Bioconversion of underutilized brewing by-products into bacterial cellulose by a newly isolated *Komagataeibacter rhaeticus* strain: A preliminary evaluation of the bioprocess's environmental impact

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Abstract

A novel *Komagataeibacter rhaeticus* UNIWA AAK2 strain was used to produce bacterial cellulose (BC), valorizing brewers' spent grain (BSG) and brewer's spent yeast (BSY). Under optimal conditions (controlled pH=6 and 30 g/L sugars), a maximum BC of 4.0 g/L was achieved when BSG aqueous extract (BSGE) was used. The substitution of yeast extract and peptone with BSY autolyzates did not show significant differences on BC concentration and productivity. The FTIR, SEM, and TGA analyses showed that the use of brewing by-products had no effect on the structure and thermal stability of the produced BC, compared to highly-pure and commercial substrates. The LCA of the developed bioprocess revealed that BSGE- and BSY-based media can reduce the carbon footprint of 1 kg dry BC by 76% compared to commercial-based-media. Beer by-products could serve as cost-effective resources to produce value-

added and sustainable biopolymers such as BC, while minimizing waste and restructuring the brewing-industry.

Keywords: Biomass residues; Sustainable biopolymers; Brewing industry; Carbon footprint

1. Introduction

Newly extracted resources are main contributors for greenhouse gas emissions (GHG) (around 50%), biodiversity loss and water pollution (over 90%) (Koundouri et al., 2021). The world's standards for fair and prosperous societies require resource-efficiency and zero/negative net emissions that reflect in transformative policies of the EU e.g., ´European Green Deal´, ´EU climate neutral by 2050´, and ´New Circular Economy Action Plan´. The sustainable management of natural resources promoting concepts of circularity, green bioprocessing, and renewability could sustain climate neutral and circular bioeconomy schemes (Culaba et al., 2023).

Cellulose is the most abundant natural biopolymer on earth. It can be primarily recovered from wood, grasses, agroforestry and agricultural residues as well as from tunicates and algae. It has been estimated that from 1 ha of eucalyptus with a basic density of 500 kg/m^3, it would take 7 years (from planting to cultivation) to produce around 80 tons of cellulose (Singhania et al., 2022b). However, one interesting alternative is the production of bacterial cellulose (BC) using acetic acid bacteria. BC biosynthesis includes the formation of cellulosic ribbons that are secreted as microfibrils in the outer bacterial membrane through extrusion pores, in the longitudinal axis of bacterial cells. The crystallization of glucan chains leads to the formation of self-assembled microfibrils that are eventually organized into clusters of cellulosic ribbons (Selianitis et al., 2021). Bacterial genera of *Acetobacter, Gluconobacter, Komagataeibacter, Agrobacterium, Sarcina, Azotobacter, Rhizobium, and Alcaligenes* can produce BC as a response against environmental threats e.g., UV radiation (Behera et al., 2022).

BC is a remarkably versatile biomaterial. It possesses high water holding capacity (WHC) and permeability to gases due to the 3D conformation of its ultrafine fibril network. Additional properties such as high thermomechanical response, purity, increased surface area, high polymerization degree and crystallinity render it valuable in active food packaging (as reinforcer, and nanocarrier), water bioremediation (adsorption of heave metals and flocculation capacity), biomedicine (tissue engineering and controlled drug delivery), additive manufacturing (3D Printing), electronics and biosensors (enhanced conductivity). So far, the major factors that limit BC scale-up production are the high cost of the fermentation media and the low productivity values that have been so far achieved. The valorization of renewable resources derived from the agri-food sector combined with newly isolated strains that can assimilate a variety of carbon sources and are tolerant to inhibitors, could improve the economics of the BC bioprocess (Singhania et al., 2022a).

Worldwide industrial attention is moving towards more efficient production practices that foster sustainability and therefore, the elimination of the negative environmental impacts of production processes. The latest report of the Intergovernmental Panel on Climate Change underlines the importance of reducing the net anthropogenic GHG (Pörtner et al., 2022). Life cycle assessment (LCA) has been used to assess the environmental impact of processes either on a lab- or industrial-scale, that are employed to produce bio-based products. So far, LCA studies have focused on the impact of different fermentation media on BC production. It has been demonstrated that 36.1 t of water are consumed per 1 kg of BC produced while most of the consumed water is spent for the formulation/production of the raw materials which inevitably causes most of the environmental impacts. Replacing petroleum-derived plastics with bio-based ones could produce 30% less CO_2-equivalents, leading to reduced global warming potential (GWP) (Forte et al., 2021).

Beer is the most consumed beverage globally, after water and tea. The beer manufacturing involves multiple steps that is, malting of barely grains, milling, mashing, lautering, boiling, wort cooling, and fermentation. Brewers' spent grain (BSG) is the solid residue (husk of barley grain) obtained after the wort separation while it accounts for 85% of the total solid residues and 30% of the initial malt weight (Mitri

et al., 2022). Given that for every 100 L of beer produced, 20 kg of BSG are obtained, and based on worldwide beer production, it can be estimated that around 36.4 million tons of BSG are generated annually (Qazanfarzadeh et al., 2023). Brewer's spent yeast (BSY) (mainly yeast cells from *Saccharomyces* sp.) is also a by-product of the brewing industry that is discarded at the end of the fermentation via flocculation. Around 17 tons of surplus yeast are generated per one million L of finished beer.

The compositional profile of BSG primarily depends on the species of barley while harvest time, brewing process, climate, and soil type can also affect its chemical composition. BSG is rich in hemicellulose (～20-40%), cellulose (～15-30%), proteins (14-46%), lignin (1.5-25%), oil (6-13%, mainly C16:0, C18:0, and C18:2) and phenolic compounds (Qazanfarzadeh et al., 2023). BSY is a low-cost source of protein (45–60%), minerals, vitamins and saccharides. Though, BSY use as a high-quality protein supplement in food systems is rather limited due to its high percentage in nucleic acids (6–15%) that can lead to hyperuricemia (Puligundla et al., 2020). Wet BSG and BSY are prone to microbial attack. BSG has a shelf life of 7 to 10 days due to its high moisture content (70-80%), and nutrients such as soluble sugars, oil, polysaccharides, and protein. BSG and BSY are mostly used in the feed and food industry while they could be considered very promising for bottom-up and top-down biorefinery development, involving sustainable fractionation and fermentation (Kumar et al., 2022).

