Biosynthesis of Xylobiose: A Strategic Way to Enrich the Value of Oil Palm Empty Fruit Bunch Fiber

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Received: February 3, 2012 / Revised: March 12, 2012 / Accepted: March 21, 2012

Xylooligosaccharides are functional foods mainly produced during the hydrolysis of xylan by physical, chemical, or enzymatic methods. In this study, production of xylobiose was investigated using oil palm empty fruit bunch fiber (OPEFB) as a source material, by chemical and enzymatic methods. Xylanase-specific xylan hydrolysis followed by xylobiose production was observed. Among different xylanases, xylanase from FXY-1 released maximum xylobiose from pretreated OPEFB fiber, and this fungal strain was identified as Aspergillus terreus and subsequently deposited under the accession Number MTCC- 8661. The imperative role of lignin on xylooligosaccharides enzymatic synthesis was exemplified with the notice of xylobiose production only with delignified material. A maximum 262 mg of xylobiose was produced from 1.0 g of pretreated OPEFB fiber using FXY-1 xylanase (6,200 U/ml) at pH 6.0 and 45°C. At optimized environment, the yield of xylobiose was improved to 78.67 g/100 g (based on xylan in the pretreated OPEFB fiber).

Keywords: Alkaline pretreatment, oil palm empty fruit bunch fiber, xylanase, xylobiose, xylooligosaccharides

Microbial xylanases (1, 4-β-β-xylanhydrolase, E.C. 3.2.1.8) are most widely used biocatalysts responsible for the breakdown of xylan, which have many industrial applications [2]. One of the recent and exciting applications of endoxylanases is xylooligosaccharides (XO) production, which has great commercial value because XO5 have potential applications in different sectors such as chemical, food, and pharmaceutical industries. For food applications, xylobiose is especially considered as this xylooligosaccharide has been used as a prebiotic, promoting the proliferation of bifidobacteria, beneficial microorganisms in the human intestine that reduce the risk of colon cancer [12]. XO5 are also known to impart physiological significance in human health mainly in reduction of cholesterol levels, maintaining gastrointestinal health, and improving the nutritional and sensory properties of food [17].

Industrially, production of XO5 was performed using different chemical and physical methods where controlling of reaction kinetics plays a significant role in synthesis of specific oligomers, which is one of the limiting factors for effective and economic production. Therefore, production of XO5 by enzymatic hydrolysis of xylan-rich lignocellulosic materials offers several advantages, like to minimize undesirable by-products, low quantities of monosaccharides production, controlling of monomeric number in oligosaccharides, and not requiring special equipment [4].

Xylan is the major constituent of hemicellulose and the second most abundant renewable polysaccharide after cellulose, and is considered as the substrate for xylooligomer synthesis [9]. To economize the process, several researchers evaluated for XO5 production from different lignocellulosic materials such as cotton stalk [4], sugar cane bagasse [9], almond shells [12], corn cobs [19], etc. Considering the fact that oil palm empty fruit bunch (OPEFB) fiber is one of the xylan-rich lignocellulosic materials containing 24% xylan [13], obtained abundantly as a residual matter from oil palm industry, the OPEFB fiber was selected for XO5 production. Moreover, the type of xylooligomer production depends majorly on the xylanase enzyme complex. Thus, a selection of a suitable microbial strain that produces preferable enzyme complex is one of the essential steps for XO5 production. Hence, in the present study, xylanases produced by five bacterial and three fungal strains were evaluated for the enzymatic hydrolysis of lignocellulosic materials for XO5 production. In addition to the source of enzyme complex, reaction process variables such as incubation time, pH, temperature, and enzyme concentration were optimized to improve the XO5 production.
Materials and Methods

Materials
OPEFB fiber used in the present study was collected from the palm oil industry located at Rajahmundry, India. The material was washed with water and dried in an oven at 60°C overnight to get constant weight and stored in a dry place until further use.

Alkaline Pretreatment of the Oil Palm Empty Fruit Bunch Fiber
Fifty grams of OPEFB fiber was placed in a 2 L Erlenmeyer flask and 500 ml of 0.5% NaOH solution added. This mixture was subjected to steam treatment at 121°C and 15 lbs pressure. After steam treatment for 20 min, the fiber was squeezed and washed with distilled water several times for neutralization. The final neutralized pretreated OPEFB fiber was dried in an oven at 60°C overnight and used for XOs production.

