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Biowaste-based biochar: A new strategy for fermentative bioethanol overproduction via whole-cell immobilization

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HIGHLIGHTS

- Biochar was produced from four types of biowaste.
- Increase in pyrolysis temperature enhanced biochars' porosity and surface area.
- Three major yeasts were immobilized on biochars exhibiting the highest surface area.
- *S. cerevisiae*- and *K. marxianus*-based biocatalysts exhibited notable productivities.
- Immobilization on vineyard prunings biochar improved biofuel production by 36–52%.

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ABSTRACT

This work explores the potential use of biochar as a microbial cell carrier enhancing the efficiency of alcoholic fermentations. Olive kernels, vineyard prunings, sewage sludge and seagrass residues were applied as biowaste for biochar production through pyrolysis at two different temperatures (250 °C and 500 °C), while a commercial type of non-biomass char was also employed for benchmarking purposes. Apart from vineyard prunings pyrolyzed at 250 °C, all other carbonaceous materials presented crystalline phases including halite, calcite, sylvite and/or silicon. Moreover, increase in pyrolysis temperature enhanced biochar's porosity and BET-specific surface area, which reached 41.7 $m^2 g^{-1}$ for VP-based biochar remaining at lower levels (0.15–5.3 $m^2 g^{-1}$) in other specimens tested. Elemental analysis demonstrated reduction in oxygen and increase in the carbon content of biochars produced at elevated temperatures, while biochar from seagrass included residues of chloride (0.3–5.14%). Three major yeasts were immobilized on materials exhibiting the highest surface areas and applied in repeated batch fermentations using Valencia orange peel hydrolyzates as feedstock. The biocatalysts developed using *S. cerevisiae* and *K. marxianus* immobilized on vineyard prunings-based biochar exhibited exceptional ethanol productivities as compared to the relevant literature, which reached 7.2 $g L^{-1} h^{-1}$ and 7.3 $g L^{-1} h^{-1}$ respectively. Although the aforementioned strains improved biofuel production by 36–52% compared to the conventional process, *P. kudriavzevii* KVMP10 was not efficient following immobilization on biochar. The approach constitutes an innovative method for bioenergy production, demonstrating a novel application of biochar in industrial biotechnology which incorporates important technological advances such as enhanced biofuel production and biomass recycling.

1. Introduction

The reduction of greenhouse gas emissions through exploitation of alternative energy sources and renewable fuels represents an area of substantial research interest. Biodiesel and bioalcohols constitute the most important biofuels, while ethanol is more preferable incorporating a series of environmental benefits [1]. However, ethanol is often produced from energy crops using agricultural land, a practice that could impact food supply, highlighting the need to identify alternative feedstocks for sustainable manufacturing [2]. Moreover, ethanol fermentations are impacted from substrate and product inhibition decreasing process productivity [3]. Immobilized biocatalysts could assist yeasts in

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the presence of inhibitors performing stable and elevated biofuel production [4]. Nevertheless, although different immobilization approaches have been employed for enhancement of ethanol fermentations, common methods (such as entrapment of cells in alginate gel beads) are limited by unstable performance due to poor mechanical properties of the carrier [5]. Thus, there is a need to identify novel eco-friendly and cost-effective carriers with rigid structure improving the stability of ethanol bioprocesses.

Biochar constitutes a carbon-rich material produced from thermal decomposition of biomass in the absence of oxygen (known as low-temperature pyrolysis), which is distinguished from charcoal through use as soil amendment [6]. A wide range of biowaste can be converted to biochar under varying pyrolysis conditions [7] yielding products characterized by different surface functional moieties (e.g. carboxyl, hydroxyl, carbonyl, alcoholic and lactone groups) and a porous structure useful for environmental and catalytic processes, where conventional support materials are commonly employed [8]. Biochar has been extensively used in environmental management applications and as soil amendment enhancing the availability of nutrients, as well as soil quality, more efficiently compared to other organic materials [6]. Based on a number of favourable properties, biochar has been successfully employed in environmental remediation constituting an advanced green sorbent for soil and water organic/inorganic decontamination [9]. Moreover, the material demonstrated effective immobilization of heavy metals, minimizing *in situ* the bioavailability of inorganic and organic contaminants to earthworms, microbes, and plants [10], whereas its use in anaerobic digestion is known to enhance methane production [11].

