Bioresource Technology 357 (2022) 127374

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Efficacious bioconversion of waste walnut shells to xylotetrose and xylopentose by free xylanase (Xy) and MOF immobilized xylanase (Xy-Cu-BTC)

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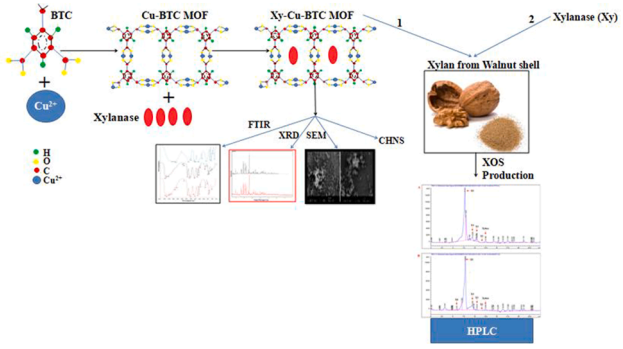
## HIGHLIGHTS

- Xylanase (Xy) extracted from *Bacillus pumilus* bacterial strain was partially purified.
- Production of Cu-BTC MOF was done at room temperature.
- Xy was immobilized onto Cu-BTC MOF to form blue crystals of Xy-Cu-BTC.
- Xylan was extracted from waste walnut shell.
- Bioconversion of xylan to xylooligosaccharides by free and immobilized enzyme system.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Keywords:*
Xylooligosaccharides (XOS)
Xylanase
Hemicellulosic
Metal organic framework
Immobilization
Xylotetrose
Xylopentose

## ABSTRACT

This study uses a cost effective and efficient method for production of higher DP (degree of polymerization) Xylooligosaccharides (XOS) from xylan extracted from the waste walnut shells. Copper based metal organic framework (Cu-BTC MOF) was prepared for immobilization of free xylanase (Xy) enzyme by green synthesis method. Both free and immobilized xylanase (Xy-Cu-BTC) were able to cause the bioconversion of xylan (87.4% yield) into XOS. Predominant production of xylotetrose (X4) and xylopentose (X5) was observed for both the methods. Percentage XOS conversion for free enzyme (Xy) was found to be 4.1% X4 and 60.57% X5 whereas these values increased in case of immobilized system where 11.8% X4 and 64.2% X5 were produced. Xylose

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https://doi.org/10.1016/j.biortech.2022.127374
Received 22 April 2022; Received in revised form 18 May 2022; Accepted 21 May 2022
Available online 24 May 2022

production was minute in case of immobilized xylanase 0.88% which makes it a better method for XOS production free from xylose interference. Xy-Cu-BTC MOF can hence be used as an attractive alternative for pure XOS production.

## 1. Introduction

Sugar oligosaccharides formed from xylose sugar are referred to as xylooligosaccharides (XOs) (Romero-Fernández et al., 2018; Liao et al., 2022). Xylooligosaccharides can be extracted from xylan polymer which forms the constituent unit of various plant hemicelluloses including shoots, vegetables, fruits, seeds, wood, and leaves. The hemicellulosic content of the various naturally occurring plant based materials can be acted upon by a special enzyme namely Xylanase which effectively breaks down the xylan backbone present in the hemicelluloses and releases xylose and xylooligosaccharides as the by product (Singh et al., 2017). Recently enough, the industrial production of xylooligosaccharides has drawn immense attention owing to their numerous applications in the fields of pharmaceuticals, food, feed and agriculture to name a few. Xylanase enzyme (Xy) typically acts on the hemicelluloses present naturally in the environment by breaking the β-1, 4- glycoside linkage in the xylan polymer which forms the constituent unit of hemicelluloses (Kaushal et al., 2021). Xylanase enzyme therefore is the main biological component required for the production of xylooligosaccharides (Gufe et al., 2022). Conventionally, the extraction of xylan is done from easily available biomass like poplar, corn cob, sugarcane bagasse, barley straw etc. by following either acid hydrolysis or simple alkali hydrolysis method (Álvarez et al., 2021). Acid hydrolysis using acetic acid/sodium acetate combination has been employed in order to extract xylan owing to the lower production of xylose as a by-product after hydrolysis along with the production of commercially important xylooligosaccharides. However, this method of xylan hydrolysis is much harsher and takes place at high temperature when compared to the simple extraction following alkali pretreatment (Liao et al., 2022). The extracted xylan is then acted upon by the xylanase enzyme which provides xylooligosaccharides of different DP (degree of polymerization) as result of the enzymatic action (Su et al., 2021).