Microorganisms
Xylanase-producing bacterial isolates, BXY-5, BXY-6, BXY-7, BXY-8, and BXY-9, were grown on nutrient agar slants for 24 h at 37°C. Inoculum was prepared by adding a loopful of each culture from nutrient agar slants to nutrient broth and incubating in a shaker at 150 rpm at 37°C. After 24 h of incubation, each 1% (v/v) of inoculum was added to the production media for xylanase production. Fungal strains FXY-1, FXY-2, and FXY-3 were grown on potato dextrose agar slants for 72 h at 30°C. Spore suspensions were prepared by adding 10 ml of sterile water containing 1% (v/v) Tween 80 to each fungal agar slant and gently scraping with a sterile inoculation loop. The obtained spore suspensions were used for the production of xylanase enzyme.

Xylanase Production
Xylanase production was carried out under submerged fermentation using the following medium components (g/l): KH₂PO₄ - 0.5, K₂HPO₄ - 0.5, MgSO₄ - 0.5, NaCl - 0.5, Beef extract - 10. For bacterial xylanase production, 2% (w/v) wheat bran was used as substrate and the medium pH adjusted to 7.0, whereas for fungal xylanase production, 2% (w/v) OPEFB fiber was used as substrate with medium at pH 5.0 and kept for sterilization. Then 1% of each bacterial suspension or fungal spore solutions was added to the sterilized medium in separate flasks and incubated under agitation at 150 rpm. Bacterial xylanase production was carried out at 37°C for 24 h of incubation, whereas fungal xylanase production was carried out at 30°C for 72 h of incubation. Xylanase activity in the culture filtrate was determined using 1% (w/v) beech wood xylan as substrate according to the method of Bailey et al. [7]. One unit of xylanase activity is defined as the amount of enzyme liberating 1.0 µmol equivalents of xylose in 1 min per milliliter of enzyme solution.

Enzymatic Saccharification
Alkaline pretreated OPEFB fiber was subjected to enzymatic hydrolysis using the following procedure: One gram of alkaline pretreated OPEFB fiber was suspended in 9.0 ml of 100 mM citrate buffer, pH 5.0, and to this mixture, 1 ml of 10 times diluted different bacterial or fungal xylanase enzymes was added and incubated at 50°C in an orbital shaker at 150 rpm. Periodically, samples were collected up to 30 h and the enzymatic reaction was stopped by boiling the samples for 5 min before they were subjected to analysis for xylooligosaccharides.

Analysis of the Xylooligosaccharides by Thin Layer Chromatography
The xylooligosaccharides produced were qualitatively determined by using thin-layer chromatography. In this method, samples were applied to silica plates and developed with a solvent system containing 2-propanol, ethyl acetate, nitro methane, and water (6:1:1:2). Then the plates were air dried and the spots were visualized with an orcinol spray reagent (10 ml of H₂SO₄, 90 ml of methanol, 0.2 g of orcinol) by heating at 100°C. The oligosaccharides in the samples were identified by comparing their chromatographic behavior with the chromatographic behavior of authentic standards.

Analysis of the Xylooligosaccharides by High-Performance Liquid Chromatography (HPLC)
The obtained xylooligosaccharides and monosaccharides were analyzed by HPLC (SHIMADZU 10A) equipped with an Aminex HPX-87H column (Bio-Rad) with a refractive index detector using acidified distilled water (14 µl H₂SO₄, in one liter) as a mobile phase with a flow rate of 0.5 ml/min and at 65°C as the working temperature. Quantitative analysis of the reaction mixture was performed using HPLC. Xylooligosaccharides, xylobiose, and xylose revealed retention times of 9.0, 11.5, and 13.0 min, respectively. The concentration of the xylooligosaccharides was quantitatively estimated by comparing the peak area of the analyzed samples with that of standards (xylobiose, xylooligosaccharides, xylooligosaccharides, and xylotriose) and expressed as mg/ml of the hydrolysate.

