Preparation of Bacterial Cellulose Using Enzymatic Hydrolysate of Olive Pomace as Carbon Source

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Bacterial cellulose has superior physical and chemical properties, biocompatibility, and purity. However, the high production cost obstructs the common use of this polymer. This study investigated the efficiency of olive pomace, an important by-product of olive oil industry in Turkey, as a carbon source for *Novacetimonas hansenii*. Olive pomace pretreatment with 1% H_3PO_4 was followed by enzymatic hydrolysis. The maximal reducing sugar concentration upon enzymatic process was 9.3 g/L with 1 enzyme: 6 substrate (dry matter) ratio. After incubation in the growth media prepared with the obtained reducing sugar as carbon source, the highest bacterial cellulose production was 0.68 g/L. Structural analysis indicated that bacterial cellulose from the enzymatic media and the conventional Hestrin-Schramm medium possess similar characteristics. The present work provides a favourable method to reduce the cost of bacterial cellulose production.

DOI: 10.15376/biores.18.2.4168-4181

Keywords: Bacterial cellulose; Carbon source; Enzymatic hydrolyzation; Lignocellulose; Olive pomace

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INTRODUCTION

Cellulose abounds widely worldwide in nature and can be synthesized both by plants and microorganisms (Geyer *et al*. 1994). Some species of bacteria (*Acetobacter*, *Acanthamoeba*, and *Achromobacter* spp.), algae (*Valonia*, *Chaetamorpha* spp.), and fungi can form this polymer naturally (Klemm *et al*. 2005).

Both plant and bacterial cellulose (BC) have identical chemical structures. However, plant-based cellulose occurs in plants as a composite with polymers such as lignin, pectin, and hemicellulose, while the BC produced in bacteria is completely pure (Cacicedo *et al*. 2016). Moreover, the superior qualifications of BC such as high crystallinity, high degree of polymerization, and great water holding capacity make it an attractive biological material in a wide variety of fields. Along with these properties, its biocompatibility and biodegradability features make it an appropriate candidate for a wide variety of applications in several areas such as biomedicine (wound dressing, drug delivery, artificial vessel), electronics (audio devices, electronic paper), and paper industry (Klemm *et al*. 2001; Czaja *et al*. 2006, 2007).

Though BC is a very attractive biopolymer owing to its outstanding characteristics,

its high production cost is an obstructive factor which makes it an unfavourable material to work with. The culture medium is the most expensive element in BC production, which could count up to 30% of the total cost (Jozala *et al.* 2016). Moreover, glucose cost represents approximately 24% of the total ingredients cost in the standard Hestrin-Schramm medium (Speretto *et al*. 2021). Therefore, replacing glucose with a cheap carbon source would provide a significant cost reduction in BC production. Various studies have been carried out so far aiming to minimize the production cost. Waste beer yeast, molasses-corn steep liquor, and rotten fruit can be counted among the carbon sources that have been tried for BC production (Jung *et al*. 2010; Lin *et al*. 2014; Jozala *et al*. 2015).

Lignocellulosic biomass can be used to produce BC. However, both cellulose and hemicellulose need to be hydrolysed for obtaining reducing sugars, which serve as carbon sources for microorganisms (Menon and Rao 2012). Various strategies have been suggested for the breakdown of lignocellulosic material, such as steam explosion (Wood *et al*. 2014), ultrasonic treatment (Nakayama and Imai 2013), and alkali treatment (Jung *et al*. 2018). Acidic pretreatment is a highly common method because of its low cost and high yield of monosaccharides. Dilute acid hydrolysis is effective to break the intractable form of cellulosic biomass when used in advance of enzymatic treatment (Mahboubi *et al*. 2017). Thus, being less corrosive compared to strong acids, phosphoric acid is effective in disengaging the hemicellulose structure and being a nitrogen source for microorganisms in fermentation (Vasconcelos *et al*. 2013).

The olive oil industry has a crucial economic impact, especially in the Mediterranean countries including Turkey. According to 2019 olive oil trade data, Turkey is in fourth place in the world in terms of olive oil production with 224.5 kilotonnes (Republic of Türkiye Ministry of Agriculture and Forestry Agricultural Economic and Policy Development Institute 2020). Olive pomace (OP) is the main by-product of the olive industry, and it is composed of pulp, stones, and skin of the fruit, which might be environmentally hazardous if not handled properly (Gomes *et al*. 2013). It comprises 30.0 to 41.6% lignin, 35.3 to 49.0% cell wall polysaccharides such as cellulose, pectin and hemicellulose, 7.5 to 14% oil, and 4.4 to 6% minerals (Miranda *et al.* 2019; Rodrigues *et al.* 2015). This material is generally burned for energy production (Vlyssides *et al*. 2004). However, the high lignocellulosic content of OP requires a more efficient way to use this material (Miranda *et al*. 2019). With this respect, using OP, which could be obtained in high amounts, might be a useful strategy. With its superior lignocellulose content, OP can be a good candidate to generate reducing sugars which would serve as a carbon source for bacterial cellulose production.

