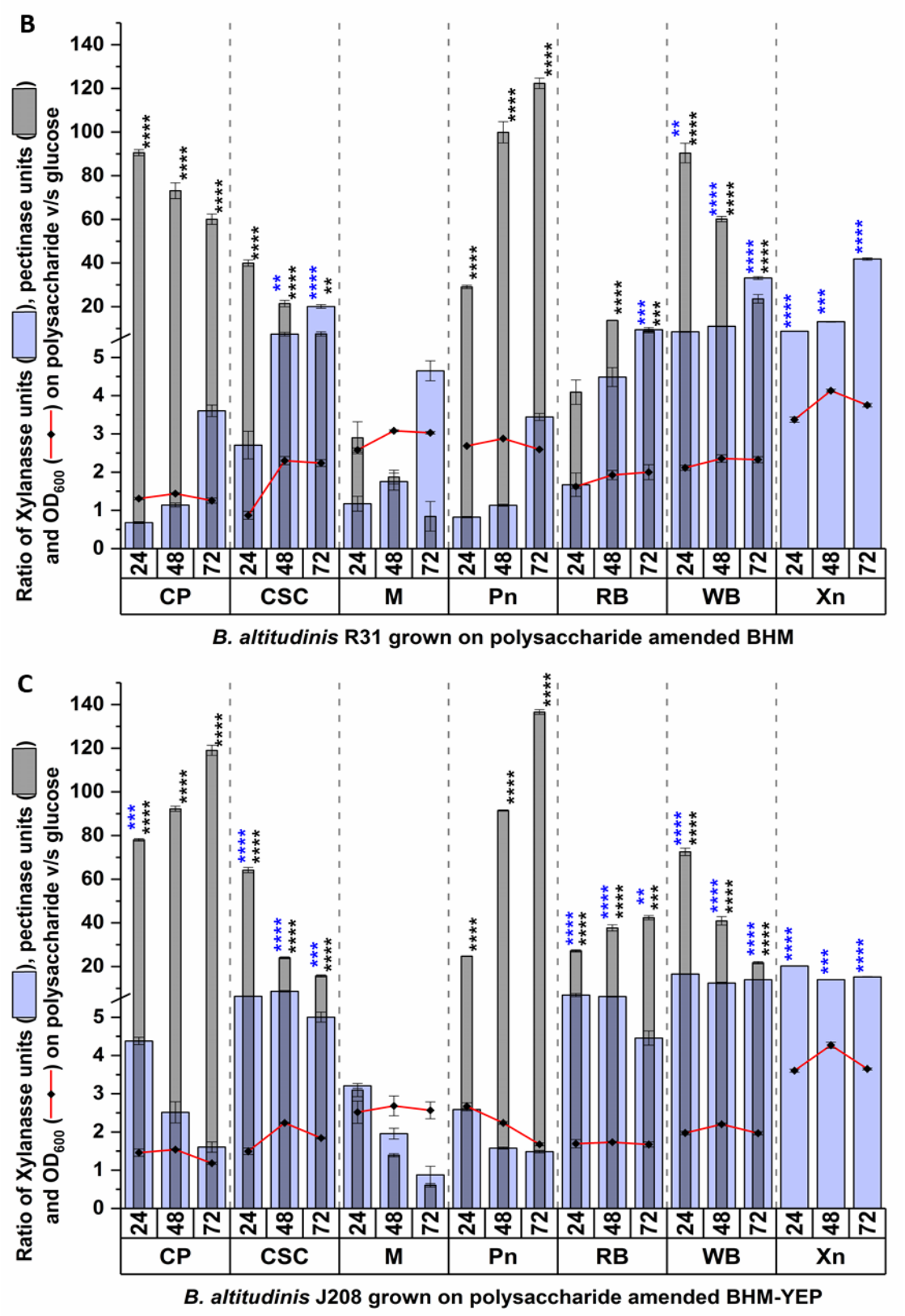



**Figure 7.4. Screening of substrates for induction of xylanases and pectinases individually:** Comparison of growth ratio with xylanase activity ratio as well as with pectinase activity ratio for **(A)** *B. safensis* M35, **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208; Values plotted are Mean ± Standard Error of Mean (SEM) for n=3; Asterisk marks depicts significant difference between (*) as well as pectinase activity ratio (*) from growth ratio (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$) for xylanase activity ratio.

The ANOVA analysis suggested that although xylan supplementation exhibited higher growth ratio, the difference in xylanase activity ratio and growth ratio was significant which suggested that xylan was a good growth enhancer as well as xylanase inducer. Similarly, significant difference in pectinase ratio and growth ratio on pectin suggested that pectin was a good growth enhancer as well as pectinase inducer. In contrast to this, though molasses exhibited higher growth ratio, there was no increase in xylano-pectinolytic enzymes suggesting the molasses as a good growth enhancer but not an enzyme inducer. CSC and RB exhibited significant difference in pectinase ratio and xylanase ratio suggesting that these substrates can be used as moderate to good xylano-pectinolytic enzyme inducers. CP exhibited significant difference for pectinase ratio making it a good pectinase inducer while WB exhibited significant difference for both xylanase and pectinase suggesting it as a good inducer for both the enzymes.

Though xylan induced xylanase, and pectin induced pectinase, the cost of using these substrates becomes limiting factor. Four plant biomass substrates, i.e., CP, CSC, RB and WB, along with xylan and pectin were taken for further studies.

### 7.3.3. Enzyme production kinetics on plant polysaccharide substrates:

Volumetric and specific rate of xylanase and pectinase production were compared to select the better inducer substrate(s) out of these four substrates for concurrent production of xylanase and pectinase. Table 7.2 and 7.3 represent the volumetric and specific rates of enzyme production respectively on four crude plant polysaccharides like CP, CSC, WB and RB as well as xylan and pectin by isolates *B. safensis* M35, *B. altitudinis* R31, *B. altitudinis* J208 individually.

**Table 7.2. Volumetric rate of enzyme production on different inducer substrates:**

| Media | | Volumetric rates ($IU*L^{-1}h^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | For xylanase production by | | | For pectinase production | | |
| | | ***B. safensis* M35** | ***B. altitudinis* R31** | ***B. altitudinis* J208** | ***B. safensis* M35** | ***B. altitudinis* R31** | ***B. altitudinis* J208** |
| BHM-YEP supplemented with | CP | 4.7 (0.9) | 5.5 (0.3) | 6.9 (0.8) | **22718.0 (2376.3)** | **55953.5 (2008.5)** | **62182.8 (1726.5)** |
| | CSC | 18.3 (3.8) | 30.9 (1.8) | 21.5 (0.8) | 3256.8 (888.4) | 3316.7 (700.2) | 8195.1 (393.7) |
| | P | 3.5 (0.2) | 5.3 (0.2) | 6.4 (0.2) | **56437.0 (1955.6)** | **73218.5 (2027.0)** | **71321.3 (782.0)** |
| | RB | 18.1 (1.7) | 14.6 (2.2) | 19.1 (1.1) | 2948.8 (269.3) | 5292.6 (740.5) | 4117.3 (712.8) |
| | WB | **48.3 (0.9)** | **51.0 (1.4)** | **60.1 (0.9)** | **5091.1 (693.3)** | **14121.6 (569.4)** | **11343.8 (391.3)** |
| | X | **83.4 (0.6)** | **64.4 (1.2)** | **65.3 (0.9)** | 0.0 (0.0) | 0.0 (0.0) | 0.0 (0.0) |

Values presented in tables are Mean ± (Standard Error of Mean) for n=3. Blue, bold values are

the highest values observed on pure and crude polysaccharide substrates.