This study valorized agro-industrial residues derived from the brewery industry to produce BC using a newly isolated and identified *Komagataeibacter rhaeticus* strain. The bioprocess was investigated in terms of initial pH values, carbon to free amino nitrogen ratio (C/FAN) and substitution of the nitrogen sources (e.g., yeast extract and peptone) with BSY autolyzates. The properties of the produced polymers were determined while the best performing conditions were evaluated regarding the sustainability potential of the developed bioprocess.

This study presents the development of an integrated bioprocess that valorize BSG and BSY to produce a generic fermentation medium suitable for BC production. To the best of the authors' knowledge, up to date, there is no report of such a study. The BC-producing potential of a newly isolated bacterial strain was also explored. The simplified LCA of the developed bioprocess presented here may serve as an

orientation of future research towards advanced biomass-based biorefineries enabling the production of high value-added products, waste minimization, and restructuring of the brewing industry.

2. Materials and Methods

2.1. Feedstock and preparation of the raw materials

BSG and BSY were kindly provided by a local craft microbrewery (Kavala's Microbrewery GP, Greece). Wet BSG was thermally treated at 121 °C for 20 min. Subsequently, it was mixed with distilled water to reach an initial solids concentration of 200 g/L. Mixtures were stirred at 500 rpm, for 1 h, at 50 °C. The liquid phase, namely BSG aqueous extract (BSGE) was separated from solids via centrifugation (10,595 RCF, 4 °C, 10 min). BSY slurry was autolyzed as follows: undiluted BSY was stirred at 600 rpm, for 24 h at 55 °C and then 1 h at 80 °C (Akermann et al., 2020). The liquid phase (BSYE) was separated from solids via centrifugation (10,595 RCF, 4 °C, 10 min). Both BSGE and BSYE were stored at -20 °C until further use.

2.2. Isolation and identification of acetic acid bacteria

The isolation of acetic acid bacteria (AAB) was realized following the method of Gullo et al. (2006) with some modifications. More precisely, 1 mL of Kombucha beverage was diluted with 9 mL of Ringer's solution, and serial decimal dilutions were spread on GYC plates (20 g/L D-glucose, 10 mL/L ethanol, 10 g/L yeast extract, 10 g/L $CaCO_3$, 15 g/L agar, at pH=6.8). Cycloheximide (0.1 g/L) was added to inhibit the possible growth of yeasts. Plates were incubated at 28 °C for one week. Twenty isolates were purified by successive streaking on GCY medium and were either subjected to DNA isolation or stored in 50% glycerol (v/v) at −80 °C. Total genomic DNA from the AAB isolates was extracted using Insta GeneTM Matrix genomic DNA extraction kit (Bio-Rad, USA). The quantity and purity of the extracted DNA were measured via a microvolume UV/Vis spectrophotometer (Epoch, Biotek, USA) at 260, 280 and 230 nm and standardized at a final concentration of 100 ng/μL. Molecular identification at species level was carried out via amplification and sequence analysis of 16 S rRNA gene using the universal primers P1V1

(5'-GCG GCGTGC CTA ATA CAT GC-3') and P4V3 (5'-ATC TAC GCA TTT CAC CGC TAC-3'). PCR amplifications were carried out in 20 μL reaction in T100 thermocycler (BioRad, United States). Thermocycling conditions were as follows: initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 30 sec, 72 °C for 90 sec and a final extension step at 72 °C for 15 min. The PCR-products were purified using Montage PCR clean up kit (Millipore) and Sanger-sequencing was performed (Eurofins, Germany). The sequence-based identification of bacteria was completed by using Microbial nucleotide BLAST https://blast.ncbi.nlm.nih.gov/Blast. One isolate, namely UNIWA AAK2, that belonged to the species of *K. rhaeticus,* was subjected to the phenotypic analysis.

2.3. Batch fermentation for BC production

Initial shake flask fermentations were carried out using commercial carbon sources (≈20 g/L) e.g., glucose, fructose, galactose and galactose, under free pH (without pH control). The fermentation media was supplemented with 5.0 g/L yeast extract, 5.0 g/L peptone, 2.7 g/L Na_2HPO_4 and 1.15 g/L citric acid. Subsequent experiments for BC production with *K. rhaeticus* were carried out using BSGE, at two initial sugars concentrations (ca. 30 g/L and 20 g/L), under free pH. Fermentations were also investigated at various initial pH values (4.5; 5.2; 6.0, 6.7) (controlled with 4 M NaOH every 24 h). The BSGE was used as the sole carbon source while commercial yeast extract and peptone were accordingly added to achieve a total FAN concentration of 350 mg/L, depending on the initial FAN concentration of the BSGE. The best performing conditions were further applied in experiments where commercial yeast extract and peptone were substituted with BSYE. Proper amounts of BSY were mixed with BSGE to obtain FAN≈350 mg/L. The fermentation medium was filter sterilized (0.22 μm Polycap TMAS, Whatman Ltd.). All fermentations were performed in Erlenmeyer flasks (50 mL working volume), inoculated with 10% (v/v) of a 24-h preculture media and statically incubated for 10 days, at 30 °C. Pre-culture preparations had the same composition with the fermentation broth while they were incubated at 180 rpm, 30 °C for 24 h. The bacterium was maintained at -40 °C in cryotubes containing bacterial culture and glycerol of 50:50 (v/v).

2.4. Physicochemical characterization of BC samples

The surface morphology of BCs from the different fermentations was investigated via a Field Emission Scanning Electron Microscope (SEM) (JEOL JSM-7610FPlus). The accelerating voltage was 15 kV. The samples were deposited on a copper grid and visualized after coating with a thin gold film. Attenuated total reflectance Fourier Transform infrared spectroscopy (ATR-FTIR) was performed on a Bruker Tensor 27 instrument equipped with PIKE MIRacle ATR and OPUS v6.5 operating software. 64 scans (525-4000 cm^{-1}) at 2 cm^{-1} resolution were performed. Samples were double measured to confirm reproducibility. TGA analysis was carried out at a TGA Q500 analysis System (TA Instruments) under a nitrogen flow of 30 mL/min with samples (5-10 mg) being heated up to 700 °C at a rate of 10 °C/min.

2.5. Analytical methods

The carbon sources determination was carried out using a High Performance Liquid Chromatography (HPLC) equipped with a refractive index (RI) detector (RID-10A; Shimadzu Corp., Japan), column oven (CTO-10ASvp; Shimadzu Corp., Japan), and a ReproGel H column (250x8 mm, 9 μm; Dr. Maisch, Germany). The mobile phase was a 5 mM H_2SO_4 aqueous solution with 0.6 mL/min flow rate at 40 °C. FAN determination, BC purification, and determination of BC weight and its water holding capacity (WHC) have been thoroughly described by Efthymiou et al. (2022b).