In the present paper, the potential of OP as a carbon source for BC production was examined with mild acidic pretreatment followed by enzymatic hydrolysis. Thus, an effective way of recycling an economically important by-product was demonstrated. BC obtained from the novel medium was characterized by scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), and X-ray diffraction analysis.

EXPERIMENTAL

Materials

Reagents

All chemicals were purchased from Carl Roth GmbH + Co. KG. and were of analytical grade. Cellic® CTec2, a mixture of cellulases, β-glucosidases and

hemicellulases, was purchased from Novozymes. Distilled water (dH_2O) was used in all cases where H_2O was stated.

Olive pomace

Olive pomace (OP) was kindly provided from ArmaGrande (Halis Toprak), a local olive oil producer in Geyikli, Çanakkale, Turkey. It contains 2.31% nitrogen, 6.16% hydrogen, 51.02% carbon, and a trace amount of sulphur. OP was kept at -20 °C and protected from light until further use.

Methods

Bacterial culture and BC production

G. hansenii strain 53582 was purchased from ATCC (American Type Culture Collection). As growth media, Hestrin-Schramm medium was used, which consists of (%, w/v) glucose, 2.0; peptone, 0.5; yeast extract, 0.5; disodium phosphate (anhydrous), 0.27; citric acid (monohydrate), 0.115. The pH was adjusted to 6.0 with diluted HCl or NaOH. The solution was sterilized by autoclaving at 120 °C for 30 min. Upon arrival, the powdered bacteria were placed in a sterilized Hestrin-Schramm (HS) medium for vitalisation. After 10 days of incubation at 30 °C, stock cultures were prepared in 20 % glycerol and kept at -80 °C until further use. For BC production, 100 mL HS medium was inoculated with 100 µl of stock culture and incubated statically at 30 °C for 5 days. This secondary culture was used for further inoculation in 24-well plates: 2 mL HS medium was placed in each well and inoculated with 100 µl of the secondary culture. The cultures were incubated statically at 30 °C for 21 days, and then the BCs were collected. They were washed with excess H_2O and then with 0.1 M NaOH solution for 1 h at 80 °C with a magnetic stirrer. After that, BCs were washed with dH_2O again until pH 7. They were freeze-dried for dry weight evaluation.

Pretreatment of the olive pomace

A total of 150 g olive pomace was mixed with 500 mL 1% phosphoric acid and placed into a 1 L stirring autoclave. The temperature was set to 170 °C and the rotational speed was adjusted to 200 rpm. The reaction mixture was stirred for 40 min once the temperature reached 170 °C with water pressure approximately 8 bar. Then the reactor was allowed to cool down to 25 °C. Liquid and solid phases were separately collected from the outlet of the reactor and stored at 4 °C until further use.

Enzymatic hydrolysis of the solid phase

Solid-phase was washed with H_2O until pH 7 to get rid of the phosphoric acid. It was filtered through a pore size 3 glass filter under vacuum and dried in low pressure (80 mbar) and a vacuum oven respectively. Once the material was completely dried, it was stored in a desiccator until the enzymatic hydrolysis.

For the enzymatic process, Cellic® CTec2 was used with a proportion of 1.5; 3; 6; 15; 30% (w/w) of the substrate. Reaction volume was modulated as stated in Martinez-Patino *et. al.* (2015) with 5 % solid concentration with respect to the total reaction volume. To have a trial estimation of the enzyme-substrate ratio, cellulose content of the olive cake was evaluated as indicated by Ouaini *et al*. 2000, which corresponds to 34% of the olive cake. Accordingly, reactions were set up in 100 mL flasks with 25 mL 0.05 M pH 4.8 sodium citrate buffer with 1.25 g solid material and varying concentrations of the enzyme as mentioned above. Reaction flasks were kept at 50 °C for 72 h, either static or stirring

conditions. In case of stirring, the rotational speed was ca. 150 rpm. All enzymatic reactions were done as triplicates.

Determination of the reducing sugar content

To determine the total reducing sugar content, dinitrosalicylic acid (DNSA) assay was applied. 1% of DNSA reagent and 40 % of potassium sodium tartrate solutions were used for the spectrophotometric assay. 1 to 10 g/L glucose standard solutions were prepared to constitute a calibration curve. All enzymatic hydrolysates were subjected to decolourization with activated charcoal in order to prevent absorbance interferences. DNSA assay was applied as stated anywhere; 200 µL of blank, each glucose standard and each enzymatic hydrolysate sample were placed in a 96-well plate. Absorbance was recorded at 575 nm in well plate reader spectrophotometer. Reducing sugar contents were calculated regarding the calibration curve equation obtained by measuring the standard solutions.

Determination of the carbohydrate content (HPLC)

Selected enzymatic hydrolysates (without bacterial inoculation) along with selected HS media and enzymatic bacterial cultures (with bacterial inoculation) were subjected to carbohydrate analysis by high performance liquid chromatography (HPLC). In case of any culture analysis, the sample was filtered through a 0.22 µm filter to prevent any bacterial residue.