One of the main challenges faced by the industries these days is the cost of production for these xylooligosaccharides from suitable materials rich in xylan content and the lack of xylanase enzyme activity after a few cycles of its treatment. Immobilization of enzymes on a suitable support can effectively enhance the enzymes stability and improve its activity when the enzyme is to be used for commercial purposes for longer durations (Fan et al., 2021). Xylanase enzyme immobilization onto a suitable support can successfully enhance the time duration of xylanase enzyme activity and enhance its various physical characteristics (optimum pH and temperature) making it more stable and easier to use for industrial xylooligosaccharide production (Romero-Fernández et al., 2018). Many different supports for immobilization of enzymes have been used in the past which have always resulted in enhancement of the enzymes activity and stability. Nanotechnology has emerged as a boon for enzyme immobilization onto nano sized supports which allow greater loading capacity of enzymes (Ariaeenejad et al., 2021; Pagolu et al., 2021). Metal organic framework (MOF) have been used effectively recently for the immobilization of various enzymes and have known to provide a suitable support because of the ease of their production depending on the enzymes characteristics. MOF's are specially structured nano coordination compounds where a metal ion interacts with an organic linker to form a cage like structure providing a very high surface area to volume ratio for the enzyme to immobilize (Wang et al., 2021). The properties of MOF's like high water stability, gentle conditions of synthesis and ease of tuneability make them a valuable candidate for the purpose of enzyme immobilization.

In the research work described below, xylanase enzyme from *Bacillus pumilus*was successfully immobilized onto green synthesized Cu-BTC metal organic framework for the purpose of xylooligosaccharide production. FTIR, XRD, SEM and CHNS analysis was performed for free and immobilized MOF in order to determine successful enzyme immobilization. Waste walnut shells collected from household were used as a source of xylan which was acted upon by free as well as immobilized xylanase enzyme for xylooligosaccharide production. Xylan extraction was carried out by simple alkali treatment (NaOH hydrolysis) which resulted in satisfactory yield of xylan polymer. The XOS production was estimated by performing HPLC for both free as well as immobilized system for comparison.

## 2. Materials and methods

### 2.1. Production of xylanase enzyme

The xylanase enzyme was produced and extracted from *Bacillus pumilus* bacterial strain which was available from UIET Panjab University and was isolated from agricultural soil from fields near Chandigarh and Panchkula and later identified by 16 S rRNA Sequencing. The media for the enzyme production had the following formulation: peptone (0.5% w/v), NaCl (0.1 % w/v), tryptone (0.15% w/v), $KH_2PO_4$ (0.04% w/v), along with 0.5 % w/v xylan acquired from beech wood (Himedia). Xylan acted as the substrate for the production of xylanase enzyme from *B. pumilus* and the reaction was carried out at a temperature of 40 °C, 155 rpm, for 48 h. After the incubation period, the resultant media was then centrifuged at 7000 rpm for 20 min at a temperature of 4 °C from which the cell free supernatant was collected as xylanase enzyme since it is an extracellular enzyme. Further, the partial purification for xylanase was done by subjecting the enzyme for ammonium sulphate precipitation (30–90% saturation). The precipitated enzyme was then dialyzed against 50 Mm phosphate buffer for 24 h at a pH of 8.0 using dialysis membrane with molecular weight cut-off between 10 and 12 kDa (Himedia) (Menon et al., 2010).