**Table 7.3. Specific rate of enzyme production on different inducer substrates:**

| Media | | Specific rates (IU/g substrate) | | | | | |
|---|---|---|---|---|---|---|---|
| | | For xylanase production | | | For pectinase production | | |
| | | ***B. safensis* M35** | ***B. altitudinis* R31** | ***B. altitudinis* J208** | ***B. safensis* M35** | ***B. altitudinis* R31** | ***B. altitudinis* J208** |
| BHM-YEP supplemented with | CP | 68.0 (13.4) | 79.9 (4.7) | 99.2 (11.7) | **327139.7 (34219.0)** | **517729.8 (28922.9)** | **895432.2 (24861.0)** |
| | CSC | 263.5 (54.9) | 445.2 (26.2) | 309.3 (11.5) | 46897.8 (12792.3) | 77760.0 (24482.8) | 118008.9 (5669.6) |
| | P | 50.8 (2.9) | 76.3 (2.9) | 91.8 (2.9) | **812693.3 (28160.1)** | **1054346.7 (29189.4)** | **1027026.7 (11260.6)** |
| | RB | 260.5 (23.8) | 209.9 (31.3) | 275.4 (16.3) | 42462.3 (3878.1) | 76213.3 (10663.1) | 98488.9 (10264.6) |
| | WB | **695.8 (13.5)** | **733.7 (20.0)** | **865.0 (12.4)** | **73312.3 (9984.1)** | **203351.1 (72999.9)** | **183351.1 (5634.3)** |
| | X | **1201.0 (8.6)** | **927.0 (16.9)** | **940.8 (13.0)** | 0.0 (0.0) | 0.0 (0.0) | 0.0 (0.0) |

Values presented in tables are Mean ± (Standard Error of Mean) for n=3. Bold values are the highest values observed on pure and crude polysaccharide substrates.

An obvious conclusion from the comparison of volumetric and specific rates in Table 7.2 and 7.3 can be drawn that xylan and pectin are the best inducers for xylanase and pectinase enzymes respectively. Whereas, CP can be considered as a best crude substrate for pectinase production which can be attributed to its pectin richness (May, 1990). Similarly, WB can be considered as a best crude substrate for xylanase production. Besides this, WB also induced more pectinase enzymes than RB and CSC which can probably be attributed to its composition of glucan, xylan and galactan. Several similar reports are available for higher xylanase production on WB than CSC, RB, oat bran etc. (Nandini and Salimath, 2001; Das et al., 2008b; Chaudhri and Suneetha, 2012; Reginatto et al., 2017). Although CSC and RB produced xylano-pectinolytic enzymes their production rate were observed to be lower than WB and CP amongst crude polysaccharides. And hence, CP and WB were the two crude polysaccharide substrates selected for further studies of inducer component optimization in media for concurrent production of xylano-pectinolytic enzymes from the three isolates, *B. safensis* M35, *B. altitudinis* R31, *B. altitudinis* J208.

### 7.3.4. Influence of different concentrations of WB and CP for xylanase and pectinase production:

Figure 7.5 A and B respectively represents the induction of xylanase and pectinase activity due to different concentrations (% w/v) of WB. A bell-shaped pattern was clearly visible for enzyme production as an effect of different concentration of WB. Both the activities increased to maximum at 2%. Above 3% concentration of WB, there

was a significant decrease in both activities making the 3% concentration as a limiting factor.

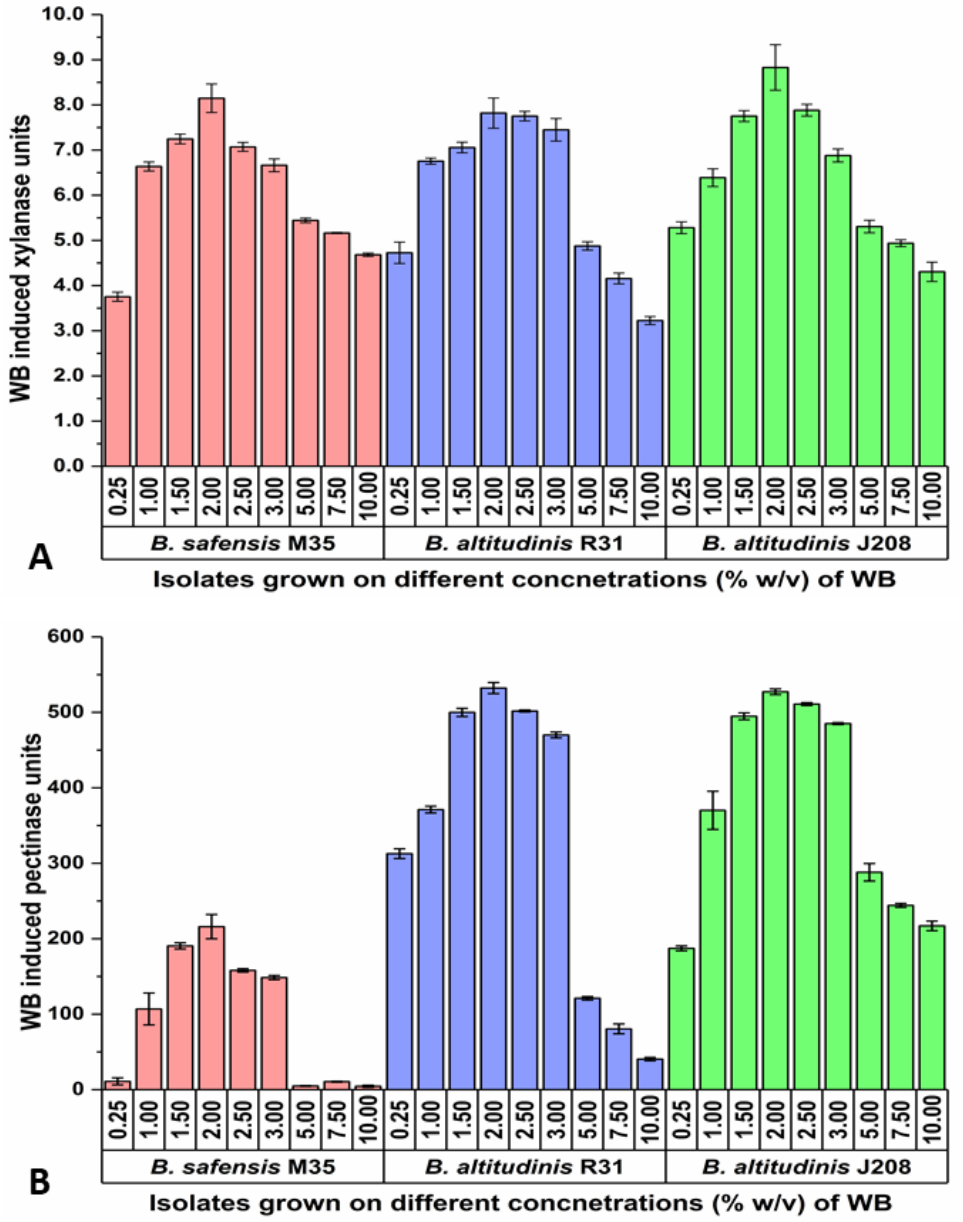



**Figure 7.5. WB induced xylanase and pectinase activities:**
**(A)** xylanase activities and **(B)** pectinase activities induced by range of different concentrations of WB from *B. safensis* M35 (□), *B. altitudinis* R31 (■) and *B. altitudinis* J208 (■); Data presented as Mean ± Standard Error of Mean (SEM) for n=3.