Carbonaceous materials (e.g. graphite, activated carbon, carbon nano-tube, biochar) can improve cell activity and growth, assisting interspecies electron transfer, buffering capacity and nutrient adsorption into their surface [12]. Moreover, biochar contains a small amount of bioavailable carbon metabolized by soil microorganisms [13] and assists electro-active strains creating syntrophic microbial interactions that improve anaerobic digestion performance [14]. The specific material has been employed as catalyst for hydrogen production and bio-oil upgrading [15] as well as in transesterification reactions for biodiesel production, while preliminary studies have explored biochar as a support for enzyme immobilization (*C. rugosa* lipases) demonstrating similar biocatalytic activity compared to commercial lipases [16]. Thus, recent advances exhibit novel biochar applications due to a number of properties that include high surface charge density enabling retention of cations through cation exchange [17], high internal porosity and surface area, as well as presence of both polar and non-polar surface sites allowing adsorption of organic molecules and other nutrients.

The production of solid and liquid fuels from biomass holds great potential for alleviating the dependence on fossil resources, facilitating application of environmental friendly processes and sustainable rural development [18]. In this context, the use of biochar produced from biowaste for enhancement of ethanol fermentations constitutes a holistic valorization approach, integrating thermal and biological methods for production of commodities with higher added-value. Thus, utilization of biowaste for biochar production and its subsequent use as carrier for efficient manufacturing of liquid transportation fuels from an abundant food waste residue, such as citrus peel waste [19], is expected to turn current liabilities into valuable assets for processors [20].

To the best of our knowledge, this is the first report evaluating the use of biochar as a renewable and low-cost support material for whole-cell immobilization in a major industrial bioprocess. Different biowaste were applied for biochar production using varying pyrolysis temperatures and the properties of the materials generated were assessed using scanning electron microscopy observations as well as surface area and elemental composition measurements. The most promising materials were used for immobilization of *S. cerevisiae*, *K. marxianus* and *P. kudriavzevii* KVMP10, which were subsequently applied for enhancing the production of ethanol from citrus peel hydrolyzates in repeated batch fermentations. All residuals generated from the entire process can be safely disposed to the environment.

2. Materials and methods

2.1. Biochar preparation from different feedstocks

According to the International Biochar Initiative (IBI), biochar should be produced through use of waste-derived biomass [21]. Thus, biochar was produced utilizing four different types of locally available biomass feedstocks comprising olive kernels (OK, *Olea europaea*, obtained from Pettemerides Olive Oil Mill Ltd, Limassol, Cyprus), vineyard prunings (VP, *Vitis vinifera*, obtained from Dafermou Winery, Larnaca, Cyprus), sewage sludge (SS, Sewerage Board of Limassol – Amathus (SBLA), Moni, Cyprus) and seagrass residues (SGR, *Posidonia oceanica*, collected from a local beach). The biochar specimens derived from OK, VP, SS and SGR will be denoted hereafter as OKB, VPB, SSB and SGRB. Upon collection, all biomass samples were stored in air tight plastic bags until application in pyrolysis. Conventional pyrolysis was performed in a furnace under controlled conditions through the supply of nitrogen gas. The temperature of the furnace was increased at a rate of 10 °C min^{-1}, while 250 °C and 500 °C (for 30 and 3 min respectively) were employed as pyrolysis temperatures. Moreover, char samples of non-biological origin (NBC) were also obtained from CBp Cyprus Ltd (Limassol, Cyprus), producing char and activated carbon from recycled car tires, and it was compared to the renewable materials selected. The production of NBC comprised a continuous pyrolysis process, using a temperature of approximately 500 °C and 1 h residence time in the reactor.

2.2. Characterization of biochar properties

An overview of the structural, physical and chemical characteristics of the biochars prepared in the present study is attempted, aiming to assess their potential use for development of new products and support materials. Thus, the specific surface area of the materials evaluated was determined by N_2 adsorption at 77 K (Brunauer-Emmett-Teller, BET method) and using a multi-point Micromeritics Gemini V System. Samples were pretreated prior to the experiment in a flowing-gas degassing unit for the removal of adsorbed contaminants. Degassing conditions included flowing N_2 gas at 180 °C (453 K) for 12 h [22].

X-ray diffraction (XRD) was used to probe the crystalline phases within biochar samples. All measurements were performed in a Rigaku Ultima IV diffractometer equipped with a Cu tube and operated at 40 kV voltage and 40 mA current. The system was equipped with a multilayer mirror for parallel X-ray beam geometry and the selected wavelength was the Cu Ka (0.15419 nm). Sample patterns were collected in Bragg-Brentano scanning mode over the 20° − 70° 2-theta range, in a sample holder without rotation [23].