### 2.2. Preparation of Cu-BTC MOF

The copper salt used for the production of Cu-BTC MOF was $CuCl_2$ along with BTC (benzene-1, 3, 5-tricarboxylate) as the organic linker both of which were obtained from Himedia. The methodology followed for the preparation of Cu-BTC MOF was as follows: Solution 1:BTC (0.135 g) was added to 10 ml of distilled water at room temperature and mixed thoroughly on a magnetic stirrer. This formed a colorless liquid of the organic linker. The pH of the linker solution was adjusted to 8.0 with the help of 0.1 M solution of NaOH which was added drop wise into the linker solution. Solution 2: For the preparation of the metal ion solution, the metal salt $CuCl_2$ (0.127 g) was added to 10 ml of distilled water and mixed thoroughly to form a light greenish blue solution at room temperature. MOF preparation was done by slowly adding the solution 2 (metal ion solution) drop wise into the first solution containing the linker BTC (Gascón et al., 2018). Instantly, the formation of a light blue viscous solution started under constant stirring conditions. The reaction was carried out for 12 h at room temperature. The solution was then centrifuged and the pellets collected. The pellets were washed thrice with distilled water and then stored in dry conditions.

### 2.3. Post synthesis immobilization of xylanase enzyme onto Cu-BTC MOF

For the immobilization of xylanase enzyme onto the prepared MOF, 10 mg of the MOF crystals were suspended in 100 ml of the enzyme solution under constant stirring conditions. The reaction was carried out

for 15 min at a temperature of 50 °C. The MOF crystals were then allowed to settle and were separated through centrifugation after which the pellets were collected and dried at room temperature. The resultant crystals (Xy-Cu-BTC MOF) were used as immobilized support for xylooligosaccharide production from walnut shells (Gascón et al., 2018).

### 2.4. Assay for evaluating xylanase enzyme activity

Xylanase enzyme activity (for both free and immobilized system) was evaluated by DNS assay (3, 5-Dinitrosalicylic acid) method where the amount of released sugars from beech wood xylan (0.5% w/v) was determined using a calorimeter at wavelength 540 nm with calibration curve of xylose (Bailey et al., 1992). 1 ml of the partially purified enzyme (or 1 mg of MOF immobilized xylanase) was made to react with 1 ml of the substrate solution (0.5 % w/v of beechwood xylan) at a temperature of 50 °C for 15 min. After the reaction, 3 ml of DNSA reagent was added to the reaction mixture and the solution was then boiled for 5 min at 100 °C. One unit of xylanase enzyme activity is equivalent to the amount of xylanase required to liberate 1 μmol xylose sugar in 1 min from xylan substrate under standard conditions (Lu et al., 2008). The enzyme activity was calculated in accordance with the xylose sugar standard curve.

### 2.5. Estimation of protein loading and immobilization yield

The total concentration of the xylanase enzyme (protein) immobilized onto the Cu-BTC MOF was evaluated by Lowry's method which uses BSA (bovine serum albumin) as a standard for determining protein concentration. For determination of bound protein, the amount of protein in the supernatant obtained after enzyme immobilization was subtracted from the initial protein concentration (Kaushal et al., 2018). Protein loading and immobilization yield were then calculated from the following formulas:

$$Protein\ Loading\ (\%) = \frac{Amount\ of\ Immobilized\ Protein}{Amount\ of\ initial\ Protein} \times 100$$

$$Immobilization\ Efficiency\ (\%) = \frac{Specific\ Activity\ of\ Immobilized\ enzyme}{Specific\ Activity\ of\ Free\ enzyme} \times 100$$

### 2.6. Extraction of xylan from waste walnut shells

Waste walnut shells were collected from household which were then crushed and churned into a fine powder. For the extraction of xylan, the powdered walnut shell was subjected to simple treatment with 40% NaOH (sodium hydroxide) aqueous solution at 80 °C for 2 h. The solid liquid ratio was adjusted to 1:20 w/v for this treatment process (Valladares-Diestra et al., 2021). The soluble fraction so obtained was neutralized to a pH of 5.0 (using acetic acid) and was then incubated for 1 h with 97% ethanol at room temperature where the ratio of xylan soluble fraction to ethanol was adjusted to 1:3 respectively. Xylan was then obtained from the precipitate obtained after ethanol reaction step and was dried at room temperature to get a powder like consistency (Romero-Fernández et al., 2018; Cebin et al., 2021).