Figure 7.6. A and B respectively represents the induction of xylanase and pectinase activity units as influenced by different concentrations of CP. Similar to WB, a bell-shaped pattern was clearly visible for production of both enzymes as a function of different concentration of CP. But in contrast to the WB, each CP had different effects on the induction of xylanase and pectinase enzymes. With increase in concentration of CP, xylanase activities increased to maximum (at 5% w/v CP) and

then decreased suggesting that the 5% w/v was the most efficient concentration of CP. In contrast to this, maximum pectinase activities were observed at 1% w/v CP and no induction was observed above concentration of 2.0 %w/v. Thus, though a broad range of CP concentrations supported xylanase induction, its higher concentration restricted pectinase induction suggesting that lower concentrations of CP was sufficient for combined induction of xylanase and pectinase enzymes. Thus, both WB and CP were capable of inducing xylanopectinolytic enzymes from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208.

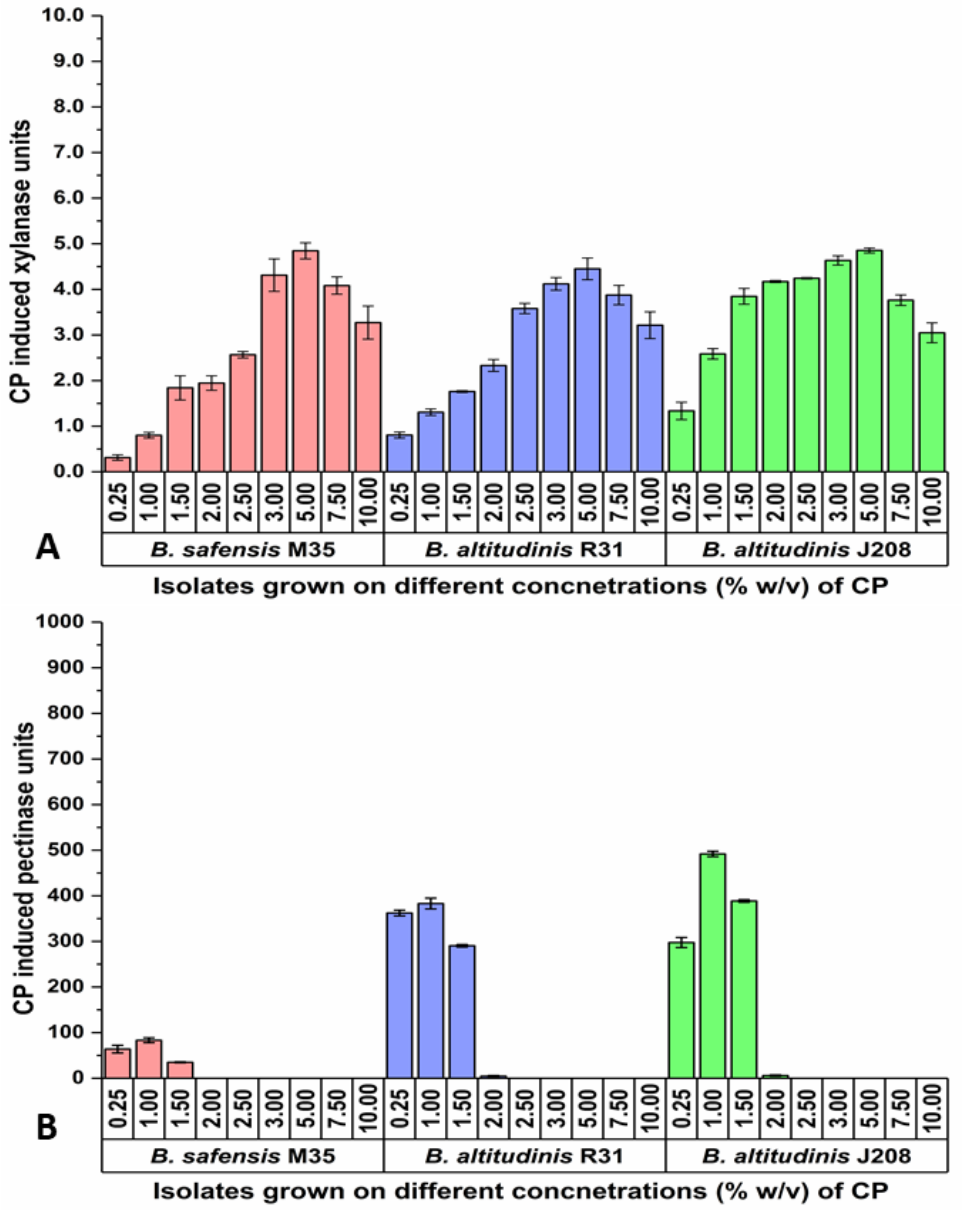



**Figure 7.6. CP induced xylanase and pectinase activities:**
**(A)** xylanase activities and **(B)** pectinase activities induced by range of different concentrations of CP from *B. safensis* M35 ( ), *B. altitudinis* R31 ( ) and *B. altitudinis* J208 ( ); Data presented as Mean ± Standard Error of Mean (SEM) for n=3.

### 7.3.5. Cumulative effect of WB and CP on concurrent xylanase and pectinase production:

Above observations revealed that WB and CP can further be used as crude polysaccharide substrates for fermentation which can induce the production of xylano-pectinolytic enzymes. In literature, enhancement of pectinase activities by *Aspergillus niger* LB-02-SF has been reported after supplementation of orange residues containing media with wheat bran (Reginatto et al., 2017). Supplementation of BHM-YEP with 0.5% of WB and CP both at the same time exhibited increased xylanase and pectinase production when compared with individually amended WB and CP substrates. Figure 7.7 represents the xylanase and pectinase production from individual and combined media. From the Figure 7.7 it is clearly visible that the combined supplementation of WB and CP significantly increased the xylanse and pectinase activity than individual supplementation of Wb or CP respectively.