The microstructural details of the samples were investigated using a Quanta 200 (FEI, Hillsboro, Oregon, USA) Scanning Electron Microscope (SEM) in various accelerating voltages. All samples were sputter coated with a thin layer of gold (few nm) prior to imaging such as to increase sample's conductivity and prevent surface charging complications. Energy-dispersive X-ray spectroscopy analysis (EDS) was also conducted along with imaging providing information about the elemental composition of the biochars prepared.

2.3. Microorganisms and cultivation of freely suspended cells

S. cerevisiae and *K. marxianus* were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), while *P. kudriavzevii* KVMP10 was previously isolated as a thermotolerant ethanologenic yeast within our research group [24]. Fermentations for ethanol production were conducted with each strain using liquid media simulating a Valencia

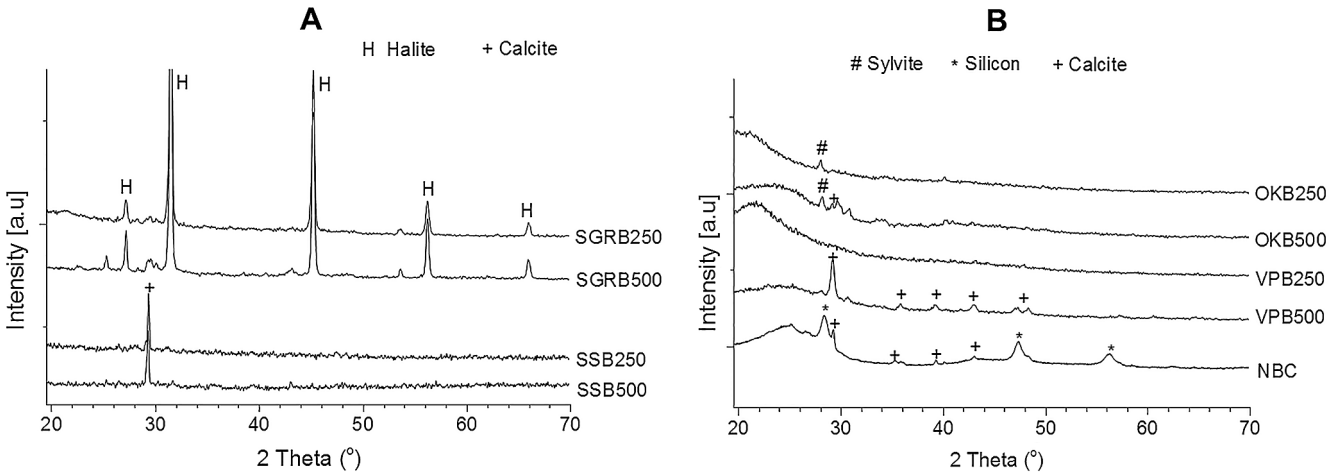

Fig. 1. X-ray diffraction pattern of the biochars produced at 250 °C and 500 °C through the use of: (A) SGR and SS, (B) OK, VP and NBC.

orange peel waste hydrolyzate, which was prepared in 50 mmol L^{-1} citrate buffer at pH 4.8 and consisted of (g L^{-1}): yeast extract 10, peptone 20, fructose 33.2, galactose 8.6, glucose 57.4, and sucrose 1.4 [25]. The microorganisms were maintained at −80 °C in glycerol stock cultures and prior to the experiment *S. cerevisiae* and *P. kudriavzevii* KVMP10 were cultured in liquid medium consisting of (g L^{-1}): yeast extract 10, peptone 20 and glucose 50. *K. marxianus* was pre-grown in media containing (g L^{-1}): yeast extract 3, malt extract 3, peptone 5 and glucose 50. The inoculums were incubated at 30 °C in an orbital shaker stirred at 100 rpm for 24 h. All chemicals were obtained from Sigma-Aldrich Company Ltd (Dorset, UK) and were of ANALAR grade.

Bioethanol fermentations were conducted in batch mode using 100 mL serum bottles, which were tightly sealed with screw caps and contained 90 mL of fermentation media and 10 mL of inoculum. Serum bottles were incubated in a water bath at a temperature according to the specifications of each experiment and reciprocal shaking at 100 rpm. All fermentations were performed in triplicate, while two samples were analyzed for each replicate constituting analyses of 6 samples at each time point.