The total yield of xylan evaluated following the formula.

$$\text{Total XylanYeild}\ (\%) = \frac{Extracted\ Xylan\ dry\ weight\ in\ gram}{Initial\ weight\ of\ walnut\ shell\ powder\ in\ gram} \times 100$$

### 2.7. Xylooligosaccharide production

For the production of xylooligosaccharides, firstly a 10 ml of walnut shell extracted xylan solution was prepared (0.3% w/v) from which 1 ml was used as substrate for free and MOF immobilized xylanase with enzyme activity of 10U for both the systems. The enzymatic hydrolysis reaction on the prepared xylan solution was then performed under mild agitation at 50 °C for half hour. After the reaction incubation time, the sample solutions were then diluted to 10% consistency and filtered for HPLC analysis (Liao et al., 2021). The filtrate was analyzed on the Agilent Hi-Plex CA (7.7x300mm) column. HPLC grade water was used as a mobile phase for the sample at a flow rate of 0.6 ml/min using a refractive index detector at a column temperature of 85 °C.

## 3. Results and discussion

### 3.1. Preparation of Cu-BTC MOF and xylanase immobilization

The Cu-BTC MOF was prepared at room temperature within 12 h of time following the method described above. An approximate yield of 12 mg of MOF crystals were recovered from the total 20 ml of solution (10 ml metal ion solution + 10 ml linker solution) which was recovered after centrifugation in the form of blue colored pallets. Xylanase enzyme was then immobilized onto the MOF post synthesis by suspending the crystals in 1:10 (w/v) ratio in the xylanase enzyme solution (Xu et al., 2021). The total protein loading and immobilization efficiency evaluated by Lowry's method was found to be 84.3 % and 95.86%, respectively (see supplementary material).

### 3.2. Characterization of Xy-Cu-BTC MOF

#### 3.2.1. FTIR analysis

The Cu-BTC MOF prepared following the method discussed above was used as support for immobilizing xylanase enzyme. The FTIR pattern for BTC, Cu-BTC MOF and the Xylanase immobilized MOF respectively is represented in the supplementary material. As evident from the figure, the OH vibration peak for BTC (organic linker) at around 917 $cm^{-1}$ is deformed in case of Cu-BTC MOF and the C = O stretching vibration owing to the interaction between metal ion ($Cu^{2+}$) and BTC linker shifted to a low wavenumber (1720 to 1712 $cm^{-1}$) when compared to that of BTC linker alone suggesting a coordination among the metal ion and linker. The bands at 1617, 1570 and 1370 $cm^{-1}$ represent the bridging interaction in the COOH group of MOF formed which are considerably deformed in the BTC FTIR spectra. Moreover, the peak at 3500 $cm^{-1}$ for Cu-BTC MOF formed shows the presence of water molecule in the MOF (Phuong et al., 2016). The free MOF FTIR pattern when compared with the enzyme immobilized FTIR pattern shows considerable changes in the chemical structure of the support. Following xylanase enzyme immobilization, the stretching band vibrations at wave number 2922 $cm^{-1}$ corresponding to the $C\text{-}H_{Aliphatic}$ band was observed in the immobilized MOF which represents the characteristic band for xylanase enzyme presence. The absorbance by the amino group present in the xylanase enzyme was made evident by addition of band at 1439 $cm^{-1}$ for enzyme immobilized Cu-BTC MOF further suggesting a successful immobilization of the enzyme on the support (see supplementary material).