2.6. Carbon footprint assessment

The environmental analysis that was performed in this study constitutes a preliminary simplified LCA approach that was carried out according to the internationally accepted methodology of LCA conforming to ISO 14040:2006 (ISO, 2006, p. 14040) and ISO 14046:2014 (ISO, 2014) guidelines and recommendations. The system boundaries (see e-supplementary materials) are defined as cradle-to-gate, since this approach is suitable for versatile building-block materials, in the research stage, with a wide range of end-uses. The functional unit (f.u.) was set as the production of 1 kg of dry BC after 10 days of fermentation. The environmental impact on climate change known as the carbon footprint (CF) or GWP (kg CO_2-eq/kg BC) was estimated using the CCalC2 software. The inputs presented in the life cycle inventory (see e-

supplementary materials) were based on the lab scale production of BC as described in this study, and the emission factors were retrieved from the publicly available CCaLC2 and Ecoinvent databases. This analysis was limited to the necessary equipment and production stages. As there is no dataset in LCA databases quantifying the emission factors of peptone, the whey protein emission factors were used instead. Production of 1 kg dry BC utilizing three different fermentation media were compared including the Hestrin and Schramm (HS) synthetic media, BSGE-based media and BSGE media supplemented with BSYE.

2.7. Statistical analysis

The statistical analysis was performed using Statgraphics. Analysis of variance (ANOVA) and Pearson's linear correlation at 5% significance level was carried out for data comparison. Significant differences between means were determined by Honest Significant Difference (HSD-Tukey test) at level of $p<0.05$. Data were reported as mean values ± standard deviation of three independent replicates ($p<0.05$, 95%).

3. Results and Discussion

3.1. Characterization of BSGE and BSYE

The ash, oil, moisture content and the pH value of wet BSG were respectively determined equal to 2.8±0.3%, 3.2±0.6%, 68.5±3.13%and 5.7±0.3. These results are very similar to BSG obtained from craft breweries in the local region of North Dakota and Minnesota (Jin et al., 2022). The moisture content of various BSGs presents considerable variation from 2-83%, that are mostly attributed to the drying process of the material including temperature, time, and technique (Qazanfarzadeh et al., 2023). High moisture content contributes to BSG high susceptibility towards microbial contamination. The common practices to increase the shelf life of BSG are the addition of preservative agents and organic acids for short-term results up to 10 days, while long-term preservation requires drying up to 10% of humidity which elevates the process cost of beer making (Qazanfarzadeh et al., 2023). Fermentable sugars can also accelerate the

degradation process of BSG, mainly due to naturally occurring bacteria, such as *Clostridium butyricum* that bioconvert the fermentable sugars into butyric acid (Akermann et al., 2020).

The BSG that was utilized in this study presented a quite high concentration of total soluble compounds. BSG was properly formulated at a solids concentration of 200 g/L to recover the water-soluble sugars, without prior drying. The total soluble sugars in BSGE were determined equal to 22.8% (g total sugars/100 g dry BSG) (45.7±3.9 g/L) with maltose accounting for 69.6% (g maltose/100 g total sugars), followed by glucose (13.3%, g glucose/100 g total sugars), maltotriose (11.8%, g maltotriose/100 g total sugars) and fructose (5.3%, g fructose/100 g total sugars). The FAN concentration was equal to 145.9±6.6 mg/L. BSG liquor has been reported to contain tri- and disaccharides that are maltotriose, sucrose, and maltose as well as monosaccharides that is, glucose, mannose, fructose, and rhamnose (Milew et al., 2022). Bjerregaard et al. (2019) demonstrated a continuous rotary drum press to process hot BSG and produce a liquid filtrate rich in maltose (33.5 g/L), glucose (7.82 g/L) and FAN (104±17 mg/L). A soluble sugar content quite similar to this study (18.2%) was reported for BSG liquor originated from a May bock brewing. The soluble sugars consisted mainly of 31.9% malto oligomers, 19.2% maltotriose, 39.6% maltose, and 9.3% glucose (Akermann et al., 2021). Much higher total carbohydrate amount (78.2 g/L) was found in BSG liquor (directly pressed by a friction press) derived from wheat bock. Maltose was found the most abundant saccharide (60.2%) while maltotriose and glucose accounted for 23.3% and 16.5% respectively (Akermann et al., 2020). A carbohydrate concentration equal to 54 g/L of BSG syrup (obtained from 400 g/L of BSG mixed with water) was reported by (Casas-Godoy et al., 2023) but they reported that glucose was the main sugar (67.2%) followed by xylose (10.9%).

The aforementioned deviations are mostly attributed to the artisanal process and the beer type produced from the local craft microbrewery that BSG was supplied from. Craft breweries typically generate two to three times more BSG compared to larger breweries. The brewhouse efficiency in small craft breweries varies between 70-80%, while industrial breweries can achieve >90% efficiencies, and thus influencing the sugar content of the resulting BSG. Other factors that can directly affect the BSG composition include the dewatering method, cereal variety, harvesting time, malting, and mashing process (Casas-

Godoy et al., 2023; Milew et al., 2022). The popularity of craft breweries is noteworthy, with the global craft beer industry expected to grow at a CARG of 14.1% from 2020 to 2025, and sales up 23% (Jaeger et al., 2020).

BSY showed a moisture content of 90.6±2.5%, oil content of 2.8±0.2%, pH of 4.7±0.4, and ash of 4.2±0.3%. The BSYE contained low levels of total soluble sugars of 6.1±0.9 g/L (6.5%, g total sugars/100 g of dry BSY) and more specifically, 4.0±0.2 g/L maltose, 1.0±0.3 g/L glucose, and 1.1±0.4 g/L fructose. This can be explained by the fact that most of BSY polysaccharides (around 83%) are insoluble while simple water-soluble carbohydrates are generally soluble in aqueous alkali (Jaeger et al., 2020). Glycerol was determined equal to 1.7±0.4 g/L. Average levels of soluble reducing sugars in BSY have been reported to be 1.3% while α-amino nitrogen of BSY extract varies within 4.1-12.9% (Jaeger et al., 2020; Puligundla et al., 2020). BSY has been applied as a cost-effective starting material to produce yeast extract via autolysis. The autolysis process initiates when the cell growth cycle is terminated. It involves the degradation of cell yeast components by endogenous enzymes e.g., proteases, nucleases, glucanases and phospholipases to release valuable components. In this study, BSYE presented a maximum FAN concentration of 1350±40.2 mg/L after autolysis at 24 h, 60 °C, using 9.4 g/L dry BSY. The application of 50 °C for 24 h during autolysis has been reported to yield considerable amounts of α-amino nitrogen which constitutes the most assimilable form of protein for microorganisms (Jaeger et al., 2020; Puligundla et al., 2020).