2.4. Immobilization of microorganisms for bioethanol production

Biocatalysts were prepared through immobilization of *S. cerevisiae*, *K. marxianus* and *P. kudriavzevii* KVMP10 on biochars derived from VP and SGR, as well as NBC. Each yeast strain was initially pre-grown in liquid medium as described in Section 2.3, while grown cells were collected through centrifugation for inoculum preparation. An amount of 2 g wet pressed yeast cells was suspended in 250 mL of fermentation media and 20 g of the support material was added. The flasks were allowed to ferment the orange peel waste hydrolyzate overnight, using 37 °C for *S. cerevisiae* and 42 °C for *K. marxianus* and *P. kudriavzevii* KVMP10. The supernatant was decanted and the biocatalyst was washed twice with 125 mL of fermentation media prior application to bioethanol production experiments. VPB, SGRB and NBC were used as support materials for bioethanol production.

2.5. Analyses

Culture samples were withdrawn aseptically, centrifuged at 13000 × g for 10 min and filtered with 0.2 μm syringe filters. During fermentations reducing sugars were analyzed by the phenol–sulfuric acid method, which is based on the phenol–sulfuric acid reaction and it is useful for determination of simple sugars, oligosaccharides, polysaccharides and their derivatives [26]. Ethanol production was monitored using gas chromatography (GC). A Shimadzu GC-2014 (Shimadzu, Milton Keynes, UK) using a flame ionization detector and a 30 m long Zebron ZB-5 capillary column (Phenomenex, Macclesfield, UK) with 0.25 mm internal diameter was employed. The mobile phase applied was nitrogen, while the stationary phase of the column was 5% phenyl and 95% dimethylpolysiloxane. Ethanol was extracted into hexane by vortexing 1 mL of the filtered sample with 2 mL of the solvent for 1 min. About 1 μL of the extract was injected and the temperature of the column was kept constant at 40 °C for 2.5 min followed by an increase of 30 °C min^{-1} up to 160 °C, while it was maintained at 160 °C for an additional 5 min [24]. Ethanol concentration was calculated interpolating from a previously established calibration curve and the coefficient of variation for 3 samples was 1.22% at a concentration level of 60 g L^{-1}.

3. Results and discussion

3.1. Characterization of immobilization carriers

Pyrolysis process conditions strongly affect the yield, morphology and physicochemical properties of biochar produced [27]. Thus, the properties of each specimen tested was determined through XRD, BET surface area, SEM and EDS to evaluate the potential of each biochar-based material for application as carrier for cellular immobilization.

3.1.1. XRD analyses of biochar

The XRD spectra of NBC and biochars produced at 250 °C and 500 °C from OK, VP, SGR and SS are depicted on Fig. 1. The XRD patterns of both seagrass samples (Fig. 1A), SGRB250 and SGRB500, demonstrate distinct peaks located at the major crystalline phase present at 27.3°, 31.7°, 45.4°, 56.5° and 66.2°. All aforementioned peaks correspond to halite (NaCl) and remain relatively unchanged independent of the pyrolysis temperature applied. Nevertheless, the XRD patterns of SSB exhibit only a single sharp peak located at 29.4°, which has been assigned to the main and strongest peak (1 0 4) of calcite ($CaCO_3$). Although calcite and quartz are considered as the two major components of sewage sludge [28], the latter was not detected in our specimens.

Fig. 1B summarizes the XRD patterns of OKB, VPB and NBC. The XRD spectra of NBC presented peaks from silicon (Si) at 28.4°, 47.3° and 56.1°, as well as calcite ($CaCO_3$) crystallites located at 29.4°, 36.0°, 39.4° and 43.1° respectively. Moreover, the broad “hump” observed between 20° and 30°, centered approximately at 26°, has been assigned to weak diffraction from an amorphous carbon network which is commonly observed in organic samples subject to pyrolysis.

Sylvite (KCl) was detected in OKB samples produced at both temperatures by the faint peak located at 28.3°. However, although the OKB500 sample (pyrolyzed at higher temperature) exhibited additional peaks, due to poor signal to noise (S/R) ratio and/or small crystallite

sizes definitive identification could not be obtained. Both OKB samples exhibited a broad peak between 20° and 30°, centered at approximately 22° for the low temperature sample (OKB250) due to the presence of cellulose, while regarding OKB500 the peak was shifted towards 25-28° resembling a peak from amorphous carbon network [29]. Similar XRD patterns have been previously observed for biochar samples obtained from other renewable feedstocks, such as the formation of calcite in biochar produced from eucalyptus [30]. Moreover, the analysis of biochar specimens derived from straws of canola at 300 °C, 500 °C and 700 °C demonstrated that although only sylvite was produced at 300 °C, the increase of pyrolysis temperature resulted in calcite generation [31].

