Summary

Chapter 2: Isolation, screening and identification of bacterial isolates for production of plant polysaccharide hydrolyzing enzymes:

- Ruminant dung, digested or decayed plant materials were sources of sampling for isolation of bacteria producing polysaccharide hydrolase enzymes. Eleven samples used for isolation and enrichment comprised six dung samples of ruminant herbivores including camel, bull, cow and buffalo fed on locally harvested dried crops and grass; three samples from farm yard manure containing grass, plant parts as well as animal dung; and two partially decayed wood samples. Other two samples were collected from Winogradsky's columns developed from vegetable and newspaper waste (Table 2.2).
- Total of 462 different isolates selected from above mentioned 13 samples, and 6 laboratory isolates were subjected to qualitative screening by plate-based assays for production of xylanase (X), pectinase (P) and cellulase (C) individually on crude and pure polysaccharide substrates. From these, 174 isolates were selected based on their ability to produce single (C, P or X), two (CP, CX or PX) as well as three (CPX) enzymes included, 116 CPX positive, 29 CX positive, 8 PX positive, 1 CP positive, 10 X positive, 9 C positive and 1 P positive isolates (Figure 2.2 and Table 2.3).
- The 174 isolates were further screened quantitatively for activities of xylanase (cut-off 3 units) and pectinase (cut-off 11 units). 136 isolates were found to produce xylanase and/or pectinase when grown on pure or crude substrates. 66 of them exhibited both activities below cut-off and were discarded. From remaining 70, seven isolates exhibited both xylanase and pectinase above cut-off values, 44 exhibited pectinases above cut-off and xylanase below cut-off, while 19 exhibited xylanases above cut-off and pectinase below cut-off. All 70 isolates were rod shaped, Gram positive and endospore forming bacteria (Figure 2.3 - 2.4 and Table 2.4 - 2.5)
- Out of these 70, 54 morphologically distinct isolates with xylanase and/or pectinase activities above cut-off values were further sorted on the ARDRA based phylogenetic analysis where these xylanase, pectinase and xylanase-pectinase producers clustered in single OTU with *B. pumilus*, *B. altitudinis* and *B. safensis* type strains. ARDRA did not give any differentiation among the isolates (Figure 2.5 - 2.6).
- Finally, seven isolates i.e., M18, M33 and M35 (from camel dung); R30 and R31 (from bull dung); as well as J208 and J216 (from buffalo dung) that exhibited highest cellulase free xylanase and pectinase activities were selected for further studies. BLAST analysis of 16S rRNA gene sequences revealed that M18, M33 and M35

isolates shared 99.7-100% homology with *B. safensis* FO-36b^T and R30, R31, J208 and J216 isolates shared 99.4-100% homology with *B. altitudinis* 41KF2b^T. Both of the above group also showed more than 99% similarity with *B. pumilus* 7061^T (Figure 2.7).

- To further clarify the identity, *gyrB* gene was amplified from the seven isolates and their RFLP analysis with amplified *gyrB* gene and BLAST analysis of *gyrB* gene sequences revealed similar pattern of clustering of these seven isolates with their respective type strains as mentioned above (Figure 2.8 - 2.10).
- The selected seven Gram positive, endospore forming, rod shaped bacteria were identified as: *B. safensis* M18, *B. safensis* M33, *B. safensis* M35, *B. altitudinis* R30, *B. altitudinis* R31, *B. altitudinis* J208 and *B. altitudinis* J216. The 16S rRNA and *gyrB* gene sequences were submitted to Gene Bank, NCBI database (Table 2.7).
- All seven isolates were strictly aerobic, able to grow at pH 5.0-10.0 with 0-7% NaCl concentrations, and at 15-45 °C. They utilized sucrose, glucose, mannitol, arabinose, trehalose, xylose, casein, xylan and pectin for growth. They were unable to utilize starch, cellulose and gelatin hydrolysis. As in case of type strains the isolates identified as *B. safensis* were sensitive to Penicillin G while those identified as *B. altitudinis* were resistant to it (Figure 2.11 and Table 2.8 - 2.10)
- *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 retained more than 50% of relative xylanase and pectinase activities between temperature range of 30-60 °C at the pH range 4.0-10.0 for xylanases and 6.0-10.0 for pectinases (Figure 2.12 – 2.13).
- These three cellulase free xylanase and pectinase producers, *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 were selected for further detailed studies. The strains were deposited to NCMR, Pune (Formerly known as MCC) and their strain accession numbers are as follows: *B. safensis* M35-MCC 3305, *B. altitudinis* R310-MCC 3308 and *B. altitudinis* J208 -MCC 3307.