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\text{XOs yield in the hydrolysate (g/100 g)} = \frac{\text{Total xylobiose/xylooligosaccharides in the hydrolysate (mg/g)}}{\text{XOs in the pretreated material}} \times 100
\]

Results and Discussion

Xylanase Production by Isolated Bacterial and Fungal Strains
In general, xylanolytic complexes produced by different microbial strains differ in the ratio of xylanase enzyme components such as endo -1,4-β-xylanases, 1,4-β-D-xylanases, α-1-arabinofuranosidase, α-1-glucuronidase, galactosidase, and acetyl xylan esterase, which play a major role on XOs production [8]. For higher xylooligosaccharide yield, the preferable xylanase enzyme complex should contain low levels of β-xylosidase activity [4], which is responsible for conversion of xylobiose to xylose. Hence, initial experimental studies were aimed to select a suitable xylanolytic complex-producing microbial strain. For this, five isolated bacterial strains (BXY-5, BXY-6, BXY-7, BXY-8, and BXY-9) and three fungal strains (FXY-1, FXY-2, and FXY-3) were screened. From analysis of the xylanase yield data, it was evident that the selected microbial strains differ in their xylanase-producing capability. Among the selected fungal strains, FXY-1 produced higher amounts of xylanase enzyme (6,207 U/ml) followed by FXY-3 (5,014 U/ml). The xylanase activities produced by
selected bacterial strains ranged from 2,448 to 3,355 U/ml. Among the different bacterial strains, the strains designated as BXY-7 and BXY-5 produced maximum (3,355 U/ml) and minimum (2,448 U/ml) amounts of xylanase. Comparative analysis revealed that the isolated fungal strains produced xylanase more effectively than all isolated bacterial strains (Fig. 1).

**XOs Production by Different Sources of Xylanase Enzymes**

To evaluate the potential of all produced xylanase complexes for XOs production, preliminary experiments were conducted using commercially procured beach wood xylan as substrate and the reaction mixtures were analyzed by thin layer chromatography. From the thin layer chromatogram (Fig. 2), it was noticed that all bacterial and fungal xylanases have potential to utilize beach wood xylan as substrate and convert this polymer to various forms of xylooligomers, especially xylobiose in major amounts and other oligomers in minor quantities (data not shown). However, the ratios of individual XOs differed with enzyme source. This data suggest that the genetic nature of the microbial strain influences the xylanase enzyme catalytic behavior with respect to variation in ratio of XOs production.

Utilization of pure xylan for production of XOs is an expensive and uneconomic process whether by the enzymatic and/or chemical method. Keeping this in view, further experimentation was aimed to use OPEFB fiber as a source of xylan. Initially, OPEFB fiber was treated with all the above bacterial and fungal xylanase complexes, and none of the enzyme complexes produced XOs from untreated OPEFB fiber (data not shown). This result is interesting, as the above xylanase enzyme complexes were produced using OPEFB fiber as substrate during fermentation and no detectable quantities of XOs were observed with OPEFB fiber as the xylan source. This may be attributed to the fact that with the use of raw OPEFB fiber, none of the enzyme complexes obtained from isolated microbial strains was able to catalyze the OPEFB xylan, which may be due to the inaccessibility of substrate to the enzyme. One of the limiting factors for this could be the lignin entanglement, or that none of the enzyme complexes were suitable for hydrolysis of the xylan present in the OPEFB fiber. The latter may not be applicable in the present study as all the enzyme complexes were able to catalyze the beach wood xylan and produce XOs, albeit in different concentrations (Fig. 2). From this study, it was noticed that production of XOs by direct enzymatic treatment is only suitable for susceptible materials such as citrus peels [6], apple pomace [11], etc.