#### 3.2.2. XRD pattern for Xy-Cu-BTC MOF

The XRD analysis was performed for both blank and Xy-Cu-BTC MOF prepared in order to evaluate the structural configuration of the MOF. As evident from the XRD patterns of both the metal organic frameworks (free and immobilized), the sharp peaks reveal that the MOF formed is crystalline in nature. The sharp peaks that confer the crystal structure of the developed MOF at $2\theta$ = 9.5, 11.5, 14.1, 18.89 and 19.95 were consistent with the literature available earlier for the similar Cu-BTC MOF. After immobilization of the xylanase enzyme, similar peaks were observed as that of the parent MOF which suggests that the crystal structure of the MOF was maintained even after the immobilization of xylanase enzyme onto the MOF (Xy-Cu-BTC MOF) (see supplementary material) (Lin et al., 2018).

**Table 1**
CHNS elemental analysis for free and immobilized Cu-BTC MOF.

| Sample ID | C% | H% | S% | N% |
|---|---|---|---|---|
| Cu-BTC MOF | 30.521 | 3.51 | 0.01 | 0.00 |
| Xy-Cu-BTC MOF | 28.607 | 4.02 | 0.97 | 0.09 |

### 3.2.3. SEM analysis

In order to testify the crystal structure formation in the MOF prepared, SEM analysis was done which successfully shows the morphological appearance of the Cu-BTC MOF. As evident from the fig., the octahedral crystal shape for both the free as well as the immobilized Cu-BTC MOF can be visualized with size of the individual particles between 10 and 20 μm. After immobilization, a stronger and more refined crystal structure formation can be visualized owing to the stabilizing and strong covalent contact between the enzyme and the MOF surface. Even the surface is seen to be more ordered and clumped after immobilization which shows that the enzyme certainly adds to the stability of the MOF crystals (see supplementary material).

### 3.2.4. CHNS analysis

CHNS elemental analysis was performed for both free and immobilized MOF in order to testify the presence of enzyme xylanase in the immobilized system. The data so obtained is presented in the Table 1. In case of the MOF without any enzyme immobilized to it, no sulphur content was found to be present. The sulphur content in the Xy-Cu-BTC MOF can be explained by the presence of this element in the protein primary structure in the form of cysteine or methionine amino acid residues. Furthermore, the presence of nitrogen in the immobilized system further suggests that the enzyme was successfully immobilized onto the MOF surface since nitrogen represents the presence of amine group characteristic of all proteins (Gascón et al., 2018; Lian et al., 2020).

### 3.3. Production of xylooligosaccharides

The xylan extracted from the waste walnut shells was used for the effective conversion of this biomass into valuable product of xylooligosaccharides. The percentage xylan extracted from the powdered walnut shell extract was found to be 87.4% which was then dissolved in water to form a substrate solution for the enzymatic reaction to take place. Both free and immobilized xylanase enzyme was used for the production of xylooligosaccharides. The reaction time (half hour) and enzyme activity (10 U) was adjusted after optimization so as to produce excess of XOS in comparison to normal xylose sugar. The HPLC results for both the methods are shown in the supplementary figure. These results were compared to the results of the standard solutions of xylooligosaccharides obtained from Megazyme for evaluation. As evident from the results, both the treatment methods were able to produce xylooligosaccharides from the walnut extracted xylan in good range. For the free enzyme reaction, the percentage of xylose sugar produced was approximately 1.43% at the retention time of 12.44 (compared to the standard HPLC values). The peak at the retention time of 10.513 represents the percentage of xylotriose produced from xylan enzymatic hydrolysis. Minimum to no production of xylobiose was found to be present in the extracts analyzed. The most predominant xylooligosaccharide produced was found to be xylopentose (retention time 7.762) with highest percentage of 60.57 %. This makes it evident that the xylooligosaccharides with high degree of polymerization were predominant in the products after enzymatic reaction.