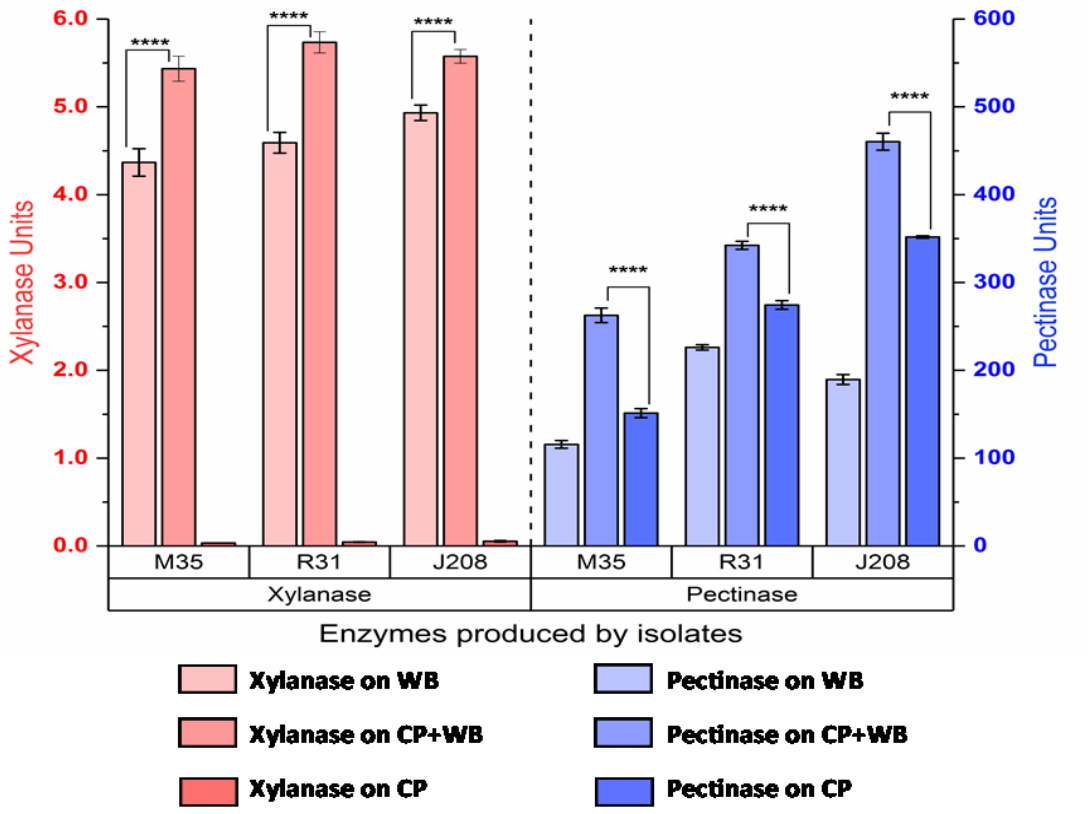



**Figure 7.7. Effect of CP and WB on concurrent production of xylanase-pectinase enzymes:**
Asterisk marks indicates the significant difference between xylanase produced by WB and CP+WB as well as between pectinase produced by CP and CP+WB (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$); The values represented are Mean ± Standard Error of Mean (SEM) for n=3.

Similarly, the values of volumetric rate and specific rate are presented in Table 7.4 and 7.5. On comparison of three different media suggested that the supplementation

of BHM-YEP media by both inducers drastically enhanced the production rates as compared individually. Kaur et al., (2017) has reported concurrent production of xylanase and pectinase from *B. pumilus* AJK using 2% WB and 2% mosambi peels.

**Table 7.4. Volumetric rate of xylanase and pectinase production on inducer substrates:**

| Inducer | Volumetric rates ($IU*L^{-1}h^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | of xylanase production by | | | of pectinase production by | | |
| | ***B. safensis* M35** | ***B. altitudinis* R31** | ***B. altitudinis* J208** | ***B. safensis* M35** | ***B. altitudinis* R31** | ***B. altitudinis* J208** |
| 0.5% WB | 91.0 (4.6) | 95.7 (3.5) | 103.3 (2.0) | 48100.4 (1931.2) | 95019.1 (1940.5) | 79601.0 (3856.4) |
| **0.5% WB & 0.5% CP** | **113.2 (4.2)** | **119.5 (3.6)** | **116.1 (2.3)** | **109877.8 (5520.9)** | **142650.0 (2702.0)** | **198742.6 (14288.5)** |
| 0.5% CP | 6.6 (0.3) | 9.1 (0.6) | 16.2 (1.5) | 63029.6 (3013.7) | 114349.1 (2893.3) | 146573.9 (829.9) |

Values presented in tables are Mean ± (Standard Error of Mean) for n=3.

**Table 7.5. Specific rate of xylanase and pectinase production on inducer substrates:**

| Inducer | Specific rates (IU/g substrate) | | | | | |
|---|---|---|---|---|---|---|
| | of xylanase production by | | | of pectinase production by | | |
| | ***B. safensis* M35** | ***B. altitudinis* R31** | ***B. altitudinis* J208** | ***B. safensis* M35** | ***B. altitudinis* R31** | ***B. altitudinis* J208** |
| 0.5% WB | 436.9 (22.0) | 459.1 (16.7) | 496.1 (9.8) | 230881.7 (9269.9) | 456091.6 (9314.4) | 382084.6 (18510.9) |
| **0.5% WB & 0.5% CP** | **543.5 (20.2)** | **573.4 (17.1)** | **557.5 (11.1)** | **527413.3 (26500.3)** | **684720.0 (12969.6)** | **953964.4 (68584.9)** |
| 0.5% CP | 31.5 (1.3) | 43.4 (2.9) | 77.7 (7.3) | 302542.2 (14466.0) | 548875.6 (13888.0) | 703554.7 (3983.5) |

Values presented in tables are Mean ± (Standard Error of Mean) for n=3.

### 7.3.6. Optimization of inducer substrate concentration on enzyme production:

Supplementation of both inducer substrates enhanced the enzyme production and the response of enzyme production versus substrate concentration was in bell shaped pattern rather than a linear one. The relationship of the experimental variables (concentration of both inducer substrates in different combinations) and the response (induction of xylano-pectinolytic enzymes) was calculated by the second order polynomial equation for response surface method (RSM).

Using CCD, RSM was carried out in order to determine the optimum concentration of two inducers i.e., WB and CP in production medium for concurrent production of xylanase and pectinase from *B. safensis* M35, *B. altitudinis* R31, *B. altitudinis* J208. Table 7.6. represents the CCD of 11 runs with corresponding responses

of xylanase and pectinase production by the three isolates.