To evaluate further and to overcome the lignin entanglement limitation, a chemical–enzymatic method was selected for XOs production from OPEFB fiber. Although lignin removal is also reported with high temperature treatment, a process known as auto hydrolysis [1], the chemical–enzymatic method is more advantageous, as this method does not produce undesirable by-products or high amounts of monosaccharides and requires little specialized equipment. In fact, many researchers focused their attention on XOs production using chemical and enzymatic methods from different lignocellulosic materials such as grape stalks [11], corn cob xylan [10], etc. Owing to the advantages associated with chemical and enzymatic methods, in the present study, initially OPEFB fiber was treated with alkali agent for lignin removal and the production of XOs was subsequently carried out with the delignified substrate. Initial experiments were conducted to select suitable conditions, were the OPEFB fiber was subjected to different concentrations of NaOH at varied solid–liquid ratios for different time intervals at 121°C. Among all tested
pretreatment conditions, pretreatment of OPEFB fiber using 0.5% (w/v) NaOH with solid-to-liquid ratio of 1:10 for 15 min of incubation at 121°C was highly accessible for xylanase for the production of XOs. This result suggested that the major limiting factor is lignin for effective conversion of OPEFB fiber xylan to XOs production by the enzymatic method. Hence for further studies, pretreated OPEFB fiber was used for XOs production.

Production of XOs from Pretreated OPEFB Fiber by Bacterial Xylanases

Fig. 3 illustrates XOs production by five bacterial xylanases from alkali-treated OPEFB fiber.

Production of XOs from Pretreated OPEFB Fiber by Fungal Xylanases

Fig. 4 illustrates xylobiose production and Table 2 represents xylotriose and xylose concentrations in enzymatic hydrolysates obtained by the three fungal xylanases. From Fig. 4, it was observed that the appreciable quantities of xylobiose was released in all three enzyme hydrolysates at 6 h of incubation, and gradually increased with incubation time and reached to a maximum at 24 h of incubation. Among the three fungal enzyme hydrolysates, the maximum (245 mg/g) xylobiose was obtained with FXY-1 xylanase and the minimum (217 mg/g) xylobiose was noticed at 24 h of incubation with FXY-2 xylanase. The enzymatic hydrolysis of the pretreated OPEFB fiber by FXY-3 xylanase produced 228 mg/g of xylobiose at a similar incubation time. The xylobiose concentration was gradually decreased in all enzyme hydrolysates after 24 h of incubation. Reduction in xylobiose production was observed with the function of time beyond 24 h of incubation.

In addition to the xylobiose, very little amounts of xylotriose and xylose were released by all bacterial enzymes (Table 1). The results showed that the maximum xylotriose (12.6 mg/g) was released with FXY-1 xylanase and 11.9, 11.5, 10.7, and 8.8 mg/g of xylotriose were noticed with FXY-2, FXY-3, FXY-8, and FXY-9 xylanases, respectively, at 30 h of incubation. The results revealed that the amount of xylotriose was nearly similar in all bacterial xylanases except FXY-9 xylanase. Among all bacterial xylanases, FXY-1 xylanase produced the maximum amount (21.1 mg/g) of xylose, followed by FXY-2 xylanase (9.1 mg/g) at 30 h of incubation, whereas FXY-7 and FXY-8 xylanases released negligible amounts of xylose at similar incubation time, and no xylose production was noticed with FXY-9 xylanase.

**Table 1.** Xylose ($X_1$) and xylotriose ($X_3$) production by different bacterial xylanases.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>BXY-5</th>
<th>BXY-6</th>
<th>BXY-7</th>
<th>BXY-8</th>
<th>BXY-9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$X_3$</td>
<td>$X_1$</td>
<td>$X_3$</td>
<td>$X_1$</td>
<td>$X_3$</td>
</tr>
<tr>
<td>6 h</td>
<td>10 ± 0.5</td>
<td>7.5 ± 0.2</td>
<td>8.9 ± 0.2</td>
<td>5.7 ± 0.1</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>12 h</td>
<td>10.5 ± 0.4</td>
<td>8.8 ± 0.2</td>
<td>9.2 ± 0.2</td>
<td>6.3 ± 0.1</td>
<td>7.5 ± 0.3</td>
</tr>
<tr>
<td>18 h</td>
<td>11.2 ± 0.5</td>
<td>9.4 ± 0.3</td>
<td>10.6 ± 0.4</td>
<td>7.1 ± 0.2</td>
<td>8.4 ± 0.4</td>
</tr>
<tr>
<td>24 h</td>
<td>11.7 ± 0.4</td>
<td>11.3 ± 0.4</td>
<td>11.4 ± 0.4</td>
<td>8.2 ± 0.3</td>
<td>10.4 ± 0.5</td>
</tr>
<tr>
<td>30 h</td>
<td>12.6 ± 0.3</td>
<td>21.1 ± 1.0</td>
<td>11.9 ± 0.4</td>
<td>9.1 ± 0.3</td>
<td>11.5 ± 0.3</td>
</tr>
</tbody>
</table>

± = Standard deviation.
xylanase from *Trichoderma viride* produced 138 and 256 mg of xylobiose and xylose, respectively, from 1.0 g of auto-hydrolyzed oil palm frond fibers [14].

