Valorisation of Citrus Processing Waste for Bacterial Cellulose Production through an Integrated Biorefinery Approach

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1. Introduction

Citrus Processing Industry (CPI) for juice and concentrate, generates substantial amounts of by-products given that only 50% of the fruit's mass is used during the production process. The worldwide production of citrus fruits accounts for 143 x 10^6 t per year, resulting in industrial generation of citrus peel waste (CPW) which exceeds 24 x 10^6 t (FAO, 2017) and mainly consists of peels, pulp, seeds and segment membranes (Marín *et al.*, 2007). Although traditional management practices include the use of CPW as animal feed or organic fertilizer (Lopez *et al.*, 2010), various studies have been conducted towards exploitation of CPW for the production of high value-added products including essential oils, pectin, succinic acid (Patsalou *et al.*, 2017), bacterial cellulose (Andritsou *et al.*, 2018), ethanol and methane (Patsalou *et al*., 2019). An additional burden of CPIs concerns the significant amounts of wastewater disposed, which constitutes mainly water used for factory cleaning, juice concentration, cooling water and water produced by essential oil extraction. Wastewater of CPIs comprises large variability of organic loads, suspended and settling solids, colloidal and settleable suspended solids and other soluble or insoluble compounds, such as sugars, phenolic compounds, essential oils and organic acids (Zema *et al.*, 2019). These molecules include valuable compounds which can be either isolated for food and pharmaceutical applications or further treated for the production of high-added value commodities.

Bacterial Cellulose (BC) constitutes a biopolymer of tremendous industrial importance owing to its numerus unique properties including high purity, high degree of polymerisation, high crystallinity, biodegradability, biocompatibility (Lee *et al.*, 2012), enhanced mechanical strength and large water-holding capacity (WHC) (Revin *et al.*, 2018). Due to these exceptional properties, it is used in various commercial applications in many industrial sectors including biomedical, electronic and food industries. BC is mainly produced via fermentation of small-chain sugars, most commonly glucose, by acetic acid bacteria of the *Acetobacteraceae* family. The Hestrin and Schramm (HS) media is often applied as culture media for BC production resulting in contents that range between 1.1 to 4 g BC L^{-1} (Mikkelsen *et al.*, 2009) depending on the strain and conditions used. The production of BC using pure cellulose incorporates several drawbacks including high operation cost and poor production yields resulting from the low productivity of the strains used. It is a relatively expensive process representing approximately one third of its total cost. Aiming to reduce BC's biosynthesis cost, the use of industrial by-products and agricultural waste rich in sugars as basic culture media are currently explored. Thus, several studies have been conducted towards BC production using orange, grapefruit and lemon peels and pulp as low-cost feedstocks producing 8.77 g L^{-1}, 6.7 g L^{-1} and 5.2 g L^{-1} respectively (Cao *et al.*, 2018; Andritsou *et al.*, 2018) .

The present work aims to develop a biorefinery exploiting green technologies processing both solid and liquid waste of CPIs for the production of essential oils, carotenoids and pectin, as well as media rich in nutrients for BC fermentations.

2. Materials and methods

A sonication system has been developed for simultaneous acid hydrolysis of citrus waste and d-limonene isolation. Following d-limonene extraction via centrifugation the remaining hydrolyzate was mixed with an equal volume of ethanol for pectin precipitation. Enzymatic hydrolysis was subsequently applied to the hydrolyzate using cellulases and β-glucosidases, while the hydrolyzate was subjected to liquid-liquid extraction for β-carotene isolation. The resulting hydrolyzate was employed in fermentation experiments using *Komagataeibacter sucrofermentans* DSMZ 15973 as cellulose producer. The pre-culture was prepared in HS media (2% glucose, 0.5% yeast extract, 0.5% peptone, 0.27% Na_2HPO_4 and 0.115% citric acid), while 10% of inoculum was added to 250 mL Erlenmeyer flasks containing 50 mL of HS media incubated at 30 °C and 150 rpm for 2 d followed by static cultivation. The total duration of fermentation experiments was 15 d.

Fermentations employing the citrus hydrolyzate were performed through adjustment of the sugar and nitrogen concentrations to HS media. The organic acids composition was analyzed through high performance liquid chromatography (HPLC). Reducing sugar, total sugar, free-amino nitrogen and d-limonene contents were determined by the DNS method using glucose as a standard, the Phenol-Sulfuric acid method using sucrose as a standard, the ninhydrin method using glycine as a standard and gas chromatography (GC) respectively.

3. Results and discussion

The CPI wastewater explored in this study was mainly composed of 73.58 g L^{-1} total sugars as well as substantial amounts of organic acids, essential oils and nutrients constituting a valuable renewable-feedstock for the development of a biorefinery for BC production. Preliminary fermentation experiments have been conducted using glucose, fructose, sucrose, galactose and xylose as carbon source, achieving final BC contents of 1.72, 2.03, 2.00, 1.76 and 1.28 g L^{-1} respectively. The results demonstrate that although according to the literature xylose can be hardly utilized by the vast majority of *Acetobacter xylinum* strains (Ishihara *et al.*, 2002), the *Komagataeibacter sucrofermentans* strain employed was capable of utilizing not only hexoses, but also pentoses yielding 0.17 g_{BC} g^{-1}_{xylose}. According to the literature, 0.21 g L^{-1} BC were produced by *Gluconacetobacter xylinus* CH001, which yielded 6.17-fold higher BC mass through addition of 3 g L^{-1} acetic acid to the culture media (Yang *et al.*, 2014*).* Tsouko *et al* (2015) reported similar performance for *Komagataeibacter sucrofermentans*, which produced 1.17 g L^{-1} and 2.05 g L^{-1} BC, utilizing glucose and fructose on static mode respectively. Higher yields have been reported for *Gluconacetobacter xylinus* ATCC 53524, which utilized glucose, fructose and sucrose producing 3.1, 2.81 and 3.83 g L^{-1} BC respectively, while the use of galactose resulted in only 0.09 g L^{-1} BC (Mikkelsen *et al.*, 2009).

The presentation will include results relevant to the enhancement of d-limonene, pectin and β-carotene isolation from the waste, while studying the effect of H_2SO_4 concentration, temperature and duration of sonication treatments. An additional parameter that will be studied concerns the amount of ethanol remaining at the hydrolysate following pectin extraction, given that previous studies have shown that the presence of ethanol enhances BC production yielding up to 15.2 g L^{-1} BC (Son *et al.*, 2001).

4. Conclusions

The present study demonstrates the development of a biorefinery for the production of d-limonene, β-carotene, pectin and BC from both solid and liquid waste streams of CPIs.

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