**Table 7.6. Xylanase and pectinase production by selected *Bacillus* spp. in individual runs of the CCD:**

| Standerd Order | Run Order | $F_1$:WB (g) | $F_2$:CP (g) | *B. safensis* M35 | | *B. altitudinis* R31 | | *B. altitudinis* J208 | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | $R_1$:X | $R_2$:P | $R_1$:X | $R_2$:P | $R_1$:X | $R_2$:P |
| 1 | 6 | 0.482 | 0.482 | 5.43 | 138.47 | 5.32 | 316.39 | 4.97 | 328.09 |
| 2 | 8 | 2.568 | 0.482 | 8.37 | 158.06 | **14.45** | 336.33 | **14.90** | 407.44 |
| 3 | 11 | 0.482 | 2.568 | 0.64 | 0.00 | 0.76 | 0.00 | 0.53 | 0.00 |
| 4 | 5 | 2.568 | 2.568 | 0.90 | 43.57 | 0.82 | 66.30 | 0.90 | 94.89 |
| 5 | 9 | 0.050 | 1.525 | 0.86 | 0.00 | 0.66 | 24.52 | 0.76 | 3.98 |
| 6 | 4 | 3.000 | 1.525 | 4.77 | 44.07 | 7.11 | 342.17 | 6.51 | 129.06 |
| 7 | 7 | 1.525 | 0.050 | 8.46 | 188.78 | 12.42 | 392.57 | 14.53 | 309.19 |
| 8 | 2 | 1.525 | 3.000 | 0.72 | 11.94 | 0.58 | 0.00 | 0.58 | 19.35 |
| 9 | 10 | 1.525 | 1.525 | **14.43** | **372.47** | 8.66 | **612.29** | 8.92 | **648.66** |
| 10 | 1 | 1.525 | 1.525 | **13.96** | **356.58** | 8.92 | **664.73** | 9.30 | **671.51** |
| 11 | 3 | 1.525 | 1.525 | **14.51** | **361.04** | 9.37 | **646.73** | 9.68 | **751.02** |

Response values presented are Mean values of units for n=3.

As can be seen from the table, out of 11 runs, combination of 9 to 11 (centre point type) with 1.525 % WB, 1.525 % CP exhibited maximum 14.51 units of xylanase, and 372.47 units of pectinase activities for *B. safensis* M35. The highest values of xylanase activities for *B. altitudinis* R31 and J208 were observed were 14.45 and 14.90 units respectively from 2.568% WB and 0.482% CP concentrations in std order 2. Whereas, the highest values of pectinase activities for *B. altitudinis* R31 and J208 were found to be 664.73 and 751.02 units respectively in 1.535% WB and 1.525% CP concentrations in std order 9-11.

During analysis of response data, selection of a suitable model becomes the important step. Design expert software, uses the fit summary report for such analysis. Where it fits linear, 2-factor interactions (2FI), quadratic and cubic polynomial model to the response data and suggests the most suitable model amongst them for further data analysis. Fit summary report includes F- value statistics, Lack of fit test, and Regression coefficient ($R^2$) analysis ($R^2$ and Predicted Residual Error Sum of Squares, PRESS) which helps in selection of most appropriate model for obtained response. Significant *p*- value, insignificant Lack of Fit value and correlation coefficient ($R^2$) values>0.7 indicate that the model fits well with the response data. Thumb rule that the difference between the predicted $R^2$ and adjusted $R^2$ should not be >0.2 applied here. Which

indicates a good correlation between the predicted and experimental $R^2$. PRESS is a measure of how a particular model fits into each design point. PRESS for the chosen model should be less as relative to other models under consideration. Adequate precision is a measure of a signal to noise ratio. It's ideal desired value should be minimum 4. Adequate precision value above 4 indicates preference for model. Based on this information, the effects of inducer substrates and their interactions on responses of xylanase and pectinase enzyme production were analyzed further.

### 7.3.7. Xylanase and pectinase response obtained from the CCD experiment:

Analysis for Fit summary plots, ANOVA analysis and model diagnostic plots suggested that for production of xylanase and pectinase both enzymes from the media containing both substrates, the response followed a quadratic model as in all six responses, lack of fit was non-significant and ANOVA analysis signified the model. This data in detail is further added in Appendix II. Contour plots representing the xylanase and pectinase responses, and further point predication analysis are as mentioned below.

- **Xylanase production response:**

Contour plots of xylanase production response for WB and CP variables are presented in Fig.7.8. The highest response values were when analyzed through axis adjustments for each contour plots, it was observed that, moderate concentration of WB (~1.6g) and CP (~1.2g) yielded highest xylanase response from *B. safensis* M35, whereas high WB (~2.3g) and low CP (~0.5g) concentrations yielded highest xylanase responses from both *B. altitudinis* isolates R31 and J208.

- **Pectinase production response:**

Contour plots of pectinase production response for WB and CP variables are presented in Fig.7.9. When the highest response values were analyzed through axis adjustments for each contour plots, it was observed that, moderate concentration of WB (~1.5-1.6g) and CP (~1.2-1.3g) yielded highest xylanase response from all three isolates.

Thus, use of raw agrowaste biomass such as CP and WB are inducers of xylano-pectinolytic enzymes, and their combination is beneficial for the cost of xylanase and pectinase production can be reduced. Hence, to achieve the production of both in an appreciable amount, the point prediction method was performed.

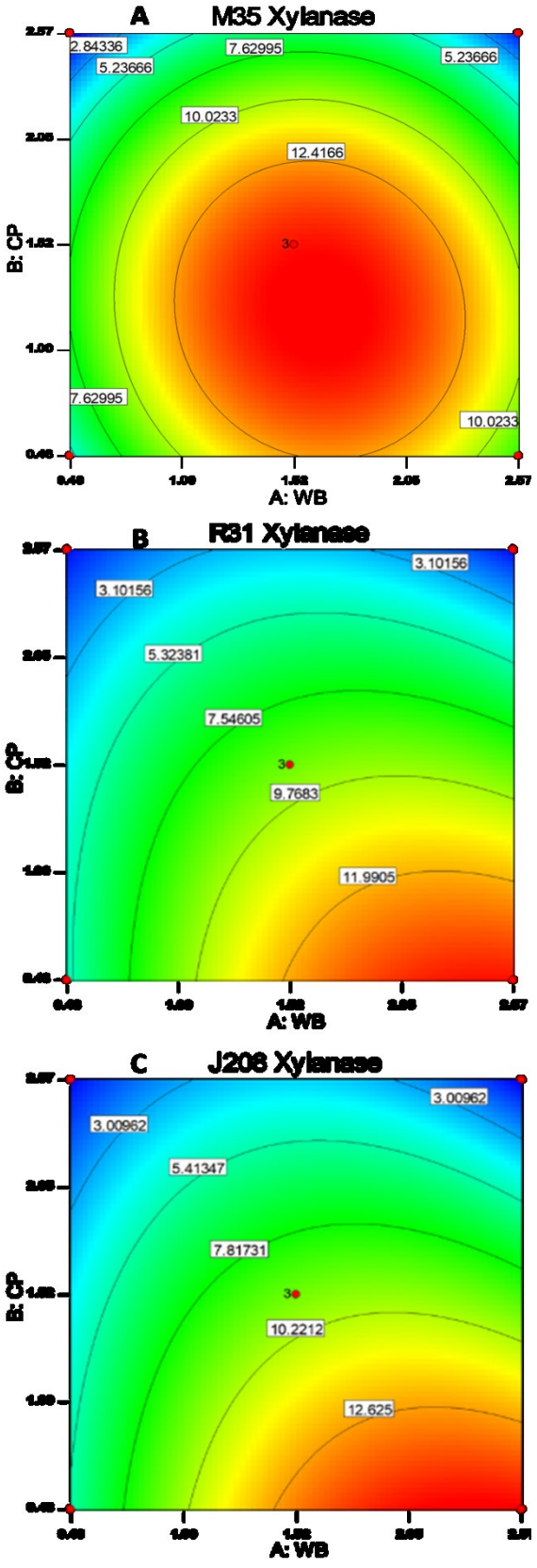



**Figure 7.8. Contour plots exhibiting effects of WB and CP on xylanase production response from three *Bacillus* strains:**
**(A)** *B. safensis* M35, **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208; Value in each box beside contour line represents the xylanase activity units.