From Fig. 4, it is also evident that the maximum xylobiose yield (73.7 g/100 g xylan) in the pretreated OPEFB fiber was obtained with the xylanase from FXY-1 at 24 h of incubation. The obtained maximum xylobiose yield using xylanases from FXY-2 and FXY-3 were 65.4 and 68.7 g/100 g xylan in the pretreated OPEFB fiber, respectively. This data further support that the isolated fungal strain FXY-1 has higher potential to produce the xylanase complex for XOs production using pretreated OPEFB fiber as substrate compared with the other isolated bacterial and fungal strains.

Further analysis of the data revealed that in addition to xylobiose, lower amounts of xylotriose and xylose were released in all three fungal enzyme hydrolysates (Table 2). This is evident from the data; a higher amount of xylotriose (12 mg/g) was noticed with FXY-3 xylanase compared with FXY-1 xylanase (11.1 mg/g) from alkali-pretreated OPEFB fiber at 24 h of incubation. The least amount of xylotriose (9.9 mg/g) was observed with FXY-2 xylanase and further increase in incubation caused a decline in xylotriose concentration and increased xylose levels in all fungal hydrolysates. The reduction in xylotriose concentration may be attributed to the degradation of xylotriose into xylose after a prolonged incubation period. Analysis of the xylose concentration indicated that the maximum amount of xylose was by FXY-1 (15.7 mg/g) xylanase, followed by FXY-3 (12.7 mg/g) xylanase. The lowest quantity of xylose (8.0 mg/g) was released by FXY-2 xylanase compared with the other fungal xylanases.

Yang *et al.* [19] found maximum XOs from corncob xylan at 6 h of incubation and reported decreased XOs production after prolonged period of incubation where xylotriose was converted into xylobiose and xylose. Ai *et al.* [3] reported XOs production in 8 h of incubation from alkaline-pretreated corncob xylan, and further increase in incubation time caused increased amounts of xylose. The above results suggested that the xylose concentration was proportionally increased with the incubation time.

### Optimization of Enzymatic Hydrolysis Conditions for XOs Production Using FXY-1 Xylanase

Preliminary comparative analysis of XOs production with pretreated OPEFB fiber as the source of xylan revealed that the hydrolytic efficiency of the fungal xylanases was higher than bacterial xylanases (Fig. 3 and 4). Among the isolated fungal strains, strain FXY-1 produced high xylanase titers under similar conditions compared with the other isolated strains (Fig. 1), and hence the xylanase enzyme complex from FXY-1 was selected for efficient XOs production. The FXY-1 strain was further identified as *Aspergillus terreus* by MTCC - Chandigarh, India and deposited with the accession number MTCC- 8661.

### Effect of pH on XOs Production

In order to determine the effect of pH on XOs production from alkali-pretreated OPEFB fiber, enzymatic saccharification was carried out for 24 h by using FXY-1 xylanase at five pH values in a range of 3.0 to 7.0. Fig. 5 illustrates the effect of pH on XOs production from OPEFB fiber. The HPLC analysis revealed that the maximum amount of xylobiose (250.8 mg/g) was released at pH 6.0. In addition to the xylobiose, maximum amounts of xylotriose and xylose (12.4 and 17.8 mg/g) were also released at this condition. The analysis of the data revealed that the xylose, xylotriose, and xylose concentrations were gradually increased with the increase of pH from 3.0 to