A JASCO device (eluent: 0.005 M H_2SO_4, flow rate: 0.5 mL/min) was used with a refractive index detector (RI-930), an intelligent pump (PU-980), and an Aminex® HPX-87H column from Bio-Rad Laboratories (length: 300 mm, inner diameter: 7.8 mm). For HPLC analysis, the polymer (*ca.* 100 mg) was treated with 70% (v/v) $HClO_4$ (2 mL) within 10 minutes at room temperature. After dilution with water (18 mL), the mixture was shaken at 100 °C for 16 hours. The samples were neutralized using a 2 M KOH. Afterwards, the samples were kept at 4 °C for 1 hour for complete precipitation of $KClO_4$. In a further step, the samples were concentrated to an amount of about 4 mL.

Detoxification and growth media preparation from the enzymatic hydrolysates

For detoxification of the hydrolysates, $Ca(OH)_2$ and activated charcoal treatments were applied as stated by Guo *et al*. 2013:

- $Ca(OH)_2$ was added to the hydrolysate until pH 11 and incubated for 3 h at 30 °C.
- Activated charcoal was added to form a 2% (w/v) suspension, then mixed vigorously and incubated at room temperature (RT) for 5 min.

Both mixtures were centrifuged at 8300 g for 5 min. Supernatants were collected and pH adjusted to 6 after adding Hestrin-Schramm ingredients except for glucose. These media were named ENZ media depending on the enzyme amount they comprise.

Preparation of control growth media

HS medium was prepared as mentioned before. Another set of media, which is here named HG (enzyme amount in µL), was designed to test the efficiency of glucose compared to a mix of reducing sugars in terms of BC production.

HG growth media were prepared with the same concentration of glucose as the reducing sugar in each enzymatic hydrolysate. Thus, each HG medium is the HS medium including the same glucose concentration that corresponds to the reducing sugar in each

enzymatic medium (*e.g.* HG 110 is a HS medium with 9.3 g/L glucose, HG 55 is a HS medium with 7.5 g/L glucose).

Calculations of BC production efficiencies in Hestrin-Schramm and enzymatic media

To compare the substrate conversion ratio, BC production rate and yield of both media, the equations given below were modified by Gomes *et al.* (2013).

Substrate conversion ratio (α) (1)

$$\alpha = \frac{S_i - S_f}{S_i} \cdot 100\,\%$$

BC production rate r_{BC} (g L^{-1} day^{-1}) (2)

$$r_{BC} = \frac{m_{BC}}{V \cdot t}$$

BC production yield $Y_{BC/S}$ (3)

$$Y_{BC} = \frac{\frac{m_{BC}}{V}}{S_i - S_f} \cdot 100\,\%$$

where S_i is the initial concentration (mg mL^{-1}), S_f is the final concentration (mg mL^{-1}) of the substrate, m_{BC} is the amount of BC produced (mg), V is the reaction volume (mL), and t is the time of cultivation (day). The HPLC column (Aminex® HPX-87H) has the same retention time for xylose and fructose. Therefore, only glucose concentration was taken into account for S_i and S_f values. Since glucose in the ENZ medium was depleted at the end of day 5 of incubation, S_f concentrations were calculated with the data at the end of day 5, for both media.

FTIR analysis

Spectra were recorded on a Nicolet iS5 spectrometer (Thermo Fisher Scientific GmbH, Dreieich, Germany) using translucent KBr pellets containing the solid PS samples. The resolution was 4 cm^{-1}, and 32 scans were collected from 4000 to 600 cm^{-1}.

X-ray diffraction

X-ray diffractograms of all samples were measured with a D8 Advance (Manufacturer: Bruker AXS GmbH) diffractometer using Cu Kα-Dublett, λ = 1,54184 Å radiation. The raw data were smoothed by means of SAVITZKY-GOLAY-filtration, whereupon the scattering background was modelled by fitting spline-functions onto significant base marks of the scan and subtracted afterwards. The measurement and evaluation were implemented as per European Standard EN 13925-13 "Non-destructive testing - X-ray diffraction of polycrystalline and amorphous materials (2003)".

Scanning electron microscopy

Scanning electron microscopy (SEM) imaging was performed with a Sigma VP Field Emission Scanning Electron Microscope (Carl-Zeiss AG, Germany) using the InLens detector with an accelerating voltage of 6 kV. The samples were coated with a thin layer of platinum (10 nm) via sputter coating (CCU-010 HV, Safematic, Switzerland) before the measurement.

RESULTS AND DISCUSSION

Reducing Sugar Determination of the Solid Phase by 3,5-Dinitrosalicylic Acid Method

To determine the efficiency of reducing sugar yield in terms of enzyme amount, various enzyme/substrate ratios were applied in static and agitated conditions, as stated in Fig. 1. Regardless of the condition (static or agitated), the concentration of reducing sugar increased as the enzyme amount increased. However, though the enzyme amount increased by around 2 folds each time from 5 to 110 µL, reducing sugar concentration did not differ dramatically. This could be due to the saturation of the substrate by the enzyme; thus, no product was formed after a certain concentration.