For the immobilized system (Xy-Cu-BTC MOF), similar results were found to be predominant (excessive production of xylotriose and xylopentose). Xylopentose was found to be present in the excess of all the other xylooligosaccharides with percentage as high as 64.2%. Unlike for the free enzyme, the percentage of xylotetrose was also high in case of immobilized xylanase reaction. Moreover, the xylose content was also as low as 0.88 percent for the immobilized system owing to a more controlled and slower reaction for the immobilized system which can be attributed to xylanase enzyme's restriction of mobility on the support surface. This can greatly help in the exclusive production of xylooligosaccharides free from xylose sugar for various applications. The detailed percentage wise production description for all the xylooligosaccharides is given in the Table 2. The results suggest the successful immobilization of xylanase enzyme onto the prepared MOF and that the immobilization process helps the enzyme to selectively produce xylooligosaccharides from the extracted xylan in large quantity when compared to the free xylanase enzyme preferably owing to the decreased mobility of the enzyme after immobilization and its specific action mechanism under controlled environment of the MOF support. Immobilization therefore was able to provide a more cost effective and efficient method for xylooligosaccharide production commercially since the immobilized system can be used for many cycles of xylan hydrolysis unlike the free enzyme which can lose its activity after a single cycle only (Gufe et al., 2022).

## 4. Conclusion

Xylanase enzyme was was immobilized onto a $Cu^{2+}$ion based MOF. The blue crystals of Cu-BTC MOF were evaluated for their chemical and physiochemical characteristics by FTIR, XRD, SEM and CHNS analysis. Both free and immobilized xylanase enzyme was employed for the bioconversion of wall nut extracted xylan into commercially important xylooligosaccharides. HPLC analysis for both the enzymatic reactions shows predominant production of high DP (xylotetrose and xylopentose) xylooligosaccharides. The immobilized system produced xylooligosaccharides with very low production of xylose showing its potential in being used as a cost effective and easy alternative to already available techniques for XOS production.

## CRediT authorship contribution statement

**Jyoti Kaushal:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Data curation, Software, Formal analysis, Investigation, Resources. **Shailendra Arya:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Data curation, Software, Formal analysis, Investigation, Resources. **Madhu Khatri:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Data curation, Software, Formal analysis, Investigation, Resources. **Gursharan Singh:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Data curation, Software, Formal analysis, Investigation, Resources. **Nur Izyan Wan Azelee:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Data curation, Software,

**Table 2**
Total Percentage of different XOS (Xylooligosaccharides) produces from walnut shell xylan.

| Sample ID | XOS Percentage Estimated | | | | | |
|---|---|---|---|---|---|---|
| | Xylose % | Xylobiose % (X2) | Xylotriose % (X3) | Xylotetrose %(X4) | Xylopentose %(X5) | Xylohexose %(X6) |
| 1)Free Xylanase | 1.4 ±0.09 | 0.03 ±0.03 | 2.3 ±0.002 | 4.1 ±0.01 | 60.57 ±0.08 | 0.00 ±0.0 |
| 2)Xy-Cu-BTC immobilized Xylanase | 0.88 ±0.07 | 0.05 ±0.01 | 0.36 ±0.01 | 11.8 ±0.05 | 64.2 ±0.09 | 0.02 ±0.001 |

Formal analysis, Investigation, Resources. **Rajinikanth Rajagopal:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Data curation, Software, Formal analysis, Investigation, Resources. **Soon Woong Chang:** Methodology, Software, Writing – original draft, Data curation. **Balasubramani Ravindran:** Resources, Methodology, Software, Data curation, Validation. **Mukesh Kumar Awasthi:** Resources, Methodology, Software, Data curation, Validation, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgement

This work is supported by Haryana State Council for Science, Innovation and Technology (HSCSIT) JRF Fellowship (Sanction number HSCSIT/ 2834), and DBT Builder Project (BT/INF/ 22/SP41295/2020) at University Institute of Engineering and Technology, Panjab University, India. This work was supported by the National Research Foundation of Korea by the Korean Government (MSIT) (2020R1G1A1012069). The authors are grateful for the financial support from the Shaanxi Introduced Talent Research Funding (A279021901 and F1020221012), Shaanxi Provincial Key R&D Plan Project (2022NY-052) and the Introduction of Talent Research Start-up fund (Z101021904), College of Natural Resources and Environment, Northwest A&F University, Yangling, Shaanxi Province 712100, China.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127374.

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