3.2. Isolation and identification of AAB

Microbial nucleotide BLAST for the sequence-based identification, using 16 S rRNA gene sequence of the strain UNIWA AAK2, showed a similarity of 99.79% *K. rhaeticus* strain ENS 9a1a chromosome (see e-supplementary materials). The majority of the species of the genus *Komagataeibacter* (family Acetobacteraceae) is implicated in extracellular cellulose synthesis through oxidative fermentation. *K. rhaeticus* was first mentioned as a great BC producer by dos Santos et al. (2014). Since then, the species,

mainly isolated from kombucha and vinegar, has been explored for the novel properties of the produced polysaccharide (Jacek et al., 2021; Machado et al., 2016).

3.3. Evaluation of commercial carbon sources for BC production

The newly isolated bacterial strain *K. rhaeticus* was initially screened on various commercial sugars to evaluate its ability to grow and produce BC (Table 1). The highest BC concentration (0.88 g/L) was observed when glucose was used as the carbon source combined with satisfying sugars consumption (69.3%). Lower BC production was determined in the case of fructose while fermentation efficiency was drastically decreased when galactose and lactose were applied (respective BC concentrations of 0.37 g/L and 0.25 g/L). The poor ability of the bacterial strain to catabolize galactose and lactose was also indicated by the sugars and FAN consumption that were found around 30%. It is well demonstrated that strains of the Acetobacteraceae family, lack the gene that encodes β-galactosidase to hydrolyze lactose into monosaccharides. BC production is a strain dependent process, while fermentation media, process parameters, and bioreactor configuration can also affect BC yield (Tsouko et al., 2015). Commercial C and N sources would rationally lead to better results since they do not contain any inhibitors and they can be properly designed to fulfill the metabolic requirements of the acetic acid bacteria. In fact, the results obtained in this study when commercial carbon sources were used, combined with the conventional HS medium, are quite low compared to relative studies that have been already reported (Tsouko et al., 2015).

3.4. BC production using BSGE

Further experiments focused on the direct valorization of BSGE. BC production was initially evaluated using ≈30 g/L and ≈20 g/L of total initial sugars. No significant differences were detected in BC amount ($p<0.05$), with the highest initial sugars concentration leading to slightly higher BC production (1.64 g/L). Glucose was the most assimilable sugar in both cases (85.7-89.1%), followed by fructose, and maltose. Maltotriose was poorly metabolized (1.8-13.6%) in all cases. FAN consumption and WHC were similar ranging within 29.6-34.2% and 65.7-68.8 g water /g dry BC respectively for the two treatments.

Sequential experiments focused on BC production applying various pH values that were maintained at their setpoints throughout the fermentation (Table 2). Only BC produced under the initial pH=4 showed statistically significant differences when compared to the other samples that derived from pH=5.2, 6 and 6,7. The most efficient fermentation in terms of BC production (4.01 g/L), and productivity (0.45 g/L/d) was the one that the pH was maintained at a value of 6. In this case, the highest consumption of total sugars (68.9%) and FAN (80.2%) were observed. The assimilation pattern of sugars was similar in all pH values. It was noticed that pH control led to higher maltose (57.5-71.6%) and fructose (65.7-81.3%) consumption in all cases compared to the uncontrolled conditions (Table 2). Similarly, to the uncontrolled pH conditions, glucose was highly catabolized by the bacterial strain (83.2-91.9%). Based on the pH monitoring throughout all fermentations, a drop in pH from the initial set value to around 4.0-4.5 occurred after 2 days of cultivation. This drop in pH might occur due to the action of dehydrogenase enzyme (that has been found in *Gluconacetobacter* species) that converts glucose into gluconic acid (Tyagi and Suresh, 2016). Optimal pH values for BC production are directly related to the type of bacterial strain. pH values within 5-5.5 have been reported to favor BC production when commercial strains of *Gluconacetobacter xylinus, K. sucrofermentans* and *K. intermedius* were employed (Chen et al., 2013; Efthymiou et al., 2022b; Santoso et al., 2020). Other studies report the pH=6 to be the most effective for enhanced BC production using *K. xylinus* and *G. xylinus* (Du et al., 2020; Tyagi and Suresh, 2016). In fact, Tyagi and Suresh (2016) reported that the production rate of BC decreased at pH 4–5 while a pH higher than 6 led to insignificant BC amounts.

As shown in Figure 1b, glucose consumption was the fastest while it was almost entirely catabolized by the bacterial strain, up to 7 days of fermentation. Considerable fructose and maltose consumption started after 5 days of fermentation while maltotriose was very poorly assimilated throughout fermentation. FAN was steadily consumed throughout fermentation. High BC production rates were observed up to 7 days, while slight variations were observed thereafter (Figure 1a).

The WHC of BC showed a slightly decreasing tendency with increasing pH up to 6, remaining almost stable thereafter (Table 2). More specifically, the WHC gradually decreased from 67.5 g water/g dry

BC to 66.5 g water/g dry BC and finally to 64.7 g water/g dry BC. Quite higher to this study WHC (>90%) have been reported by several scientific studies that evaluated BC production under static conditions using fermentation media that derived basically from renewable resources (Du et al., 2020; Efthymiou et al., 2022b; Tyagi and Suresh, 2016). WHC is directly related to the surface area and pore size of the polymer. High WHCs indicate that high water amounts are captured in the three-dimensional matrix of BC with water being retained via hydrogen bonding between water molecules and BC fibrils (Efthymiou et al., 2022b).

Although BSGE is rich in soluble sugars, there are very few studies that have employed it for value-added valorization e.g., microbial bioconversion (Akermann et al., 2020). Most studies have focused on the hydrolysis of the hemicellulose and/or cellulose which are the major fractions of BSG (Agrawal et al., 2023). In fact, most cited publications remove the soluble components from BSG, mainly glucose, maltose and malto oligomers by several washing steps (normally three times) prior to the pretreatment stages e.g., organosolv pretreatment with screw-pressing (Amraoui et al., 2022), or microwave-assisted technique with alkali (Weiermüller et al., 2021). They perform sequential enzymatic hydrolysis to produce fermentable sugars that can be assimilated by microbes and produce value added compounds such as 2,3-butanediol, itaconate and polyhydroxyalkanoates.