Apart from the broad cellulose peak, the presence of any crystalline phases was not observed in the XRD spectra of VPB250. However, the increase of pyrolysis temperature to 500 °C resulted in crystallization of the calcite phase. Furthermore, the amorphous peak of cellulose was shrunk and shifted towards higher angles demonstrating the conversion to amorphous carbon. The significant crystallization process of the specific sample at 500 °C is expected to increase the specific surface area, since crystallization increases density and subsequent shrinkage can generate cracks and porous within the material. The crystalline mineral phases detected in the biochar samples tested could serve as important factors affecting cell immobilization and culture performance. Calcite crystals provide a safe microenvironment against stressful or hazardous conditions in encapsulated microalgae cultures [32] and facilitate flocculation of microalgae enabling easy recovery of the biomass produced following culture completion [33]. Moreover, immobilization of enzymes on silicon surfaces with high porosity enables significant applications such as decontamination, microbial fuel cells, microreactor healthcare and biological sensing [34], demonstrating the potential favorable effect of the specific crystalline phases when employed as constituents of microbial carriers in ethanol fermentations.

3.1.2. BET specific surface area

The effect of pyrolysis temperature on the BET specific surface area of the biochar samples produced is shown in Table 1. The increase of pyrolysis temperature between 250 °C and 500 °C resulted in increase of the BET specific surface area. All biochars processed at 250 °C exhibited low surface area values. Nevertheless, when pyrolysis was conducted at 500 °C, significant differences between the BET surface areas of the samples were observed. The materials produced from VP and SGR achieved the highest specific surface area that reached 41.7 and 5.3 $m^2 g^{-1}$ respectively, while the BET values of OKB and SSB remained at lower levels (1.5 and 1.4 $m^2 g^{-1}$ respectively). Although NBC demonstrated the highest specific surface area (73 $m^2 g^{-1}$), the aforementioned material constitutes a commercial product incorporating different processing conditions (e.g. pyrolysis temperature, heating rate), and thus, it was applied in the present study for comparison against the renewable feedstocks tested.

Table 1
Surface area and elemental composition of biochars derived from different feedstocks at 250 °C and 500 °C.

Sample	Temperature (°C)	Specific surface area ($m^2 g^{-1}$)	C (%)	O (%)	Ca (%)	Cl (%)	Si (%)
OKB	250	0.15	61.72	21.42	1.14	0.30	–
	500	1.5	65.73	20.02	2.23	0.64	0.23
VPB	250	0.5	69.62	24.65	3.04	–	–
	500	41.7	71.65	21.59	1.24	–	–
SSB	250	0.7	33.11	34.38	6.30	–	3.48
	500	1.4	55.78	23.47	3.76	–	1.73
SGRB	250	1.9	60.52	24.07	0.61	3.90	0.18
	500	5.3	60.97	22.86	0.6	5.14	–
NBC	–	73.0	88.0	3.16	0.32	0.12	1.26

Pyrolysis studies of biomass-based feedstocks have previously demonstrated that the resulting surface area of biochar can be low. However, although biochars produced from safflower seed press cake at temperatures between 400 and 600 °C exhibited BET values lower than 4.2 $m^2 g^{-1}$ [35], the specific surface area of VPB at 500 °C was substantially higher. The type of feedstock constitutes an additional important factor demonstrated by the high BET values (376–401 $m^2 g^{-1}$) obtained for hickory wood, bagasse and bamboo at 600 °C [36], while optimization of pyrolysis conditions is also crucial considering that the specific surface areas were 30 times higher compared to the values achieved at 450 °C using the same raw materials. Moreover, there is a strong relationship between pyrolysis temperature and the biochar's surface area, which is known to increase at elevated pyrolysis temperatures [37]. Thus, similarly to the findings of the present work the specific surface area of biochar produced from SS was enhanced with an increase in pyrolysis temperature from 350 °C to 650 °C, while the porosity can be also improved in higher pyrolysis temperatures [38].

3.1.3. SEM and EDS analyses

The microstructural features of each specimen were evaluated through SEM imaging, while EDS was applied to determine the elemental composition of biochars produced. Observations confirmed the presence of significant differences among the biochar samples produced at each temperature. The morphology of OKB, SGRB and VPB produced at 500 °C (Fig. 2E–G) included formation of smooth surface and porosity. However, the aforementioned biochar samples produced at 250 °C did not demonstrate porosity (Fig. 2A–C), while SSB remained non-porous at both temperatures applied (Fig. 2D and H). Moreover, similarly to the renewable carbonaceous materials formed at 250 °C, no porosity was observed for the commercial NBC tested (data not shown). Thus, SEM analysis confirmed the significant increase of specific surface area which occurs at elevated temperatures for some of the biochars produced (e.g. VPB) highlighting their potential for application in cellular immobilization.