### Table 2. Xylose (X₁) and xylotriose (X₃) production by three fungal xylanases at different time intervals.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>FXY-1 X₁ (mg/g)</th>
<th>FXY-2 X₁ (mg/g)</th>
<th>FXY-3 X₁ (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>6.3 ± 0.2</td>
<td>6.2 ± 0.2</td>
<td>6.9 ± 0.2</td>
</tr>
<tr>
<td>12</td>
<td>8.9 ± 0.4</td>
<td>8.0 ± 0.4</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>18</td>
<td>10.3 ± 0.5</td>
<td>10.6 ± 0.5</td>
<td>8.0 ± 0.3</td>
</tr>
<tr>
<td>24</td>
<td>11.1 ± 0.5</td>
<td>15.2 ± 0.6</td>
<td>9.9 ± 0.5</td>
</tr>
<tr>
<td>30</td>
<td>9.3 ± 0.3</td>
<td>15.7 ± 0.4</td>
<td>9.7 ± 0.3</td>
</tr>
</tbody>
</table>

± = Standard deviation.
6.0, and further increase in pH caused reduction in these xylooligomers and xylose concentrations. At pH 3.0, 4.0, 5.0, and 7.0, 192.3, 231.3, 246.8, and 221.4 mg/g of xylobiose; 5.2, 8.4, 10.5, and 9.8 mg/g xylotriose; and 8.4, 11.4, 15.2, and 14.6 mg/g of xylose were released respectively. This data suggest that acidic conditions are more favorable for enzymatic saccharification, which may be attributed to the catalytic properties of xylanase enzyme, as this enzyme complex is more active in the pH range of 4.0 to 6.0 and any variation in this environment negatively influenced the catalytic function [15].

Table 3 presents the pH effect on xylobiose production in terms of yield. The maximum xylobiose yield (75.31 g/100 g xylan) in the pretreated OPEFB fiber was obtained at pH 6.0 followed by pH 5.0 (74.11 g/100 g xylan in the pretreated OPEFB fiber) and the minimum (57.74 g/100 g xylan in the pretreated OPEFB fiber) was noticed at pH 3.0. It is evident from the data that pH 6.0 was the optimum condition for enzyme-substrate interaction that caused higher amounts of xylobiose production. The result revealed that enzymatic saccharification of OPEFB fiber was influenced by the alteration in the buffer pH. After evaluation of the pH conditions, xylobiose yield was increased from 74.11 (pH 5.0) to 75.31 g/100 g xylan in the pretreated OPEFB fiber (pH 6.0). Hence, in further studies, enzymatic saccharification was carried out at pH 6.0 instead of pH 5.0. This result coincides with the literature reports on xylooligosaccharide production from cotton stalks and *Populas tomentosa*, which have shown maximum product yield at pH 5.4 [4, 18].

**Effect of Enzyme Loading on XOs Production**

Enzymatic saccharification depends mainly on the amount of the enzyme used for the hydrolysis. Hence, to optimize the enzyme concentration, FXY-1 xylanase enzyme (6,200 U/ml) was diluted to 10, 20, 40, and 80 times and enzymatic hydrolysis was carried out for 24 h of incubation for XOs production using pretreated OPEFB fiber. Based on xylobiose analysis, it is evident that 20 times diluted FXY-1 xylanase was the optimum enzyme concentration for higher amounts of xylobiose (254.4 mg/g) production (Fig. 6). In addition to the xylobiose, low quantities of xylotriose and xylose were released and maximum xylotriose and xylose were produced with undiluted xylanase enzyme. The amounts of xylotriose and xylose were gradually decreased with decreased xylanase concentration. From Fig. 6, it was noticed that using undiluted xylanase, and 10, 40, and 80 times diluted xylanase, the released xylobiose was 182.9, 249.6, 236.5, and 217 mg/g, respectively. The results indicated that the studies.
high and very low concentrations of xylanase enzyme were not effective for xylobiose production.