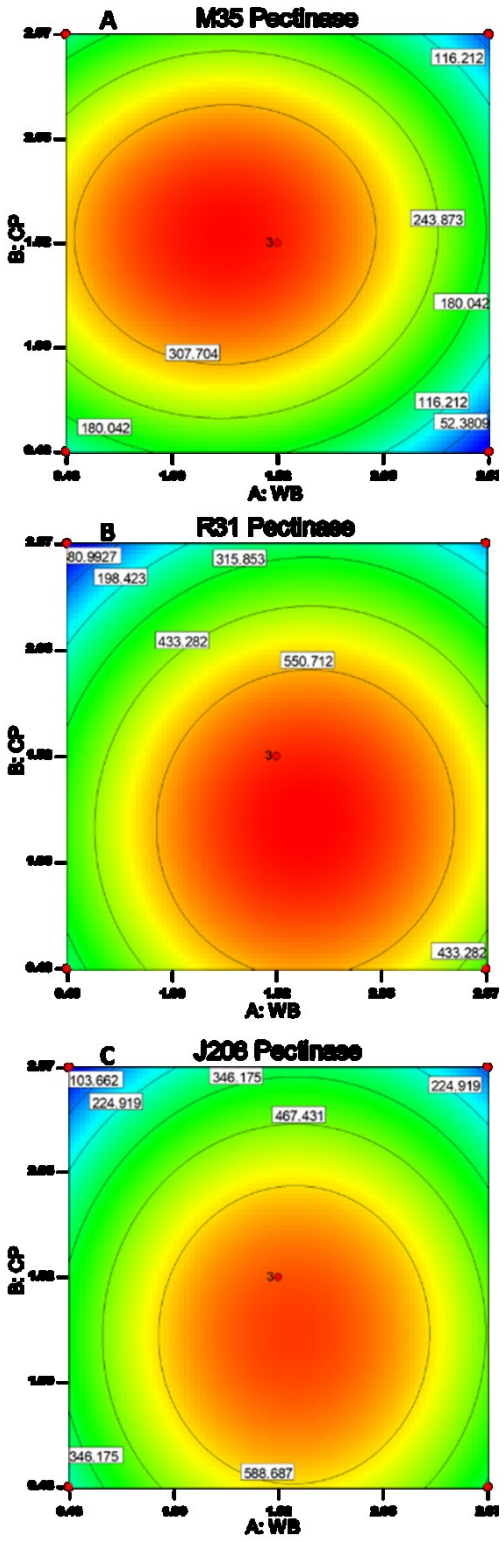



**Figure 7.9. Contour plots exhibiting effects of WB and CP on pectinase production response from three *Bacillus* strains:**
**(A)** *B. safensis* M35, **(B)** *B. altitudinis* R31 and **(C)** *B. altitudinis* J208; Value in each box beside contour line represents the pectinase activity units.

## 7.3.8. Optimization, point prediction and validation of predicted points:

Further, based on the above data and analysis using the Design Expert Software,

for optimized production of xylano-pectinolytic enzymes from the single media amended with both WB and CP, initially numerical as well as graphical optimization for xylanase and pectinase production responses were carried out and based on this further point prediction was performed.

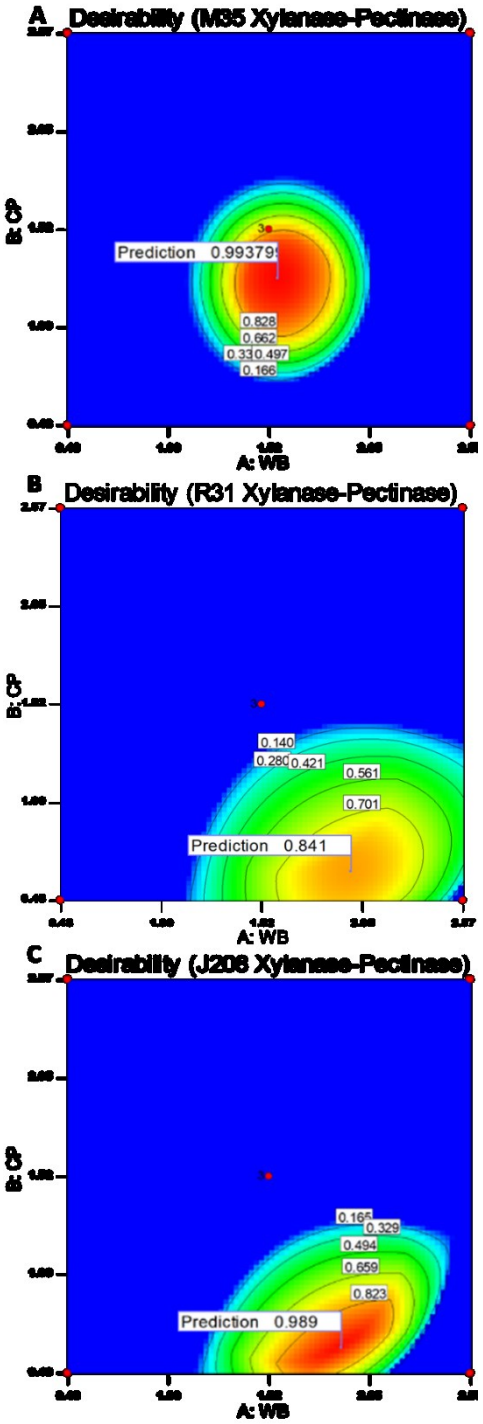



**Figure 7.10. Desirability plots for optimized responses of xylanase and pectinase production from three *Bacillus* strains obtained through numerical optimization:**
(A) *B. safensis* M35, (B) *B. altitudinis* R31 and (C) *B. altitudinis* J208; X and Y axis represent concentration (in %w/v) of WB and CP respectively; Value in each box beside contour line represents the desirability value.