Contrary to previous findings (Gunjikar *et al*. 2001; Ye *et al*. 2012), the static condition led to a higher reducing sugar yield compared to the agitated condition in each case. This result was particularly convenient in terms of reducing energy consumption. Because the static condition led to higher reducing sugar concentrations, it was determined as the principle method for further growth media preparation.

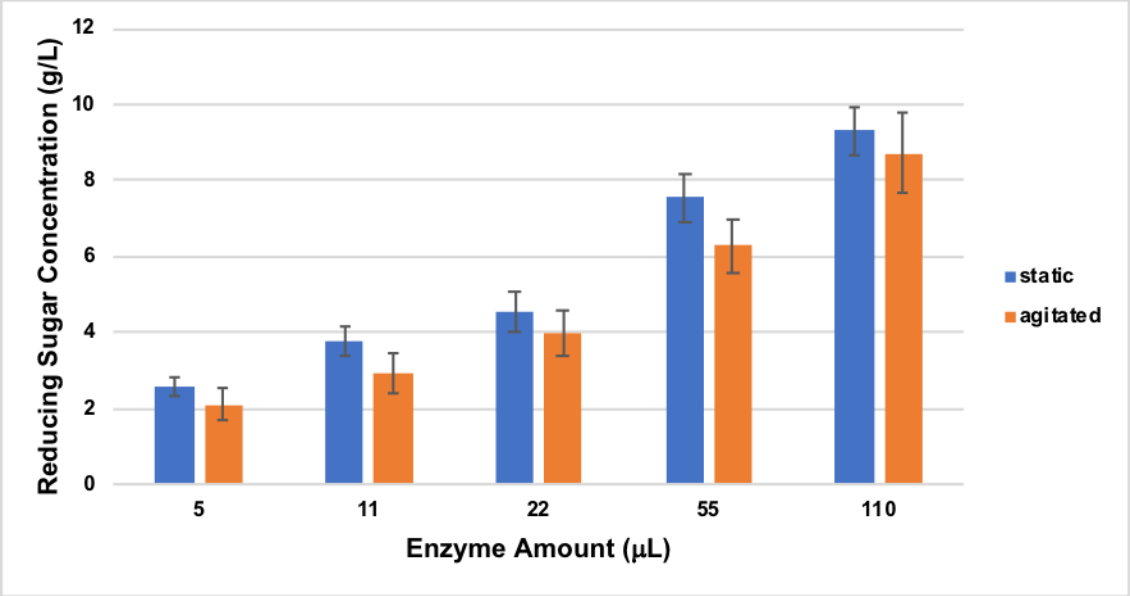

Fig. 1. Reducing sugar concentration obtained from enzymatic hydrolysis of olive pomace (OP) in static and agitated conditions (time: 72 h, temperature: 50 °C for both experiment sets; agitation speed: ~150 rpm)

BC Production in Enzymatic Hydrolyzates

The BC production in HG growth media increased as the amount of glucose increased in the medium (from HG5.5 to HG110), as shown in Fig. 2. For enzymatic hydrolysates, BC production followed a rising trend until ENZ22, but the amount was reduced in ENZ55 and to a higher degree in ENZ110 media, albeit with higher sugar concentration. Previous studies stated that the BC production is dependent on the sugar concentration along with the inhibitor quantity (Hong *et al.* 2011; Kiziltas *et al.* 2015). Thus, the reduction in BC amount was caused by the higher amount of inhibitors in ENZ55 and ENZ110 growth media due to the increased amount of enzyme.

Sugar Content Analysis of Enzymatic Hydrolysates

Enzymatic hydrolysates were analysed with HPLC to investigate the amount of glucose and xylose+fructose in samples. In Fig. 3, the amount of sugars (%) in each sample is shown. Both glucose and xylose+fructose yield increased as enzyme amount increased. The glucose amount was affected more drastically by enzyme content.