3.5. Effect of BSYE on BC production

In this set of experiments the entire substitution of commercial yeasts extract and peptone with BSYE was evaluated (Table 2 and Figure 1c, d). No significant differences were detected on BC concentration (decreased by only 4%) and productivity when compared to the fermentation with commercial N sources (pH=6, controlled). The catabolic profile of individual sugars (Figure 1d), and FAN (Figure 1c) during 10-day fermentation was very similar to the BSGE-based experiment (controlled pH) (Figure 1a, b). Glycerol was very quickly and entirely consumed by the bacterial strain. The WHC of BC was considerably higher (98.6 g water/g dry BC) compared to all the previously examined samples. The WHC is affected by the fermentation media. For instance, Tsouko et al. (2015) reported WHCs within 102–138 g

water/g dry BC when *K. sucrofermentans* DSMZ 15973 was grown on biodiesel-derived glycerol and confectionery waste. WHC is an important feature of BC when food and biomedical applications are considered.

Tyagi & Suresh (2016) demonstrated that BC production using *G. intermedius* SNT-1 was comparable when corn-steep liquor or yeast extract were used as the N sources combined with heat pretreated molasses, while BC yield decreased by 30% when polypeptone was not added. The cost of yeast extract presents great variations within 50–350 $/kg, while BY cost has been reported to be 6 $/kg. Accordingly, the complete replacement of N and C sources with BSGE and BSYE that was suggested in this study, can substantially decrease the cost of the fermentation media which represents 30% of the total production cost (Pejin et al., 2019).

Pejin et al. (2019) employed BSG enzymatic hydrolysates (FAN before autoclaving: 30-76 mg/L; reducing sugars prior to BSY addition: 252 g/L) supplemented with various amounts (5-50 g/L) of BSY to replace yeast extract with alternative nitrogen sources and finally to produce lactic acid with *Lactobacillus rhamnosus*. They added different amounts of dry BSY to the BSG hydrolysate followed by autoclaving at 121 °C for 15 min. The maximum FAN concentration of 393.6 mg/L combined with high total reducing sugars (44 g/L) were achieved when 50 g/L of BSY were added. In this case, a lactic acid yield of 89% with a productivity of 0.89 g/L/h were reported. Several attempts have been made to substitute (partially or entirely) commercial nitrogen sources e.g., yeast extract and peptone in fermentation media. BSY has been proven as an efficient alternative supplement to produce enzymes, ACE-inhibitory proteins from *Ganoderma lucidum* mycelia and succinate (Puligundla et al., 2020).

Several studies have developed a two-stage bioprocess to maximize BC production with the strain *K. sucrofermentans* DSM 15973 using waste streams such as wheat milling by-products (static shake flasks) (Natsia et al., 2022), sunflower meal and crude glycerol from the biodiesel industry (in 6 L static tray bioreactor) (Efthymiou et al., 2022a) and confectionery waste (static shake flasks) (Efthymiou et al., 2022b). More specifically, they involved solid state fermentation to produce crude enzymatic complexes with *Aspergillus awamori*. The latter were sequentially used to break down macromolecules contained in

these waste streams e.g., starch, disaccharides, and proteins into simple sugars, amino acids, and peptides. The produced enzymatic hydrolysates could fulfill the requirements of the bacterial strain for proliferation and BC production (5.2-12 g/L). In the study of Heydorn et al. (2023), a fermentation media containing molasses as the C source and mixtures of vinasses and waste beer fermentation broth as the N source resulted in high concentrations of BC (8.1 g/L) with crystallinities around 58% and DP_n of 3343. Another study proposed mechanically pretreated corncob and sugarcane bagasse as effective feedstock to produce enzymatic hydrolysates rich in C6 and C5 sugars. Agitated shake flasks of the newly isolated strain *Komagataeibacter* sp. CCUG73629 could produce BC amounts within 1.2-1.6 g/L after 10 days of fermentation (Akintunde et al., 2022). Varying BC concentrations have been reported when oil palm frond juice (2.9 g/L, in static containers for 10 days using *A. xylinum* 0416) (Said Azmi et al., 2021), tobacco waste extract (5.2 g/L, in agitated shake flasks for 7 days with *A. xylinum* ATCC 23767) (Ye et al., 2019), tomato juice RSV-4 (7.8 g/L, in 10-L static trays for 7 days using *A. pasteurianus*) (Kumar et al., 2019) and orange peel hydrolysates (0.67 g/L, in static shake flasks, with *K. sucrofermentans* DSM 15973) were utilized as the fermentation substrate (0.67 g/L) .

Moving towards a circular bioeconomy and being in line the Sustainable Development Goals that have been established by the United Nations, renewability and sustainability concepts as well as material circularity should be seriously taken under consideration. Extensive cellulose extraction from plants should be avoided, while extra requirements for cellulose in several sectors should be covered via alternative resources. Advanced multi-product biorefineries based on BSG and BSY combined with other renewable feedstock of similar nature, could be developed to produce a variety of valuable products, combining fractionation and extraction processes (to obtain lignin, pectin, polyphenols, oil, etc.) followed by microbial fermentation (to produce biobased chemicals and polymers) to maximize resource efficiency. Though, studies similar to the present one can make substantial contribution to the aforementioned.

3.6. Characterization of BC samples

SEM micrographs (see e-supplementary materials) revealed cellulose fibrils with a typical 3D network of ultrafine and randomly organized fibers and porous structure. The average fiber diameters ranged between 25-80 nm. The presence of such ultrafine fibers in BC are responsible for the materials' increased tensile strength and elongation at break, compared to typical cellulose fibers.