The elemental EDS analysis of the biochars formed at different pyrolysis temperatures are shown on Table 1. An increase in temperature from 250 °C to 500 °C enhanced the carbon content and reduced that of oxygen in all biochars tested. Thus, increased pyrolysis temperature resulted in more carbonaceous materials, which has been previously demonstrated for other agricultural residues [39]. The material exhibiting the highest increase in carbon content was SSB containing 55.78% of carbon at 500 °C, while at the lower temperature carbon remained at 33.11%. Moreover, the specific material exhibited a more pronounced shift in oxygen content, which was reduced from 34.38% to 23.47% between 250 °C and 500 °C respectively. The content of other elements such as calcium, chloride and silicon was also monitored (Table 1) demonstrating that SGRB comprised elevated chloride quantities (5.14% at 500 °C), which could potentially affect the efficiency of the material for biocatalyst development. Overall, the elemental composition of the biochars formed was similar to that of biochar generated from other biomass-based feedstocks including orange peel [40] and pinewood sawdust [41].

3.2. Bioethanol production using freely suspended cells of S. cerevisiae, K. marxianus and P. kudriavzevii

S. cerevisiae constitutes an industrial workhorse strain for bioethanol production using a wide range of sugar-rich feedstocks [42], while *K. marxianus* is an important bioethanol producer demonstrating elevated growth rate, ability to consume a wide range of sugars and thermotolerance [43]. The latter characteristic, which ensures lower contamination risk and reduced requirements for cooling to maintain fermentation temperature between 25 and 35 °C, is also exhibited by *P. kudriavzevii* KVMP10, a yeast isolated from our research group holding the capacity for elevated bioethanol production from citrus peel

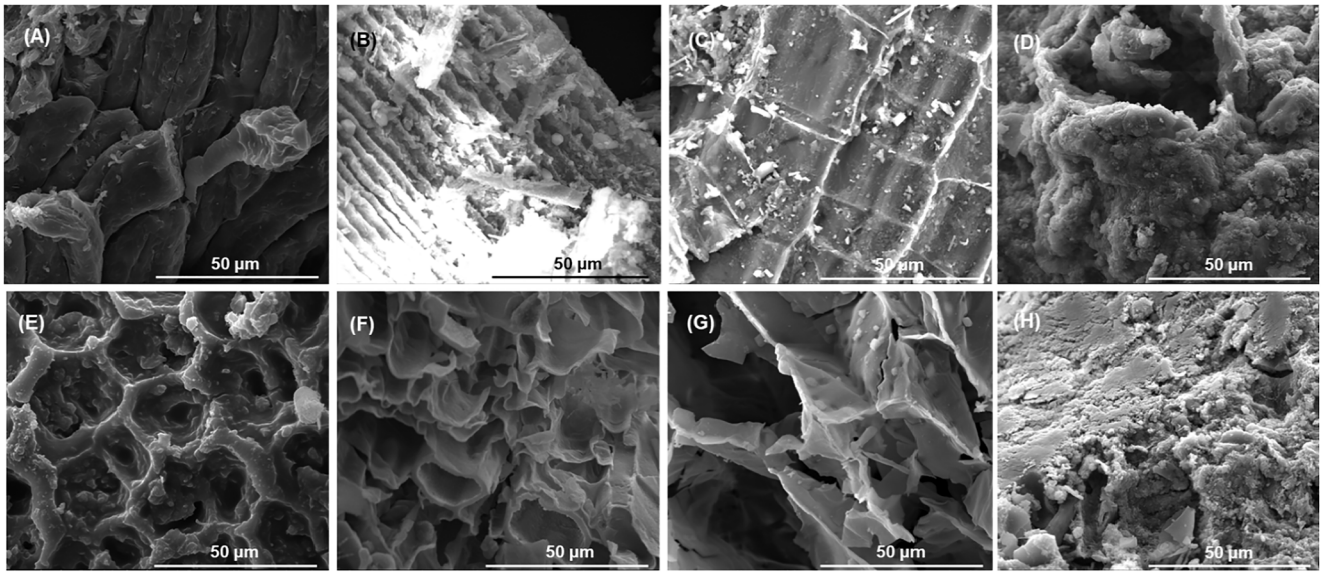

Fig. 2. SEM images of biochar specimens at 3000× magnification. The materials produced using 250 °C comprised: (A) OKB, (B) SGRB, (C) VPB, and (D) SSB. The products formed at 500 °C included: (E) OKB, (F) SGRB, (G) VPB, and (H) SSB.