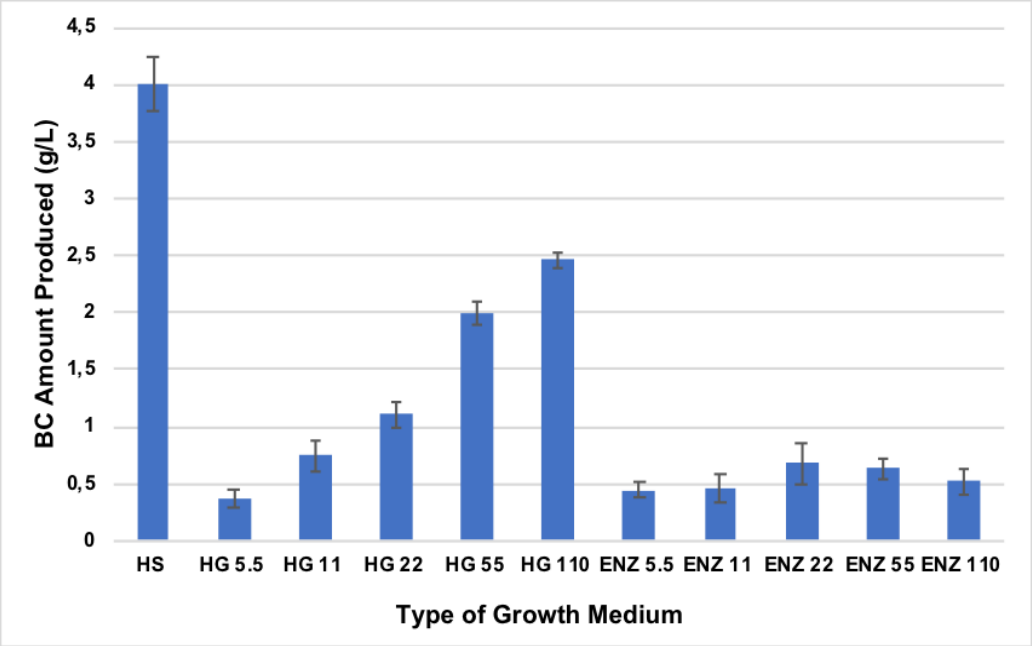

Fig. 2. Bacterial cellulose amounts produced in varying media (g/L). HS: Hestrin-Schramm medium; HG: HS media containing the same concentration of glucose as the reducing sugar concentration of the related enzymatic solution, ENZ: media prepared with enzymatic hydrolysates, enzyme amount stated for each condition (incubation time: 21 days, temperature: 30 °C)

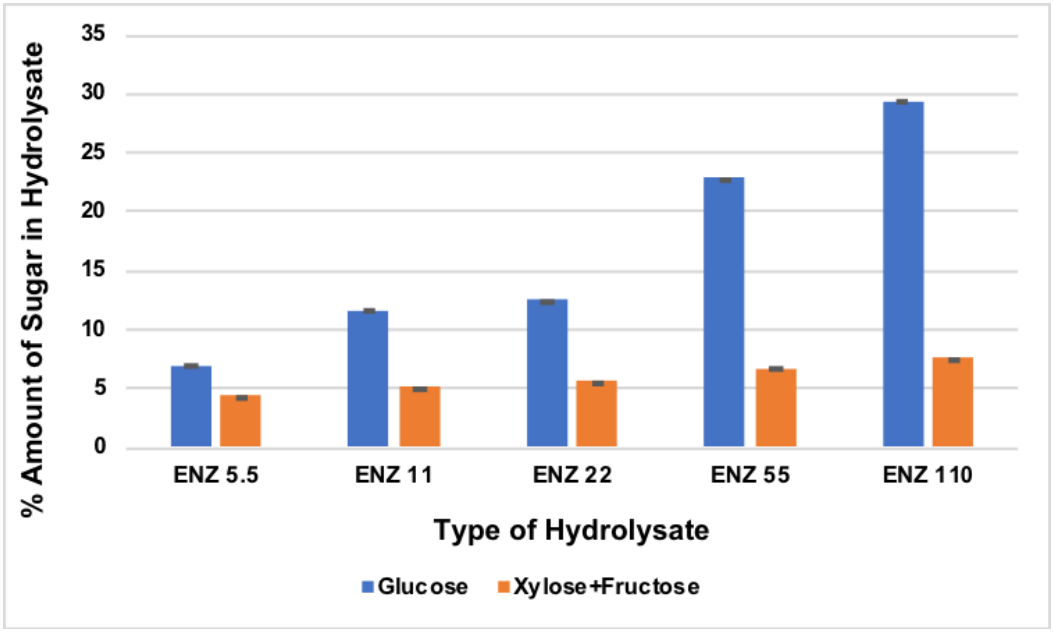

Fig. 3. Sugar composition of hydrolysates obtained by enzymatic hydrolysis of OP

The yield of xylose+fructose and glucose were 4.3% and 7%, respectively, for ENZ 5.5, while they were 7.5% and 29.2% for ENZ 110. Thus, the enzyme was more effective in terms of converting cellulose to glucose rather than hemicellulose to xylose, though fructose was detected along with xylose. The progress of glucose yield by means of enzyme amount was in correspondence with the findings summarized in Fig. 3; glucose yield did not show a dramatical increase although the enzyme amount increased by 20-fold, glucose content increases by only around 4-fold from ENZ 5.5 to ENZ 110, probably because of the substrate saturation.

Sugar Consumption in Hestrin-Schramm and Enzymatic Media

To determine the sugar content of ENZ 110 and HS media, another HPLC analysis was performed. Sugar consumption of *G. hansenii* during the incubation time was monitored in both media. While the glucose amount remained almost constant during the first 2 days in ENZ 110 media, it sharply decreased from day 2 on and was completely consumed at the end of day 5, as can be seen in Fig. 4a.