Chapter 3: Synergism of crude xylanases and/or pectinases with commercial cellulase in enzyme cocktail mediated saccharification of raw agrowaste biomass:

- Comminuted raw Barley Husk (BH), Sugarcane Bagasse (SCB) and Wheat Husk (WH) were used in saccharification by individual enzymes C, P and X; cocktail of two enzymes CP, PX and CX and cocktail of three enzymes CPX.
- In individual application pectinase, loading yielded least saccharification than xylanases or cellulase. In spite of higher cellulose content (34.8, 41.4 and 36.0%) than

xylan (30.4, 28.2,18.0%) in raw BH, SCB and WH biomass respectively, the saccharification yield by crude xylanases (400 µg loading) was more than that of commercial cellulase (Figure 3.1).

- Highest saccharification obtained by crude xylanases, crude pectinases, commercial cellulase and 1N HCl were as follows:

% Saccharification of raw biomass by individual enzyme or chemical agent			
Enzyme / Chemical agent	**BH**	**SCB**	**WH**
C	**2.20**	**2.58**	**4.83**
P_4	1.38(R31)	1.54 (R31)	1.65 (R31)
X_4	**4.36 (J208)**	**4.69** (J208)	**7.01** (J208)
HCl	14.94	7.69	10.14

- All the cocktails CX_4, CP_4, P_4X_4 and CP_4X_4 from M35, R31 and J208 *Bacillus* cultures individually exhibited positive synergism as the practical yield (PY) exceeded the cumulative yield (CY) after 60 h application (Figure 3.2-3.5 and Table 3.1-3.4). CP_4X_4 cocktail mediated enzymatic saccharification of raw biomass yield was comparative to the HCl mediated saccharification of respective biomasses.

- **The maximum saccharification, observed in case of CP_4X_4 cocktail was 10.58, 9.68 and 16.14% from raw BH, SCB and WH biomass respectively as depicted below:**

% Saccharification of raw biomass by enzyme cocktail									
Enzyme Cocktail	**Raw BH**			**Raw SCB**			**Raw WH**		
	PY	**CY**	**Isolate**	**PY**	**CY**	**Isolate**	**PY**	**CY**	**Isolate**
CX_4	8.42	6.67	(J208)	10.08	7.22	(J208)	14.71	11.84	(J208)
CP_4	6.45	3.63	(J208)	6.33	3.72	(M35)	11.72	6.20	(J208)
P_4X_4	9.37	5.69	(J208)	6.69	5.67	(J208)	9.88	8.38	(J208)
C P_4 X_4	**10.58**	7.99	(J208)	**9.68**	8.20	(J208)	**16.14**	13.21	(J208)

- The practical yield of saccharification of raw BH, SCB and WH increased in the order as follows: CP_4X_4 > CX_4 >CP_4 > P_4X_4. This was in accordance with the proportion of cellulose, xylan and pectin components present in the biomass.
- Structural analysis by SEM of commercial cellulase treated raw biomass showed structural changes giving flaky appearance, surface roughness and unevenness of the parenchymatous pith cells which was further enhanced after CPX cocktail treatments (Figure 3.6 -3.8).
- The peaks attributed to cellulose and hemicellulose (1430, 1376, 1320, 1251,

1160, 1120, 1061, 997 and 897 cm^{-1}) components decreased in FTIR analysis of raw agrowaste biomass with commercial cellulase treatment and further decreased with CP_4X_4 cocktail treatment indicating gross chemical changes in the biomass (Figure 3.9).

Chapter 4: Physicochemical pretreatment of sugarcane bagasse for biomass deconstruction:

- Out of three different raw agro-waste biomass, i.e., BH, SCB and WH, based on the industrial importance, cellulose content and bulk biomass availability, SCB was selected for further pretreatment and enzyme cocktail mediated saccharification studies.
- Morphological analysis of raw and pretreated SCB biomass revealed several visible differences. Hydrothermal pretreatments (autoclave and steam explosion) enhanced softness and opacity of biomass. Steam explosion imparted more fluffy, bright and whitish appearance to biomass. Chemical pretreatments separated the fluffy pith and vascular bundles giving biomass more fibrous appearance. Alkali pretreatment imparted yellow coloration to biomass while acid pretreatment imparted pink coloration to biomass (Figure 4.1).
- Among all pretreatments highest amount of soluble reducing sugar was released by H_2SO_4 pretreatment (3.0-8.0%), where 1.25% SCB loading at 3.0% H_2SO_4 loading released maximum ~8% of soluble reducing sugar. Further NH_4OH (0.4-0.8%), Steam explosion (0.2-0.6%), Autoclave (0.2-0.5%) and NaOH (0.1-0.4%) was the order in which the release of % SRS decreased (Figure 4.2).
- The released SRS by TLC and HPLC was below detectable range for Autoclave and Steam explosion pretreated filtrate while negligible amount of glucose was detected in Alkali and NH_4OH pretreatment filtrate. Largest peak for xylose was observed in acid treatment filtrate suggesting breakdown and removal of hemicellulosic mass from SCB (Figure 4.3-4.4).
- Among all pretreatments highest amount of loss in dry wright of biomass was observed for NaOH pretreatment, where 1.25% SCB loading at 3.0% NaOH loading imparted highest loss of ~45% in dry weight. Further H_2SO_4, NH_4OH, Autoclave and Steam explosion was the order in which highest values of loss of % dry weight in biomass decreased from 38% to, 16%, 13% and 7% respectively (Figure 4.5).
- Presence of additional peaks, other than glucose and xylose, in HPLC chromatogram from NaOH, NH_4OH and H_2SO_4 pretreatment filtrate also suggested

release of soluble components other than reducing sugars, like lignin from SCB biomass. Number and area under the peaks decreased in order of NaOH, NH_4OH and H_2SO_4 pretreatments (Figure 4.4).

- Theoretically calculated amount of soluble compounds other than reducing sugars were maximum in case of NaOH pretreatment followed by H_2SO_4, NH_4OH, Autoclave and Steam explosion (Table 4.2).
- NaOH treatment gave the best results among all the pretreatments since it released least reducing sugar and highest loss of dry weight in terms of other solubles as depicted below.

Pretreatment of SCB biomass	% Released SRS in filtrate		% Soluble other than SRS	% loss in Dry Weight
	Glucose	Xylose		
Raw	-	-	-	-
Autoclave	-	-	++	++
Steam explosion	-	-	+	+
Alkali (NaOH)	**+**	**-**	**++++**	**++++**
AFEX (NH_4OH)	+	-	++	++
Acid (H_2SO_4)	+	+++	+++	+++

- Since the H_2SO_4 pretreatment removed the major hemicellulosic fraction of biomass thereby easing the cellulose accessibility, however due to loss of hemicellulose component, H_2SO_4 pretreatment was not considered for further saccharification studies.
- Chemical characterization by FTIR of raw and PSCB revealed drastic differences in SCB after NaOH and NH_4OH pretreatments. Peaks observed at wavenumbers 1734, 1630, 1600, 1510, 1325, 1270, 1070, 833 cm^{-1} etc. were attributed to chemical nature and bond properties present in lignin which significantly decreased after NaOH pretreatments. NH_4OH pretreatment gave moderate decrease (Figure 4.7).
- Structural characterization by SEM of raw and PSCB revealed drastic changes in morphology of biomass like enhanced deformations in the cell walls of the parenchymatous pith regions giving it scaly and damaged appearance, enhanced roughness on the surface of cell wall as well as unevenness and sharpness of cell wall margins (observed at higher magnification). NaOH pretreatment affected biomass severely and parenchymatous pith, soft tissue cells were shattered and separated from the vascular bundles giving the biomass more fibrous appearance (Figure 4.8).
- All pretreatments enhanced the cellulose, xylan and pectin accessibility to commercial cellulase, M35, R31 as well as J208 crude xylanase and M35, R31 and J208

crude pectinase respectively suggesting that the pretreatments efficiently increased the biomass saccharification. **Commercial cellulase yielded highest saccharification of 38.81% from the 1% NaOH PSCB. M35 xylanase and pectinase gave maximum saccharification of ~15.6% and 5.88% respectively from 2% NaOH PSCB** (Figure 4.9-4.11)**.**

- In general, the accessibility was higher for cellulase than xylanase and pectinase as it is evident from the following data:

% Saccharification of different PSCB biomass by individual enzyme		
Saccharifying agent	**NaOH PSCB**	**Other PSCB***
C	**38.81**	**9.62**
P_4	5.18 (M35)	1.79 (M35)
X_4	**15.59** (M35)	**5.17** (M35)

*Other PSCB included Autoclave, Steam explosion and NH_4OH treated SCB

- Fluorescence microscopy of safranin stained sections, of cellulase treated stele region of PSCB showed ruptured inner most circles of pith cells surrounding the vascular bundle in case of hydrothermal pretreatment of steam explosion and autoclave pretreatment. In NH_4OH pretreatment the innermost pith cell layer was intact. After NaOH pretreatment, the cell wall of soft pith cells was completely separated from vascular bundles which gave fibrous appearance to PSCB (Figure. 4.12 - 4.17).