hydrolyzates [24]. Thus, the capacity of these yeasts for bioethanol production at high rates was evaluated through immobilization on biochar. Bioethanol fermentations of the three selected yeasts were initially conducted applying freely suspended cells at two different temperatures (37 and 42 °C) in an attempt to determine suitable fermentation conditions using the media simulating Valencia orange peel hydrolyzate. *S. cerevisiae* produced 51 g L^{-1} of ethanol at 37 °C and 42 g L^{-1} at 42 °C following 40 and 64 h of incubation respectively. The elevated temperature of 42 °C enhanced ethanol production from *K. marxianus* which yielded 46 g L^{-1}, while *P. kudriavzevii* produced 45 g L^{-1}. However, the use of 37 °C reduced biofuel formation from *K. marxianus* and *P. kudriavzevii* producing 39 g L^{-1} and 24 g L^{-1} of ethanol respectively.

The production of ethanol observed was similar to previous studies employing the specific strains in fermentations of citrus peel hydrolyzates [24]. Thus, the preliminary fermentations conducted using freely suspended yeast cells demonstrate that biofuel production was enhanced at 37 °C in *S. cerevisiae* fermentations, while *K. marxianus* and *P. kudriavzevii* KVMP10 performed elevated ethanol formation at 42 °C. Thus, the capacity of each strain for the development of immobilized biocatalysts was tested at the aforementioned conditions maximizing ethanol formation.

3.3. Development and evaluation of immobilized biocatalysts for ethanol production

3.3.1. Immobilization of yeasts on selected carriers

Specific surface area constitutes a major factor for the development of immobilized biocatalysts, considering that elevated surface area would enhance the formation of porous structures within biochar, thus controlling the material's capacity for adsorption of nutrients and cell attachment [36]. VPB and SGRB produced at 500 °C, as well as NBC, demonstrated the highest specific surface area values holding significant potential for effective immobilization of *S. cerevisiae, K. marxianus* and *P. kudriavzevii* KVMP10 for optimal bioethanol production. Therefore, biocatalyst development was evaluated employing the selected materials summarized above. The electron micrographs obtained from fermentations of the three yeast strains following immobilization on each material are depicted on Fig. 3. The results confirmed that the yeasts adhered densely and homogenously to the surface of each carrier, as a result of either physical adsorption by electrostatic forces or due to natural cell entrapment into the porous or covalent binding between the membrane and the support. Moreover, apart from the SEM images presented, effective immobilization was further established by the ability of biocatalysts (following thorough washing to remove free cells) to perform efficiently in repeated batch fermentations as discussed below.

3.3.2. Repeated batch fermentations of S. cerevisiae for ethanol production

S. cerevisiae immobilized on VPB, SGRB and NBC was employed in two repeated batch fermentations to evaluate the capacity of the developed biocatalysts for enhancing the production of ethanol as compared to freely suspended cells (Fig. 4). During the first batch, the developed *S. cerevisiae*-based biocatalysts employing VPB and NBC achieved faster kinetics as compared to free cells and those immobilized on SGRB. Nevertheless, recycling of the biocatalysts in a subsequent batch demonstrated that cells immobilized on VPB exhibited the highest productivity generating net production of 72 g L^{-1} within 10 h of fermentation, while the NBC-based biocatalyst also promoted net ethanol production reaching 60 g L^{-1} over the same period. The maximum net ethanol concentrations formed using cells immobilized on SGRB and the suspended culture remained at lower levels reaching 48 g L^{-1} and 53 g L^{-1} respectively. Moreover, although cells immobilized on VPB exhibited significantly higher ethanol production over the first 10 h of the second batch experiment, as compared to suspended cells, the consumption of sugars was similar in both fermentations. Thus, higher substrate quantities were potentially utilized by immobilized cells for product formation rather than yeast growth as opposed to the suspended culture. The results presented demonstrate that the VPB-based biocatalyst produced 36% more ethanol compared to the conventional process, serving as an efficient cell carrier that elevates substantially the production of the biofuel, while enabling easy recycling of yeast cells in subsequent batch experiments and improvement of biocatalytic efficiency.