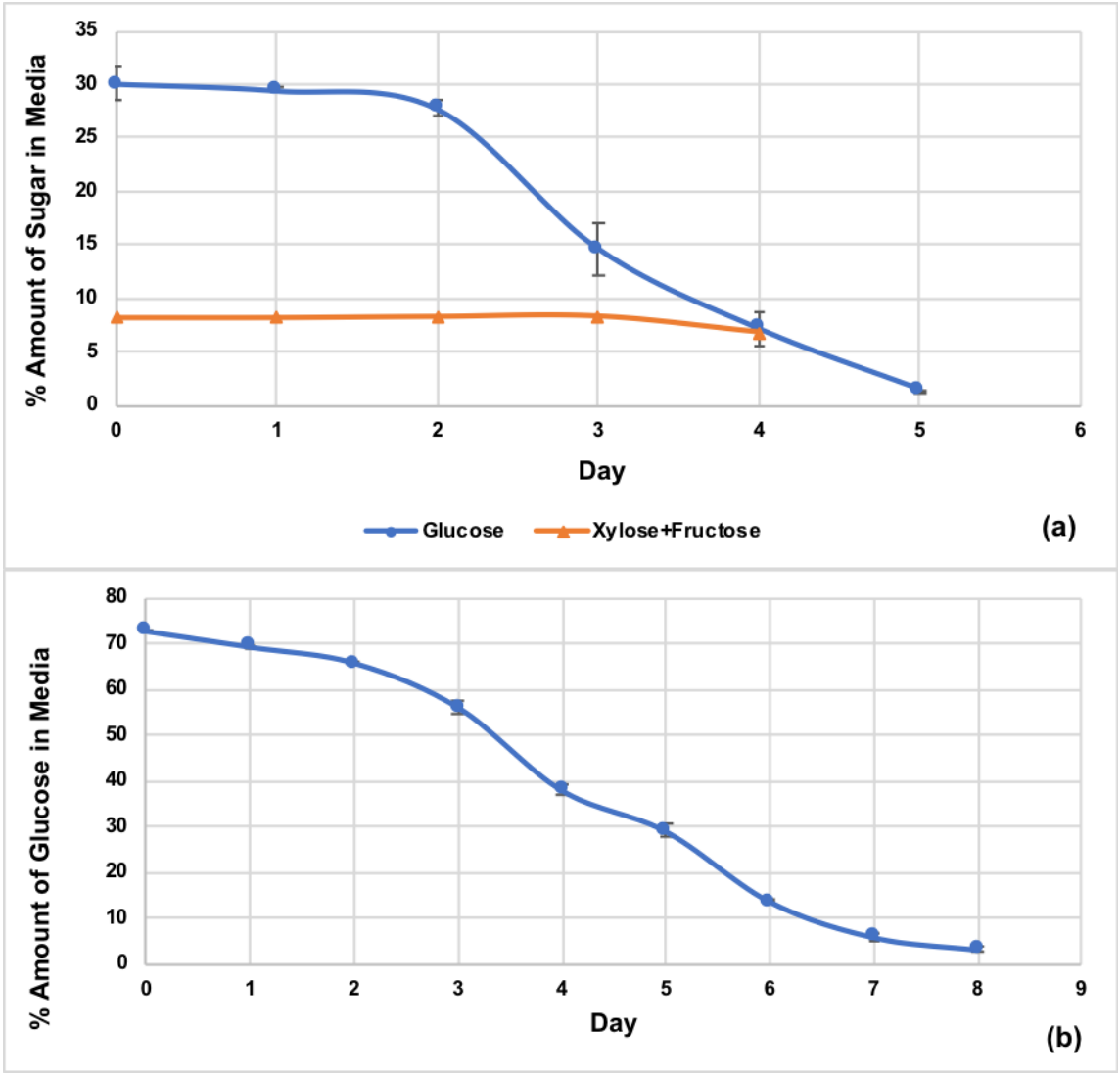

Fig 4. (a) Monosaccharide consumption during BC growth in ENZ 110, **(b)** Glucose consumption in HS medium

The xylose+fructose amount was almost steady until day 4; however, it was diminished between day 4 and day 5 and totally depleted at the end of day 5, albeit a relatively low amount. This may be due to the need of bacteria to use an alternative carbon source when growth medium lacks glucose, which corresponds with the previous studies (Ishihara *et al.* 2002; Keshk and Sameshima 2005).

Glucose in HS media followed a rather constant trend in terms of glucose consumption by bacteria, as shown in Fig. 4b. Contrary to the ENZ 110 media, glucose was not consumed totally until the end of day 8, apparently due to the higher initial concentration.

BC Production Efficiencies in Hestrin-Schramm and Enzymatic Media

For the calculation of substrate conversion ratio, BC production rate and BC production yield of ENZ 110 and HS media, HPLC data from the previous step were employed. As shown in Table 1, at the end of day 5 of incubation, substrate conversion ratio (α) was 95% in the ENZ 110 medium, while α was 60% in the HS medium. Hence, the HS medium still contained glucose, whereas almost all glucose was consumed in ENZ 110 medium after 5 days. Therefore, the BC production yield of ENZ 110 medium seemed to be higher compared to the HS medium, due to the total consumption of glucose. However, the BC production rate of HS medium was higher than ENZ 110 medium upon the superior amount of BC production.