Chapter 5: Enzyme cocktail mediated saccharification of pretreated Sugarcane bagasse (PSCB):

- Additive approach exhibited positive synergism as the practical yield (PY) by each of CP, CX, PX and CPX cocktail exceeded the cumulative yield (CY) from PSCB. **Among all pretreatments, the additive M35-CPX, R31-CPX and J208-CPX cocktails gave highest saccharification of 81.30%, 72.24% and 71.42% respectively on NaOH pretreated SCB biomass (**Figure 5.1 - 5.4 and Table 5.1 - 5.3**).**
- The maximum saccahrification given by other additive cocktails like CP, CX and PX were as shown below.

Maximum % saccharification of different PSCB biomass by additive enzyme cocktails						
Enzyme Cocktail	**NaOH PSCB**			**Other PSCB***		
	PY	**CY**	**Isolate**	**PY**	**CY**	**Isolate**
CP	60.52	53.90	(R31)	12.55	11.29	(R31)
CX	76.56	61.10	(M35)	15.64	14.77	(M35)
PX	25.42	21.47	(M35)	7.94	6.83	(R31)
CPX	**81.30**	**65.70**	**(M35)**	**17.27**	**16.43**	**(R31)**

*Other PSCB included Autoclave, Steam explosion and NH_4OH treated SCB

- The substitutive cocktails of M35, R31 and J208 xylanase together and M35, R31 and J208 pectinase together gave 14.3 and 5.98 % saccharification with NaOH pretreated biomass which is higher as compared to the saccharification by individual M35, R31 and J208 xylanases and pectinases. Saccharification values of individual xylanases and pectinases with substitutive xylanase (X_{sub}) as well as substitutive pectinase (P_{sub}) are given below.

% Saccharification by individual M35, R31 and J208 accesosry enzymes and their cocktail								
Raw and pretreated SCB biomass	**Xylanases**				**Pectinases**			
	M35-X	**R31-X**	**J208-X**	**(X_{sub})**	**M35-P**	**R31-P**	**J208-P**	**(P_{sub})**
Raw	2.62	2.55	2.67	**2.9**	1.16	1.12	0.98	**1.32**
Autoclave	3.88	3.93	4.03	**4.21**	1.79	1.72	1.76	**2.01**
Steam Explosion	4.25	4.30	4.51	**4.74**	1.39	1.45	1.48	**1.72**
NaOH	15.59	13.60	12.90	**14.30**	5.88	5.56	5.59	**5.98**
NH_4OH	5.17	5.13	5.02	**5.47**	1.73	1.68	1.74	**1.92**

- Application of substitutive PX, CX, CP and CPX cocktails also yielded highest saccharification from 1.25% SCB biomass loading treated with 1.0% NaOH. **The substitutive CPX cocktail containing seven enzyme preparations of M35, R31 and J208 xylanases as well as M35, R31 and J208 pectinases with commercial cellulase Primafast®200 exhibited 84.02%** Saccharification. Further, CX, CP and PX was the order of substitutive cocktails in which the saccharification yield decreased i.e., 77.89, 65.45 and 26.44% respectively (Table 5.6)
- The crude M35, R31 and J208 xylanases and pectinases performed on par with each other as the additive CPX cocktail values suggest. Rather the six enzymes (three xylanases and three pectinases) complemented each other to enhance the cellulase mediated saccharification as the substitutive CPX cocktail suggests (Figure 5.6).
- FTIR spectra of substitutive CPX cocktail treated PSCB revealed decrease in the peaks attributed to cellulose and hemicellulose components as discussed in Chapter 3 which was more in comparison to the FTIR analysis of substitutive CPX treated raw SCB (Figure 5.7).
- SEM analysis of pretreated biomass with substitutive CPX cocktail pointed structural damages such as the flaky appearance, surface roughness and unevenness of the parenchymatous pith cells like in the case of raw biomass discussed in chapter 3. However, as compared to CPX hydrolyzed raw biomass as discussed in Chapter 3, the damage in this case seemed to be more pronounced (Figure 5.8).
- The salient results of enzymatic saccahrification of SCB are as follows:

- Commercial cellulase gave only **2.58%** saccharification from raw SCB which increased to **38.81%** from SCB after pretreatment with NaOH. The additive M35-CPX, R31-CPX and J208-CPX cocktails of commercial cellulase, xylanase and pectinase from *B. safensis* M35, *B. altitudinis* R31, and *B. altitudinis* J208 enhanced saccharification to **8.13**, **7.84** and **9.68%** respectively from raw SCB which further enhanced after to pretreatment to **81.30**, **72.24** and **71.42%** respectively from SCB after pretreatment with NaOH. The substitutive CPX cocktail enhanced the saccharification further to **84.02%** suggesting that substitutive cocktails work better than additive cocktails.

Chapter 6: Studies on physicochemical properties of crude xylanases (xylan hydrolases) from selected isolates:

- CP induced CFS of M35, R31 and J208 exhibited highest units of pectinase activities i.e., polygalacturonate hydrolase or PGase (11.3-14.8 units), pectate lyase and pectin lyase (9461-14221 units) along with negligible xylanase activities. The saccharification activity therefore was contributed by PGase, PNL and PL enzymes from the three *Bacillus* cultures. WB induced CFS of M35, R31 and J208 exhibited highest amount of xylan hydrolase activities (4.8-5.5 units) and some amount of PGase (4.1-4.9 units), PNL (578-2261 units) and PL (701-2848 units) activities. In no case the CP and WB induced supernatant exhibited cellulase activity, confirming the cellulase free xylanase and pectinase production by M35, R31 and J208 (Table 6.2).
- Besides, FPase activity (5.0 units), the commercial cellulase Primafast®200 also exhibited, comparative amount of xylanase (4.8 units) and PGase (4.6 units) activity and 122.6 and 170.1 units of PNL and PL activities respectively (Table 6.2).
- As the complexity of xylan substrate increased, the xylanase activity decreased, yet the crude xylanases from all three isolates were able to act on both birchwood and beechwood xylan polymers. Relative to 100% activity on birchwood xylan, M35 xylanase demonstrated ~95% activity, whereas R31 and J208 xylanases respectively demonstrated 72% and 82% activity, on beechwood xylan (Figure 6.1).
- As the complexity in terms of degree of esterification (DE) of pectic polymer increased the activity of pectinases decreased. The hydrolase activity showed wide specificity than lyase activity on complex pectic substrates. Relative to 100% activity on PGA, hydrolase activity on DE35 pectin and DE65-DE70 pectin were found to be in range of 85-97% and 66-80% respectively by three M35, R31 and J208 pectinases

and lyase activity on DE35 pectin and DE65-DE70 pectin was found to be in the range of 81-93% and 15-38% respectively by the M53, R31 and J208 pectinases (Figure 6.2).

- Zymography of crude M35, R31 and J208 xylanases revealed only single band in the gel from each crude CFS. Approximate MW of M35, R31 and J208 xylanase was calculated to be 100 kDa each. The reference strain whole genome analysis suggested that the xylanase from all three isolates belonged to hydrolase class (Figure 6.3).
- The crude pectinase showed a single protein band from CFS of the three *Bacillus* cultures in zymogram on both PGA and pectin individually. The molecular weight for this band was determined as ~32kDa for M35 pectinase and ~34kDa for R31 as well as J208 pectinase. The whole genome analysis of reference strain suggested that the pectinase from all three isolates included pectin lyase enzyme belonging to class lyase as the pectin hydrolase sequence was absent (Figure 6.4).
- Since xylan hydrolases have a significant role as accessory enzymes further characterization of only xylan hydrolases was undertaken. Study of xylanase enzyme kinetics revealed that the M35, R31 and J208 xylanase had more affinity for birchwood xylan (K_m 4.48, 4.40 and 5.76 mg/ml) than beechwood xylan (K_m 5.15, 6.02, 5.96 mg/ml). Similarly, the catalytic efficiency was also higher for birchwood xylan i.e., 6.23, 5.07 and 3.98 respectively than beechwood xylan viz., 4.85, 3.37 and 3.80 respectively (Table 6.4).
- The studies on combined effect of temperature and pH on crude xylanase activity revealed that the three xylanases have different optimum pH values (pH 7.0 for M35, between pH ~7.5 for R31 and pH 8.0 for J208), and temperature range (40-60 °C) for activity. Thus, if applied together in a cocktail, the working pH range of the xylanases will be expanded from acidic to alkaline (pH 5.0-9.0) conditions at temperature of 40-60 °C (Figure 6.5).
- Effect of temperature on stability of crude xylanase exhibited that, $t_{1/2}$ of M35 xylanase was highest (24 h) followed by J208 xylanase (13 h) and R31 xylanase (11 h) at 40 °C. Hence, although 45–60 °C was the range of maximum activity for the three xylanases. Extended exposure of the enzymes to temperatures above 50 °C resulted in decline in stability as well as residual activity (Figure 6.6).
- Effect of pH on crude xylanase stability revealed that all three xylanases were comparatively more stable in pH range 6.0 -9.0 than pH 4.0, 5.0 and 10.0 (Figure 6.7).
- Effect of crude xylanase activities on viscosity of birchwood xylan substrate