TGA was performed to determine the water retention capacity and obtain information on thermal decomposition behavior of BC and thermal profiles are shown in Figure 2. Thermal stability can be a critical parameter for study, depending on the application of BC, and might also provide some indications on BC fibre interactions. Previous studies have shown that the thermal stability of BC samples depends on parameters such as the molecular weight, crystallinity, BC fiber orientation. Two significant mass losses can be observed in Figure 2, that corresponded to: (i) the initial weight loss (30-120 °C) due to dehydration/evaporation of absorbed moisture from the BC films and (ii) the thermal degradation due to depolymerization, dehydration and decomposition of glycosyl units and formation of carbonyl and carboxyl groups and (120–400 °C). Carbonaceous residues of between 11.7-25.2% was observed at 700 °C. For the thermal degradation phase where significant weight loss occurred for the BC samples, onset and end temperatures ranged between 294.6-323.5 °C and 375.6-399.5 °C respectively, while normalized weight changes were between 69.9-77.0%. In this study, the weight loss profiles obtained via TGA analysis (see e-supplementary materials) are similar to the majority of TGA analysis previously reported for BC. For example, BC pellicles produced by *G. xylinus* BNKC 19 using HS media as well as pineapple peels and over-ripened banana hot extracts showed a significant mass loss of between 70-80% at around 310°C, whereas carbonaceous residues at 550°C were between 10-30% (Jittaut et al., 2023). In another study, a kombucha consortium consisting of *K. saccharivorans LN886705, Brettanomyces bruxellensis MH393498* and *B. anomalus KY103303* with a ratio of (4:1:1) produced BC utilizing black tea media.

TGA analysis revealed an initial weight loss of 8%, from room temperature to 225 °C, and a second weight loss of around 61% from 225 °C to 350 °C. The carbonaceous residue at 650 °C was around 25% (Avcioglu et al., 2021). TGA analysis on BC produced by an isolated thermophilic *Bacillus licheniformis* strain ZBT2 utilizing modified HS media showed an initial weight loss of 22% at a temperature range of 41 to 113 °C, followed by a 52% mass loss due to degradation around the temperature range of 259-375°C with a solid residue of around 25% present at 800 °C (Bagewadi et al., 2020). Moreover, BC produced by *A. xylinum* ATCC 23767 using tobacco waste extract and HS media showed a 60% weight loss between 250-340 °C and a residue of between 10-20% at 500 °C (Ye et al., 2019). In conclusion, all BC samples produced in this study showed very good thermal stability, which was in general higher compared to several previously published studies that report degradation ranges between 250-375 °C.

FT-IR spectra provided information on the functional groups present in the BC samples (see e-supplementary materials). The spectra were similar for all BC samples and quite typical to those previously reported in the literature, confirming the structure of bacterial cellulose type I. The peak at 3340 cm^{-1} indicates the hydroxyl (-OH) group stretching of BC, while peaks originated from 2800 cm^{-1} to 2900 cm^{-1} indicate C-H stretching vibrations of BC (including $-CH_2$ and $-CH_3$). Peaks at 1645 cm^{-1} and 1333 cm^{-1} are attributed to hydroxyl group bending and in-plane -OH bending. Other characteristic peaks are observed at 1167 cm^{-1} and 1060 cm^{-1} that belongs to C-O-C group of the carbohydrate skeleton and C-O-H bond of BC (Avcioglu et al., 2021; Bagewadi et al., 2020).

Overall, upon SEM, TGA and FTIR analysis it can be concluded that no major difference was observed between BC samples produced by highly pure substrates and brewing by-products. As thermal stability is known to be dependent on the molecular weight, crystallinity and fibril orientation, these results indicate no severe changes due to the use of BSGE and BSYE.

3.7. Carbon footprint of BC production

This study employed a simplified LCA methodology to give insight into the environmental impact and hotspots of the BC production process. Figure 3 and Table 3 present the CF of each production stage, the total CF, and the carbon savings for the three fermentation media that were investigated. The HS medium resulted in the highest carbon footprint, equal to 573 kg CO_2-eq/kg f.u. while the BSGE- and the BSGE+BSYE-derived media led to 4.2-4.4-fold lower CO_2-eq/kg f.u. The significant decrease in the CO_2 emissions is attributed to the higher BC concentration achieved and the substitution of commercial carbon and nitrogen sources in the medium with BSGE and BSYE.

In all the examined cases, the most carbon-intensive stage of the bioprocess was the fermentation due to the high energy consumption of the incubator. More specifically, the contribution of the fermentation stage to the total CF for 1 kg dry BC varied within 89-92% when HS medium, BSGE, and BSGE+BSYE were used. The raw materials (water, glucose, yeast extract and peptone) that were used in the HS medium corresponded to 5.8% of the total CF while their substitution with brewery-derived sidestreams led to reduced total CF. The medium sterilization and inoculation accounted for 3-1% of the CF in all cases.

The conventional landfill practice of BSG leads to the release of 514 kg CO_2-eq/ton of disposed BSG, while the treatment of BSY into municipal waste treatment plants results in 83 kg CO_2-eq/ton of waste treated (Mitri et al., 2022). GHGs that would otherwise be emitted if BSG and BSY were disposed in the landfill, are avoided when these streams are employed as fermentation feedstock, leading to carbon savings. Examining the BSGE-based production scenario, the total CF minus carbon savings was equal to 106.25 CO_2-eq/f.u., representing a significant 81% reduction compared to the conventional HS fermentation medium. Similarly, the BSGE+BSYE-based media showed an impact of 100.95 kg CO_2-eq/f.u, which is a 82% reduction of CF compared to the HS medium.

There are limited cited publications performing cradle-to-gate LCA for BC production. BC production can be less carbon-intensive compared to cellulose extraction from plant biomass demonstrates that. The CF of BC is reported within the range of 34-296 kg CO_2-eq/kg BC (Silva et al., 2020; Forte et al.,

2021; Martínez et al., 2023). These high variations are due to differences in the production methods, product yields, equipment, LCA software applied, inventory databases and the system boundaries considered. Indicatively, Martínez et al. (2023) reported the lowest CF (39 kg CO_2-eq/kg BC) when BC was produced valorizing nata-de-fique agro-waste. However, their analysis did not consider electricity emissions of the equipment used in the process. Forte et al. (2021) performed a meticulous attributional LCA using Gabi Pro software, carefully modelling, and including all processes and emissions to calculate the CF of BC produced in HS medium. They reported a much higher CF equal to 296 kg CO_2-eq/kg BC. Silva et al. (2020) used the SimaPro® software to compare BC production in three fermentation media e.g., HS, soybean molasses and cashew juice. The CF of HS medium was the highest (255 kg CO_2-eq/kg BC) while the BC produced on hydrolyzed soybean molasses was the least intensive carbon (100.6 kg CO_2-eq/kg BC), which is in agreement with the findings of this study. Additionally, Forte et al. (2021) and Silva et al. (2020) determined the effect of BC production in multiple impact categories, illustrating a minimal environmental impact regarding acidification, freshwater and marine eutrophication, and freshwater ecotoxicity. The methods used to obtain cellulose from plant biomass are typically energy intensive and require strong acids and bases to deconstruct plant cell walls, leading to higher overall environmental impact. Microcrystalline cellulose extracted from sugarcane bagasse, via acid-alkali pretreatment and depolymerization, was the lowest calculated CF (261 kg CO_2-eq/kg of cellulose) (Katakojwala and Mohan, 2020) coup-scalmpared to nano-cellulose derived from wood pulp via acid-alkali treatment and homogenization (810 kg CO_2-eq/kg) and nano-fibrillated cellulose derived from thermo-groundwood via acid etherification and sonication (1160 kg CO_2-eq/kg of cellulose) (Li et al., 2013; Turk et al., 2020). The environmental hotspots concerning the CF of these processes were mainly the electricity consumption and the embodied energy inherent to chemical modification, while significant scores are reported for the impact categories of acidification, eutrophication and ecotoxicity of freshwater.