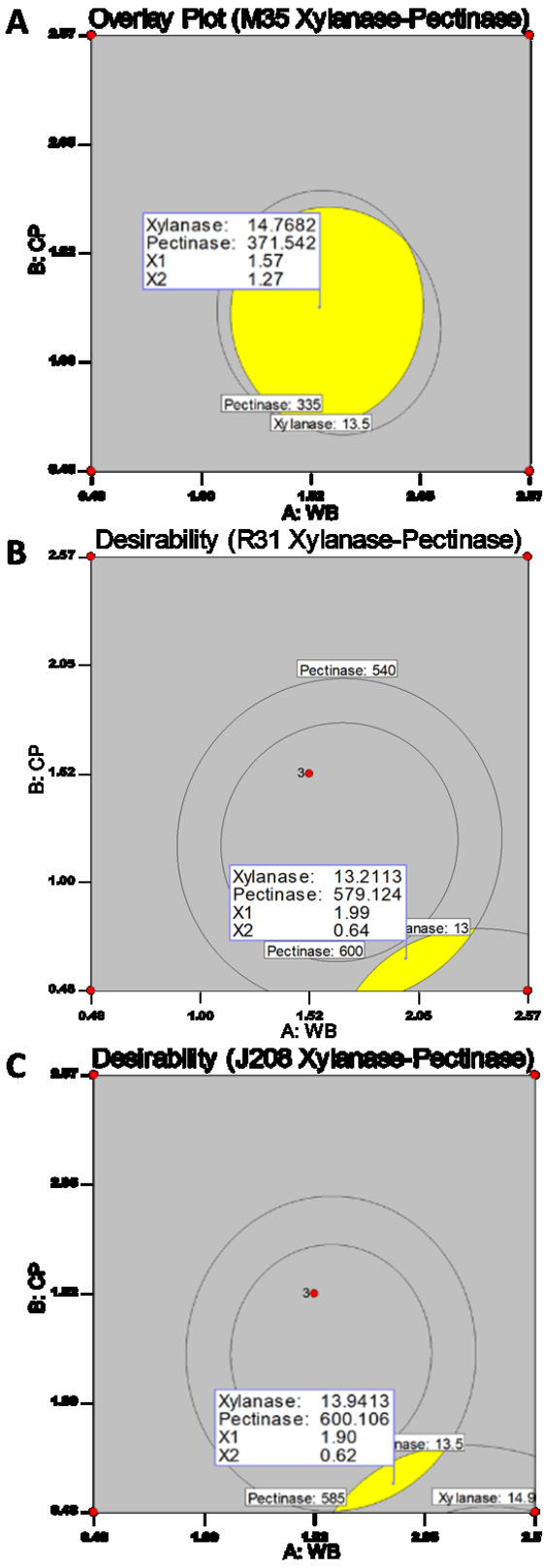



**Figure 7.11. Overlay plots for optimized responses of xylanase and pectinase production from three *Bacillus* strains obtained through graphical optimization:**
(A) *B. safensis* M35, (B) *B. altitudinis* R31 and (C) *B. altitudinis* J208; X and Y axis represent concentration (in %w/v) of WB and CP respectively; Yellow coloured region indicates the range of predicted highest response; Value in large box represents the predicted maximum responses at mentioned variable concentrations.

Numerical optimization was performed with the same range of WB and CP concentrations which was used in CCD experiments for maximized production response of xylanase and pectinase. And desirability plots for combined production of xylanase and pectinase are presented in Fig 7.10. Desirability values approaching 1.0 are most preferable. Analysis through axis adjustments of each contour plots suggested desirability value >0.84 for both optimized responses from a single media. The corresponding concentrations of WB and CP variables for X and Y axis values at the highest desirability value, on combination help to generate the maximum xylanase and pectinase production response.

The region of highest desirability values was observed in range of variables which produced 90-100% of maximum response observed during CCD studies. And hence, 90-100% of maximum response obtained was considered as margin of xylanase and pectinase production response for graphical optimization of media and the overlay plots for both responses were generated. As shown in Figure 6.16, the limits of both responses were marked as contours in graphs. Both xylanase and pectinase production responses for each isolate were overlapped. The overlapped graph for both responses for individual isolates are presented and the yellow coloured region from the overlapped graphs corroborated with the region of highest prediction from the figure 7.11.

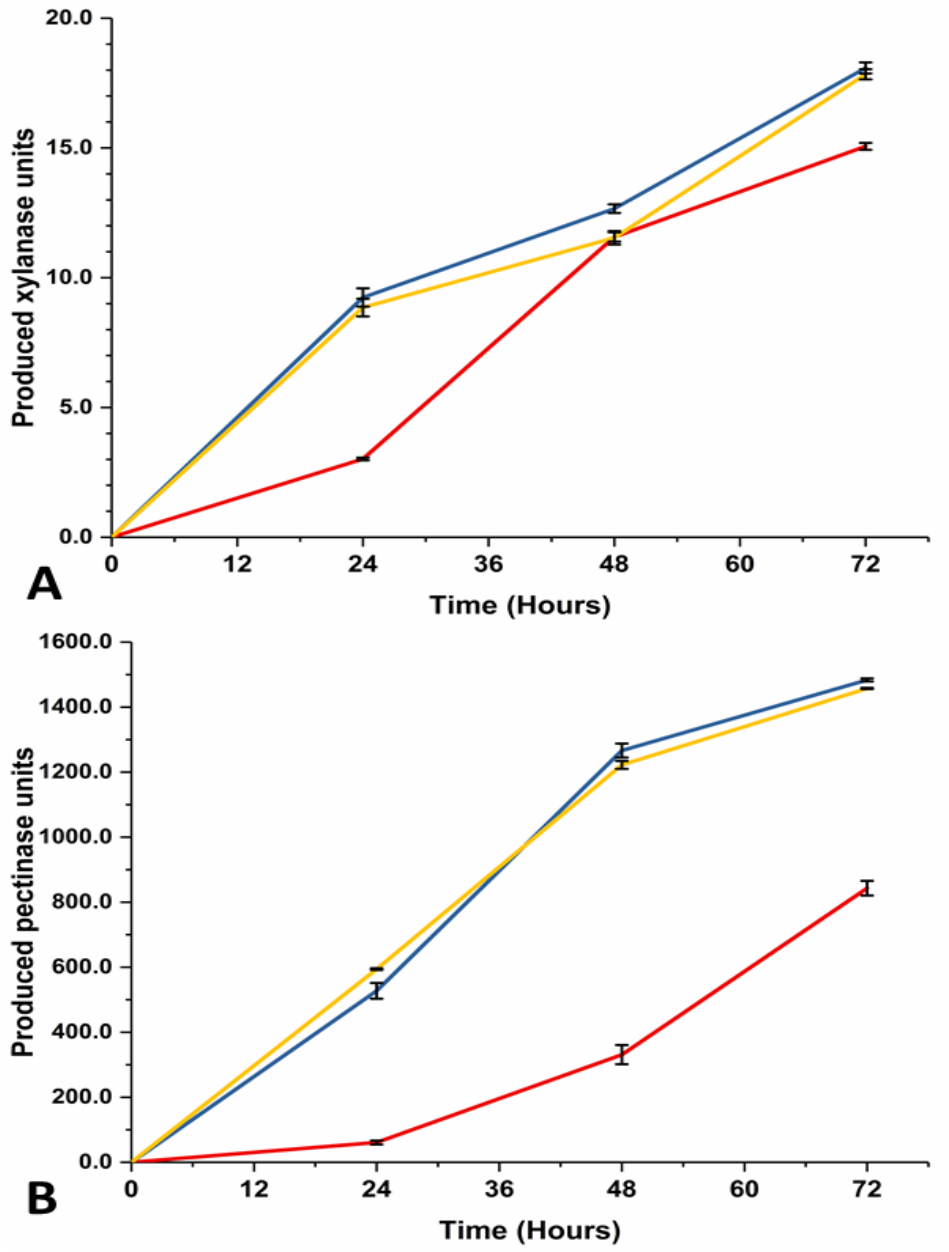
From numerical and graphical optimization along with overlay plots, the prediction of the point for optimized xylanase and pectinase production was done with help of Design Expert software and values are presented in the Table 7.7.