Table 3 demonstrates xylobiose yield at different enzyme concentrations. Initially, 10 times diluted xylanase enzyme was used for enzymatic hydrolysis of OPEFB fiber, which showed a xylobiose yield of 74.95 g/100 g xylan in the pretreated OPEFB fiber. In order to find out the optimum substrate-to-enzyme ratio, further experiments were conducted with altered concentration of enzyme. The maximum xylobiose yield (76.39 g/100 g xylan in the pretreated OPEFB fiber) was observed with 20 times diluted xylanase enzyme, whereas 54.92 g/100 g xylan in the pretreated OPEFB fiber was obtained with the undiluted xylanase enzyme, indicating an increase of enzyme concentration reduced the yield. This may be attributed to the fact that at higher enzyme concentration, the produced xylobiose may be further converted into xylose. Hence, in further studies, enzymatic saccharification of the OPEFB fiber was carried out by using 20 times diluted xylanase enzyme.

**Effect of Temperature on XOs Production**

To evaluate the impact of reaction temperature on xylobiose production, enzymatic hydrolysis of the OPEFB fiber was carried out at different temperatures from 35 to 60°C. Fig. 7 illustrates the production of xylobiose, xylotriose, and xylose at different temperatures from pretreated OPEFB fiber. The results showed that the xylobiose concentration was gradually increased from 35°C and reached to a maximum at 45°C (262 mg/g), and further increase in the temperature led to a slight decrease in xylobiose concentration. From 35 to 45°C, xylobiose concentration was increased from 214 to 262 mg/g and then slowly decreased to 257, 252, and 248 mg/g at 50, 55, and 60°C, respectively. The analysis of the data revealed that incubation at 45°C was favorable for xylobiose production and any further increase or decrease in temperature caused reduction in xylobiose production. In contrast to the xylobiose concentration, low amounts of xylotriose and xylose were released, similar to the earlier studies. Fig. 7 has shown that the maximum amounts of xylotriose (16.58 mg/g) was released at 45°C and then slowly decreased with increasing temperature. The released xylotriose at 35, 40, 50, 55, and 60°C was noticed to be as 10.37, 15.38, 13.12, 10.23, and 9.35 mg/g, respectively. Xylose concentration was gradually increased from 35 to 55°C and reached to a maximum (21.23 mg/g) at 55°C. An increase in temperature from 55°C to 60°C caused reduction in xylose production (18.79 mg/g). The analysis of the data revealed that both xylotriose and xylose were released in nearly similar quantities up to 45°C, and thereafter, xylotriose concentration was decreased and xylose concentration was increased with increasing temperatures.

From Table 3, the maximum and minimum xylobiose yields were 78.67 and 64.26 g/100 g xylan in the pretreated OPEFB fiber at 45 and 35°C, respectively. Hence, in further studies enzymatic hydrolysis of OPEFB fiber was carried out using 20 times diluted FXY-1 xylanase at 45°C with buffer of pH 6.0. Overall, after optimization of the pH, enzyme concentration, and temperature, the xylobiose yield was increased from 73.7 to 78.67 g/100 g xylan in the pretreated OPEFB fiber.

In contrast, Brienzo et al. [9] reported 31.5% of XO yield from alkali-pretreated sugarcane bagasse using 120 U/g of *Thermoascus aurantiacus* xylanase enzyme at 24 h of incubation. XO production compared among the other literature reports suggested that the production of XOs varied with the type of the lignocellulosic material, xylanolytic complex, and different pretreatment methods. Xylanase from *Aspergillus oryzae* MTCC 5154 hydrolyzed alkali-treated corn cobs and yielded 81% of XOs [1] whereas Teng et al. [16] obtained 28.6 g of XOs from 100 g of steam-treated liquor of corn cobs. Akpinar et al. [5] obtained 8 and 13 g of XOs from 100 g of alkali-treated cotton stalk xylan by enzymatic and acid hydrolysis methods. Ai et al. [3] reported 81% of XO yield from alkali-treated corn cobs by immobilized xylanase from *Streptomyces olivaceoviridis*.

In the present study, xylooligosaccharides production was carried out by a chemical–enzymatic hydrolysis method using OPEFB fiber as substrate. To obtain higher yields of XOs, different microbial sources of xylanases were used, and among these, FXY-1 xylanase produced higher quantities of xylobiose. Irrespective of the source of xylanase, with all tested different sources of xylanases.
Acknowledgments

The Authors are thankful to the Council of Scientific and Industrial Research, New Delhi for financial support, and the Director, Indian Institute of Chemical Technology, Hyderabad for his encouragement.

References