5. Conclusions

In this work, a newly isolated bacterium, *K. rhaeticus* UNIWA AAK2, was efficiently cultivated on brewing-manufacturing by-product to produce BC. Substitution of commercial C and N sources with BSGE and BSYE reduced CF 5.4-fold compared to conventional HS-fermentation media. The fermentation stage was pinpointed as the hotspot in this lab-scale-based LCA, indicating that the energy-efficiency of the incubators is essential for scaling up the process. The substitution of pure substrates with brewing by-products did not seem to affect the thermal stability and structure of the produced BC indicating that biopolymers with similar thermal profiles may be produced using renewable resources.

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Conflicts of 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.

Appendix A. Supplementary data

E-supplementary data for this work can be found in e-version of this paper online.

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Figure captions

Figure 1 Evaluation of (a, c) BC production (■) and FAN consumption (▷) as well as (b, d) carbon source consumption when fermentations with *K. rhaeticus* were performed on BSGE as the sole carbon source and BSGE as the sole carbon course combined with BSYE as the sole nitrogen source. Total carbon (□); maltose (○); maltotriose (△); glucose (▽); fructose (◇); glycerol(◁).

Figure 2 Thermogravimetric analysis (TGA) of BC samples that were produced using commercial carbon sources (glucose, fructose, galactose, lactose), BSGE as the sole carbon course, under free pH conditions, controlled pH at 6 and combined with BSYE as the sole nitrogen source

Figure 3 Total carbon footprint from 1 kg of dry bacterial cellulose production utilizing HS, BSGE, and BSGE+BSYE-based media.

Figure 1

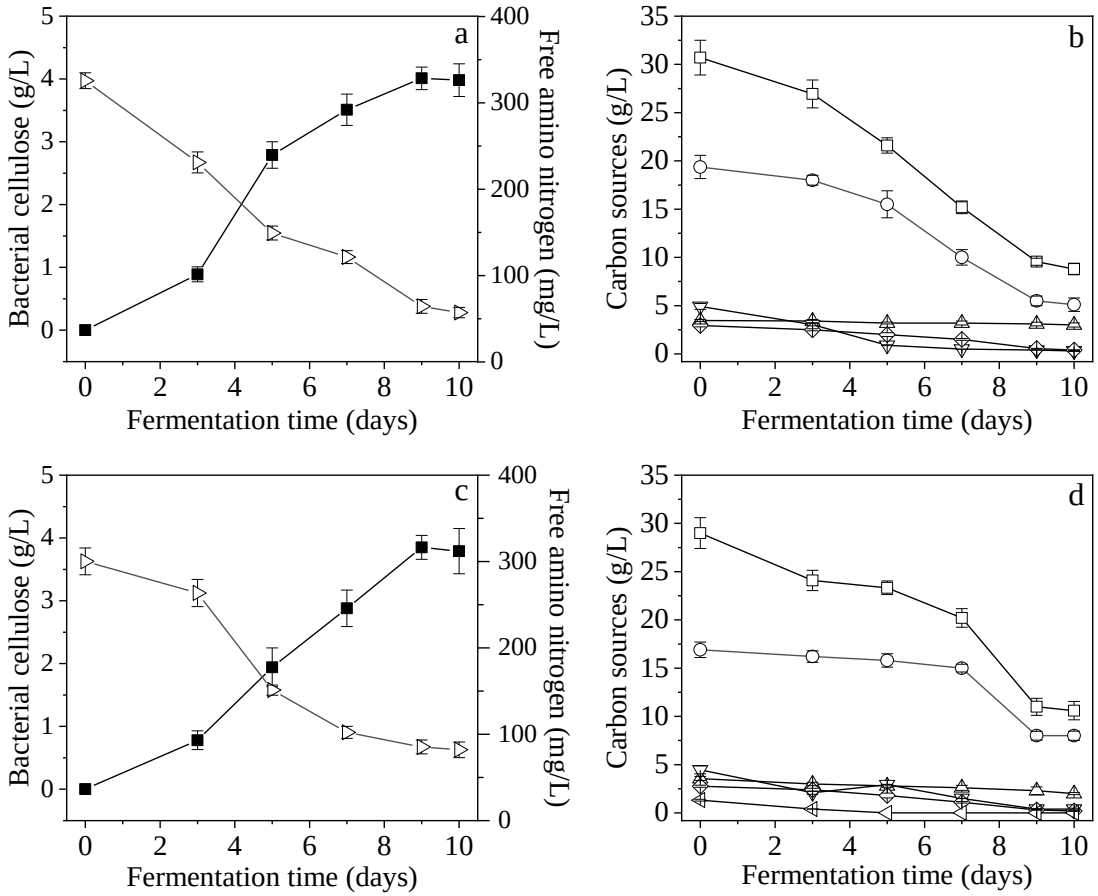
a
Bacterial cellulose (g/L)
Free amino nitrogen (mg/L)
Fermentation time (days)
b
Carbon sources (g/L)
Fermentation time (days)
c
Bacterial cellulose (g/L)
Free amino nitrogen (mg/L)
Fermentation time (days)
d
Carbon sources (g/L)
Fermentation time (days)