revealed that ~50% or more decrease in viscosity in unit time by the enzyme. Further Thin Layer and High-Performance Liquid Chromatography analysis revealed that the end product xylose was not detected in any of the hydrolysate system even after 10 h of hydrolysis (Figure 6.8 – 6.10).

- Studies on effect of different modulators on enzyme activities revealed that different monovalent cations like (1 mM) Na^+, Li^+, K^+, $(NH_4)^+$ slightly enhanced xylanase activities while 2 and 5% concentration of Na^+ decreased the activities of the three xylanases by ~9-21% and 10-28% respectively. Divalent cations like (1 mM) Ca^{2+}, Cu^{2+}, Mn^{2+} and Pb^{2+} enhanced activity while (1mM) heavy metals like., Hg^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+} decreased the xylanase activities of the three xylanases. Presence of 2-Mercaptoethanol was found to double the xylanase activity and other detergents and reducing agents also significantly enhanced activity of all three xylanases (Table 6.9).

Chapter 7: Selection and optimization of inducer substrate in media for concurrent production of xylanase and pectinase enzymes:

- Since xylanases and pectinases have been proven to be important accessory enzymes, their industrial application required low-cost and effective production process. Hence, use of crude polysaccharide from agro-waste as substrate for production of both enzymes simultaneously was studied.
- 0.5% of citrus peel (CP), cotton seed cake (CSC), molasses (M), pectin (Pn), rice bran (RB), wheat bran (WB) and xylan (Xn) were screened as crude or pure polysaccharide substrate by supplemting in BHM-YEP medium for production of xylanases and/or pectinases. Among them, Xn and Pn were commercial substrates while CP, CSC, M, RB and WB were raw substrates. Screening of these seven substrates for growth and enzyme production by *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 revealed highest growth on Xn. But Pn, WB, M, RB, CSC and CP in this order showed decrease in growth (Figure 7.1).
- M35, R31 and J208 produced the highest xylanase i.e., 5.3, 5.2 and 6.2 units respectively on Xn followed by 4.1, 4.8 and 5.1 units respectively on WB. M35, R31 and J208 produced the highest pectinase units i.e., 222, 275 and 255 respectively on Pn followed by 173, 222 and 252 units respectively on CP (Figure 7.2 – 7.3).
- For xylanases and pectinases, the Xn and Pn substrates respectively induced the highest enzymes but vice-versa was not so. WB, CSC and RB substrates produced decreased levels of xylanase in this order while CP, CSC and RB substrates produced

decreased levels of pectinase in this order. WB for xylanases and CP for pectinases were good inducer substrates

- When growth ratio, xylanase activity ratio and pectinase activity ratio were compared, BHM-YEP media supplemented with 0.5% molasses exhibited higher growth ratio than both the activity ratios suggesting that though molasses enhanced the growth, it did not induce either of the enzyme production. While 0.5% CP supplemented BHM-YEP media exhibited more pectinase ratio than growth ratio suggesting CP as an efficient pectinase inducer. Use of WB supplemented BHM-YEP media exhibited both xylanase and pectinase ratio higher than growth ratio suggesting that, though pectinase induction by WB was moderate, it can be an inducer for both xylanase and pectinase as can be seen from following data.