Table 1. Substrate Conversions, BC Production Rates and Yields for the Enzymatic and HS Media at the End of Day 5

Type of Growth Medium	Substrate Conversion Ratio - α (%)	BC Production Rate - r_{BC} (mg mL^{-1} day^{-1})	BC Production Yield ($Y_{BC/S}$)
Enzymatic	95.11	0.14	9.6
HS	60.01	0.84	2.53

Characterization of BC

Scanning electron microscopy (SEM)

SEM results indicated that BC samples produced in enzymatic hydrolysates of olive pomace had a nano-sized fibrillar structure as shown in Fig. 5.

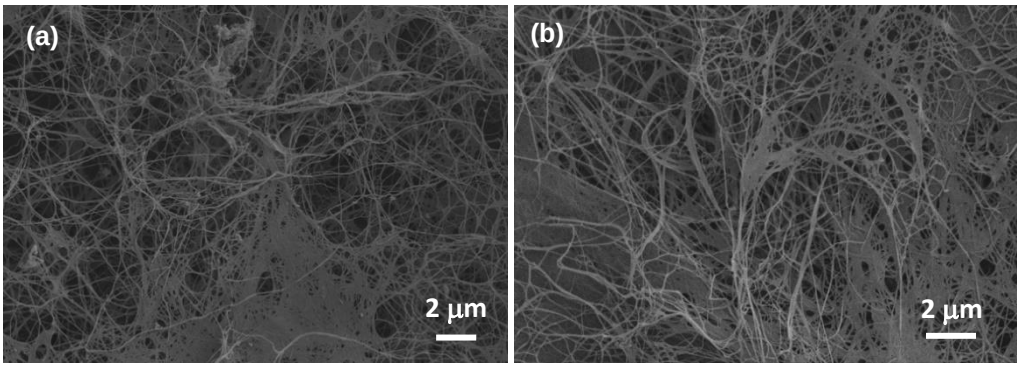

Fig. 5a. (a) SEM image of the BC produced in HS standard medium, **(b)** SEM image of the BC produced in HG medium (9,637 g/L glucose), **(c)** SEM image of the BC produced in enzymatic medium. (EHT: 6.0 kV; Magnification: 2.71, 5.18, 5.78 10^3 respectively)

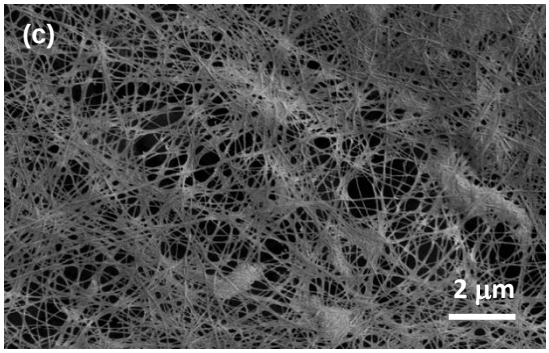

Fig. 5b&c. (a) SEM image of the BC produced in HS standard medium, **(b)** SEM image of the BC produced in HG medium (9,637 g/L glucose), **(c)** SEM image of the BC produced in enzymatic medium. (EHT: 6.0 kV; Magnification: 2.71, 5.18, 5.78 10^3 respectively)

These findings are in accordance with the previous studies of BC production conducted with agricultural waste materials (Lin *et al.* 2014; Yang *et al.* 2014; Li *et al.* 2015).

FT-IR analysis

The absorption bands at 3344, 2895, 1654, and 1032 cm^{-1} correspond to O-H, C-H, C-O-C, and C-O stretching, respectively, which are the characteristic spectrum of cellulosic material as presented in Fig. 6. The results are consistent with former studies (Yang *et al*. 2014; Kiziltas *et al*. 2015; Jahan *et al*. 2018). Bands corresponding to impurities were not detected.