**Table 7.7. Point prediction for validation of the selected quadratic model:**

| Media composition and incubation conditions | | Organisms | | |
|---|---|---|---|---|
| | | ***B. safensis* M35** | ***B. altitudinis* R31** | ***B. altitudinis* J208** |
| Media components (g/100ml) | BHM | 0.327 | 0.327 | 0.327 |
| | Yeast Extract | 0.025 | 0.025 | 0.025 |
| | Peptone | 0.075 | 0.075 | 0.075 |
| Inducer substrate (g/100 ml) | WB | 1.57 | 1.99 | 1.90 |
| | CP | 1.26 | 0.64 | 0.62 |
| Physical parameters for incubation | Initial pH | Default (5.8) | Default (6.2) | Default (6.2) |
| | Temperature (°C) | 37 | 37 | 37 |
| | Agitation rate (rpm) | 160 | 160 | 160 |
| Inoculum (%v/v) | | 0.5 | 0.5 | 0.5 |
| Xylanase | Predicted response | 14.7 | 13.2 | 13.9 |
| | **Observed response** | **14.9** | **17.0** | **18.2** |
| Pectinase | Predicted response | 371.5 | 579.6 | 601.8 |
| | **Observed response** | **695.9** | **1464.9** | **1465.8** |

The values presented are Mean value obtained from Suggested CCD experiment.

In other words, the optimised medium for individual *Bacillus* spp. for concurrent xylanase and pectinase production was as per Table 7.7. Further to validate the prediction of the model, experiments were performed, where the optimized concentrations of WB and CP in media for individual isolate was used. And the response obtained of xylanase and pectinase production were recorded in Table 7.7 for each isolate. Experimental values exceeded the predicted values for all xylanase and pectinase responses. These results verified the validity of the model and existence of the optimal points indicating the adequacy of the RSM data.



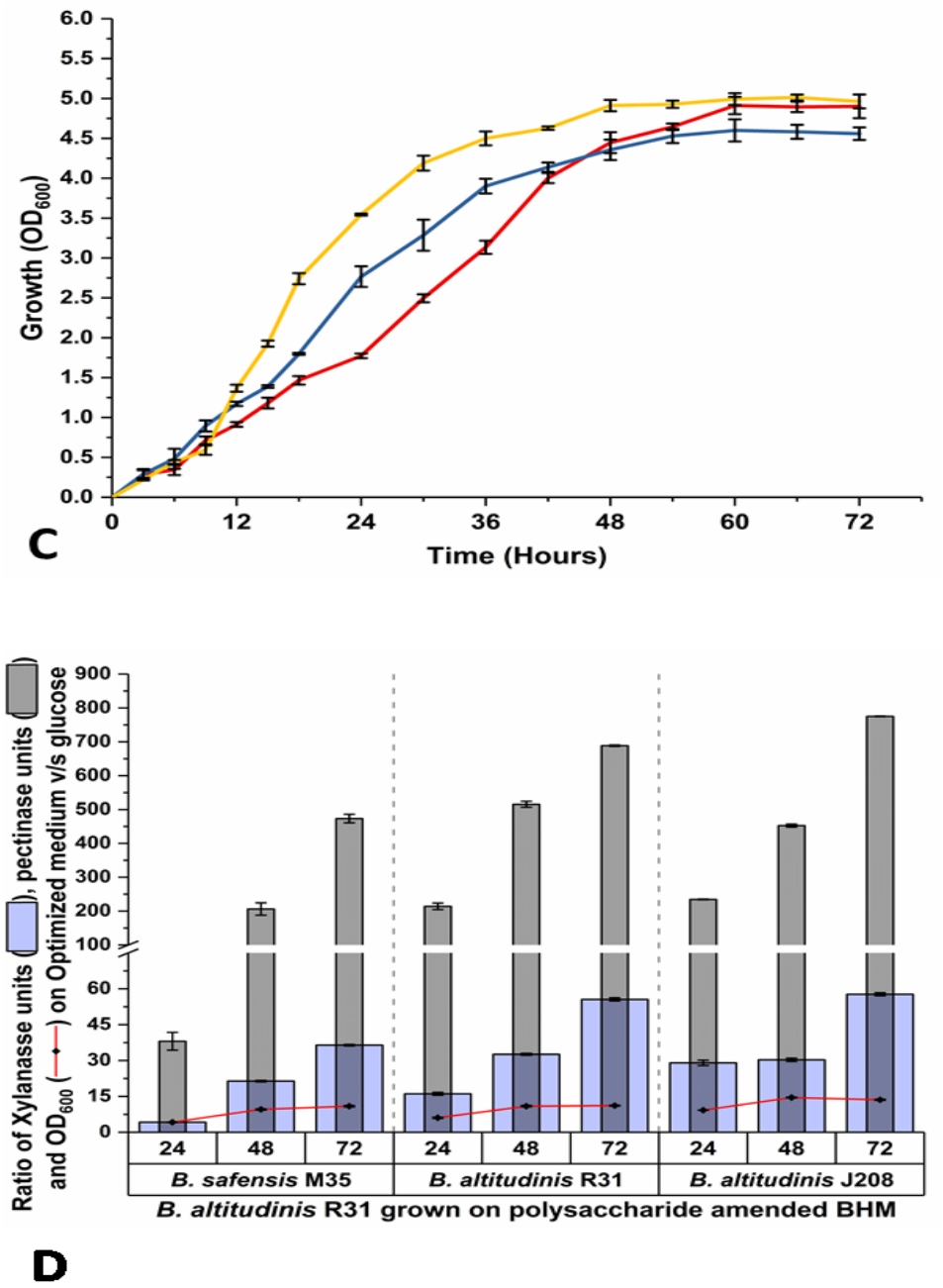



**Figure 7.12. Xylanase-pectinase production by the three *Bacillus* strains and their growth on optimized production medium.**
**(A)** Growth **(B)** Xylanase and **(C)** Pectinase. *B. safensis* M35 (red), *B. altitudinis* R31 (blue) and *B. altitudinis* J208 (yellow); **(D)**: Comparison of growth ratio (red line) with xylanase activity ratio (blue column bars) as well as pectinase activity ratio (dotted column bars) for modified production media (MPM). Values plotted are Mean ± Standard Error of Mean (SEM) for n=3;

Figure 7.12 represents the xylanase and pectinase activities obtained on optimised production media (OPM) as well as the activity ratios and growth ratios for (OPM) versus glucose for each isolate. Comparison of $OD_{600}$ and growth ratios of OPM with those on WB and CP containing media revealed that the OPM enhanced the growth of bacterial isolates several folds than individual WB or CP containing medium. Further

comparison of xylanase and pectinase ratios confirmed that though the OPM acted as a growth medium, the ratio of xylanase production was 3 to 4 times more than the growth ratio and ratio of pectinase production was 25-50 times more than the growth ratio. Hence, it can be concluded that the optimization of inducer substrate concentrations for concurrent as well as enhanced production of xylanase and pectinase was effectively carried out for each of the isolate, i.e., *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208.