Maximum growth, xylanase and pectinase ratios as influenced by the inducer substrate

Inducer substrate	Growth (OD_{600}) ratio	Xylanase unit ratio	Pectinase unit ratio
CP	1.3	1.6	**109.5**
CSC	2.3	4.0	12.5
M	2.5	0.8	0.8
P	1.6	1.1	**136.4**
RB	2.0	4.5	8.8
WB	2.2	**13.5**	**15.2**
X	3.7	**17.2**	0.0

- Estimation of process kinetics parameters, i.e., volumetric rate of enzyme production and specific rate of enzyme production also supported the above observations (Table 7.2 - 7.3)
- Among all used crude substrates highest production rates were observed on WB and CP supplemented BHM-YEP medium as shown below.

Maximum production kinetic parametres observed on different crude inducer substrate

Inducer substrate	volumatric rate of enzyme production		Specific rate of enzyme production	
	Xylanase	Pectinase	Xylanase	Pectinase
CP	6.9 (J208)	**$6.2x10^4$ (J208)**	99.2 (J208)	**$8.9x10^5$ (J208)**
CSC	30.5 (R31)	$8.1x10^3$ (J208)	445.2 (R31)	$1.1x10^5$ (J208)
RB	19.1 (J208)	$5.2x10^3$ (R31)	275.4 (J208)	$9.8x10^4$ (J208)
WB	**60.1 (J208)**	**$1.4x10^4$ (R31)**	**865.0 (J208)**	**$2.0x10^5$ (R31)**

- At 2% WB concentration, M35, R31 and J208 gave 8.2, 7.82 and 8.83 units of xylanase activities respectively where as 216, 552 and 537 units of pectinase activities respectively (Figure 7.5).

- At 5% CP concentration maximum of 4.8, 4.4 and 4.6 xylanase units were produced by M35, R31 and J208 respectively whereas, at 1% concentration of CP highest pectinase activities were obtained as 83,397 and 491 units respectively by M35, R31 and J208.At lower concentration of 0.5-1.0% CP induced pectinases while higher concentration of 5.0% induced xylanase production. 2% or more WB decreased both xylanase and pectinase production (Figure 7.6).
- When 0.5% CP and 0.5% WB were combined in media production of xylanase and pectinase was increased in comparison to same amount of CP and WB supplementation to BHM-YEP medium individually (Figure7.7).

Enzyme	0.5% WB	0.5% CP	0.5% WB + 0.5% CP
Xylanase units	**4.3-4.9**	0.3-0.5	**5.4-5.7**
Pectinase units	115.6-226.1	**151.2-351.7**	**262.5-460.3**

- Central Composite Design of Response Surface Methodology was used to study the production response of xylanase and pectinase towards two variables of CP and WB. Hence, 0.25-3.0 g CP and WB were used to optimize their concentration for concurrent production of xylanases and pectinases.
- Two Factor model generated the table with 11 runs containing 3 repeated central values. 3 runs with central point type with 1.525 % WB, 1.525 % CP exhibited highest values of 14.51 units for xylanase, and 372.47 units for 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 in response of 2.568% WB and 0.482% CP combined concentrations. Whereas, the highest values of pectinase activities for *B. altitudinis* R31 and J208 were observed as 664.73 and 751.02 units in response of 1.535% WB and 1.525% CP combined concentrations (Table 7.6)
- 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 (Appendix-III).
- Contour plots generated from the selected quadratic model, were further used to determine the optimum values of concentrations of the WB and CP concentration for concurrent production of xylanase and pectinase from individual *Bacillus* spp. Using numerical and graphical optimization methods, concentration of CP and WB were

determined as: *B. safensis* M35: 1.57% WB and 1.26% CP; *B. altitudinis* R31: 1.99% WB and 0.64% CP; *B. altitudinis* J208: 1.90% WB and 0.62% CP (Figure 7.8 – 7.9)

- Using this composition of WB and CP as inducers in BHM-YEP medium, the predicted values and experimental response values of xylanase and pectinase obtained were as follows:

Response	Isolates	Predicted unit values	Experimental unit values
Xylanase	*B. safensis* M35	14.7	14.9
	B. altitudinis R31	13.2	17.0
	B. altitudinis J208	13.9	18.2
Pectinase	*B. safensis* M35	371.5	659.9
	B. altitudinis R31	579.6	1464.9
	B. altitudinis J208	601.8	1465.8

- **The optimized medium for concurrent production of xylanse and pectinase from each individual culture was as follows:**

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**