Figure 2

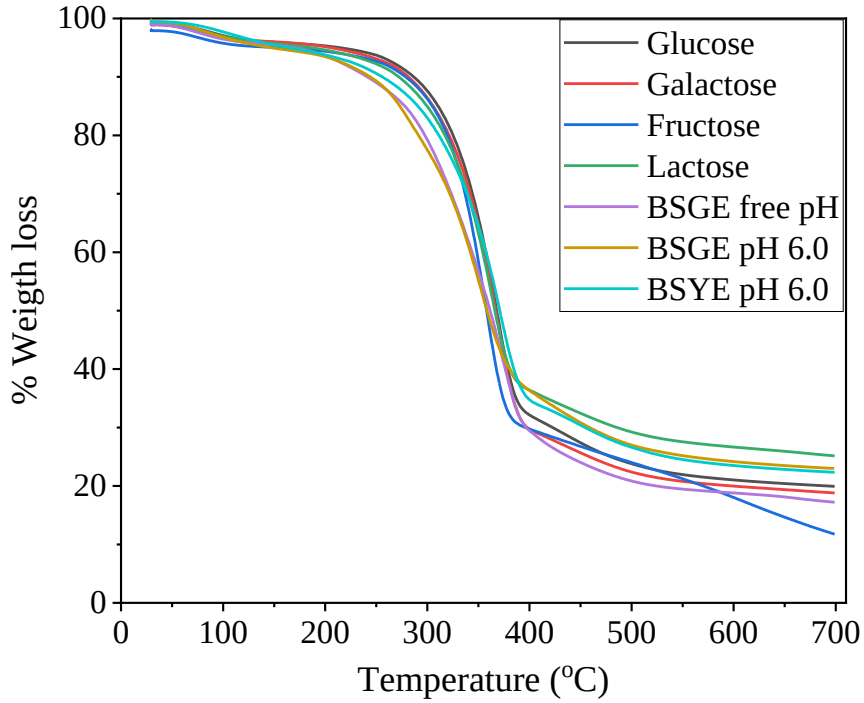
100
80
60
40
20
0
% Weigth loss
0
100
200
300
400
500
600
700
Temperature (°C)
Glucose
Galactose
Fructose
Lactose
BSGE free pH
BSGE pH 6.0
BSYE pH 6.0

Figure 3

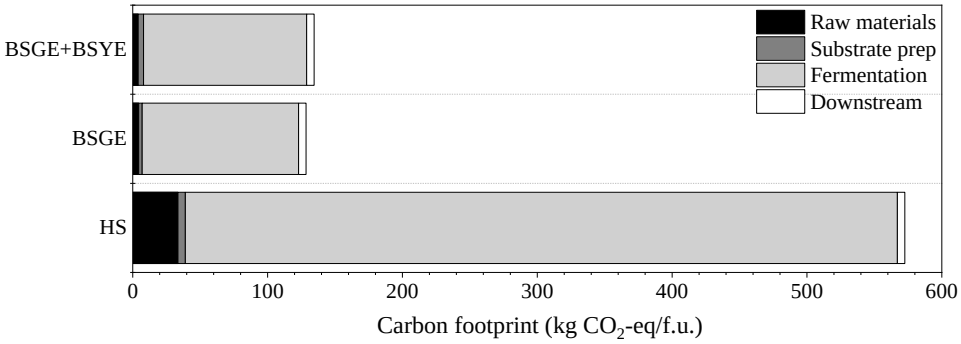
BSGE+BSYE
BSGE
HS
Raw materials
Substrate prep
Fermentation
Downstream
0
100
200
300
400
500
600
Carbon footprint (kg CO2-eq/f.u.)

Table 1 BC fermentation efficiency cultivating *K. rhaeticus* on synthetic carbon sources, using the Hestrin and Schramm fermentation medium.

Sugars	BC (g/L)	Productivity (g/L/d)	Sugar consumption (% w/w)[1]	FAN consumption (% w/w)
Glucose	0.88±0.09[a]	0.088±0.005	69.3	39.7
Fructose	0.49±0.03[b]	0.082±0.003	40.9	27.9
Galactose	0.37±0.05b[b]	0.062±0.001	32.5	30.3
Lactose	0.25±0.02[b]	0.025±0.001	31.3	28.0

[1] g of consumed sugars/g initial sugars. Different superscript letters within same column indicate statistically significant differences ($p<0.05$)

Table 2 BC fermentation efficiency cultivating *K. rhaeticus* on BSGE as the sole carbon course under different initial carbon concentration and pH values.

BSGE	pH	BC (g/L)	Productivity (g/L/d)	Sugar consumption (% w/w)[1]					FAN consumption (% w/w)	WHC (w/w)[2]
Initial sugars (g/L)				Maltose	Maltotriose	Glucose	Fructose	Total		
≈30	Uncon-trolled	1.64±0.11[a]	0.23±0.02	20.4	1.8	85.7	49.7	31.6	34.2	68.8±5.43[a]
≈20	Uncon-trolled	1.35±0.08[a]	0.19±0.01	29.8	7.2	89.1	30.7	37.5	29.6	65.7±3.92[a]
Initial pH_BSGE-based media										
4.5	Controlled	2.40±0.32[a]	0.24±0.01	57.5	13.6	91.6	72.0	59.4	60.2	67.5±4.80[a]
5.2	Controlled	3.70±0.24[b]	0.37±0.02	66.4	13.0	89.8	71.1	64.6	75.2	66.5±3.78[a]
6.0	Controlled	4.01±0.29[b]	0.45±0.02	71.6	10.4	91.9	81.3	68.9	80.2	64.7±5.56[a]
6.7	Controlled	3.24±0.15[b]	0.32±0.02	62.6	13.3	83.2	65.7	60.7	68.5	65.1±5.79[a]
Substitution of commercial N_BSGE & BSYE-based media										
≈30 g/L C sources, pH=6.0	Controlled	3.85±0.21[b]	0.43±0.04	52.7	35.3	91.0	89.1/100[4]	62.1	71.6	98.6±7.12[b]

[1]g of consumed sugars/g initial sugars, [2]g water/g dry BC, [4]refers to glycerol

Different superscript letters within same individual columns (initial sugars, initial pH) and between the initial pH of 6 and substitution of commercial N, indicate statistically significant differences ($p<0.05$). WHCs have been compared overall.

Table 3 Total carbon footprint from 1 kg of dry BC production utilizing HS, BSGE, and BSGE+BSYE-based media and carbon savings.

(kg CO_2-eq/f.u.)	HS	BSGE	BSGE+BSYE
Raw materials	33.5	4.4	3.99
Substrate preparation	5.53	2.6	4
Fermentation	528	116	121
Downstream	5.5	5.5	5.5
Total carbon footprint	573	129	135
Carbon savings	0	22.75	34.1
Total CF minus carbon savings	573	106.3	100.9