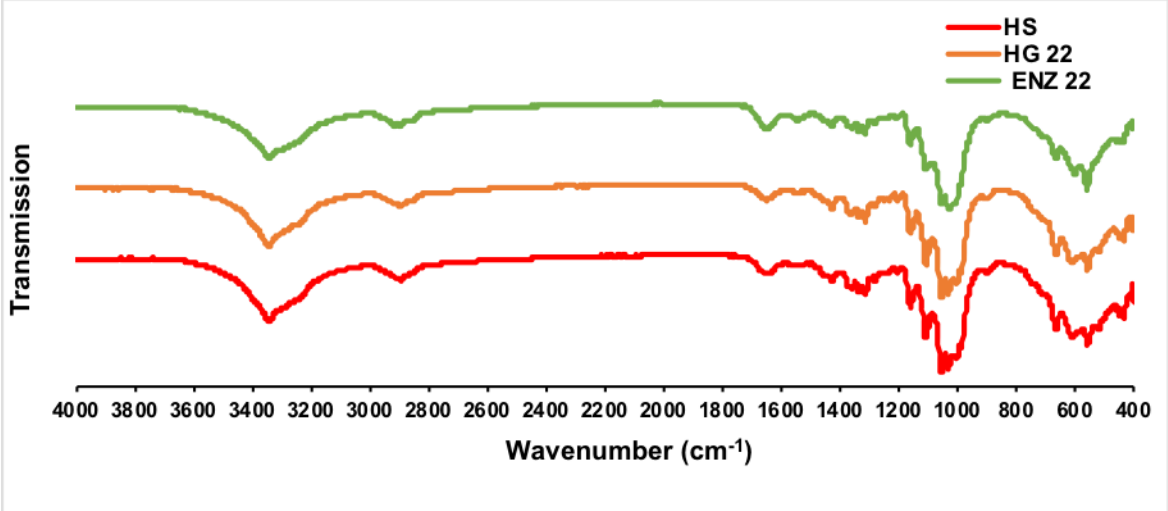

Fig. 6. FT-IR spectra of HS-BC (red), HG-BC (orange) and ENZ-BC (green) samples

X-ray diffraction analysis

BC samples of enzymatic hydrolysates and HS medium were analysed in terms of their X-ray diffraction patterns. They all exhibit the typical cellulose I peaks at 2θ angles 14°, 18° and 22°, as shown in Fig. 7. Nevertheless, all specimens displayed a broad signal at 2θ=11°, which did not assign to any known cellulose structure (not shown). The samples were analysed with SEM/EDX. These signals are based upon salts of phosphate, which are natural ingredients of the growth medium.

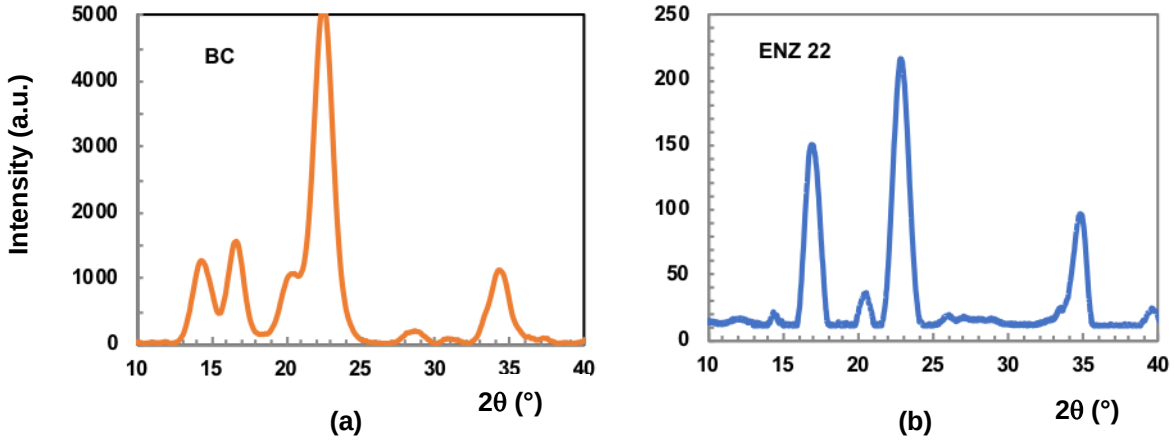

Fig. 7. X-ray diffractograms of **(a)** HS-BC and **(b)** ENZ-BC samples

CONCLUSIONS

1. Olive pomace was used as a carbon source for production of bacterial cellulose (BC).
2. Enzymatic hydrolysis led to an effective yield of reducing sugars without the necessity of harsh chemical pretreatment steps such as concentrated acid.
3. Though the BC amount obtained from the enzymatic growth medium was much lower than the one from the control medium, the production of BC without additional carbon sources was achieved, which is a promising step for further studies in terms of reducing the production cost and making use of olive pomace.

FUTURE PERSPECTIVES

To increase the BC production yield, the growth medium could be supplied with additional glucose, which would also contribute to the mechanical quality of the BC polymer, however, at the expense of cost increase. Besides, finding a more effective strategy to detoxify the microbial inhibitors will enable the use of higher reducing sugar amounts by bacteria in relevant enzymatic hydrolyzates. Further studies are required to elucidate the impact of growth medium content and inhibitors on BC production in the enzymatic hydrolyzates.

ACKNOWLEDGEMENTS

The research was partially supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK) [Scholarship No. 1059B141800242], Council of Higher Education of Turkey (YUDAB), and Istanbul Technical University Scientific Research Projects Coordination Unit [Project no: 41942]. The authors wish to thank Dr. Thomas Schulze, Thuringian Institute for Textile and Plastics Research (TITK), Rudolstadt, for his kind support with X-ray Diffraction Analysis and data evaluation. The authors are also

indebted to Steffi Stumpf and the SEM/TEM facility of the Jena Center for Soft Matter (JCSM) that was established with a grant from the German Research Council (DFG) and the European Regional Development Fund (ERD).

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Article submitted: January 9, 2023; Peer review completed: January 28, 2023; Revised version received: March 19, 2023; Accepted: April 6, 2023; Published: April 27, 2023.
DOI: 10.15376/biores.18.2.4168-4181