S. cerevisiae constitutes the most widely used yeast for industrial ethanol production based on a number of favorable characteristics which include among others osmotolerant, inexpensive, high ethanol production, low generation of by-products as well as toleration of elevated ethanol and sugar concentration [44]. Thus, numerous studies have previously investigated immobilization of the yeast to other support materials through application of different immobilization techniques [45]. *S. cerevisiae* produced 50 g L^{-1} of ethanol from 120 g L^{-1} of

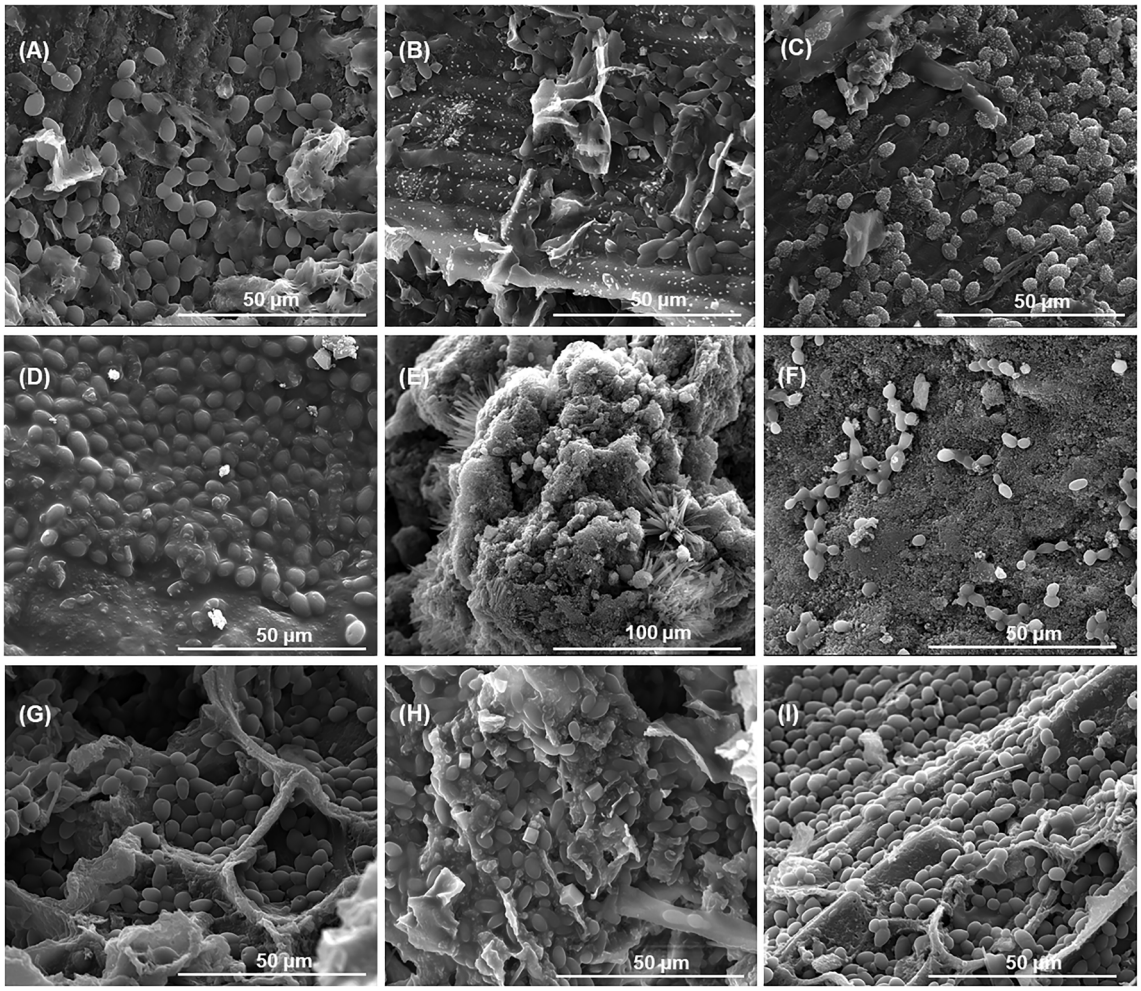

Fig. 3. SEM images of immobilized biocatalysts at 3000× magnification. (A) *S. cerevisiae*, (B) *P. kudriavzevii*, and (C) *K. marxianus* KVMP10 cells following immobilization on SGRB obtained at 500 °C. (D) *S. cerevisiae*, (E) *P. kudriavzevii*, and (F) *K. marxianus* KVMP10 immobilized on NBC. (G) *S. cerevisiae*, (H) *P. kudriavzevii*, and (I) *K. marxianus* KVMP10 immobilized on VPB (500 °C).

glucose in an immobilized cell reactor using calcium alginate as a support [39]. The same carrier was employed for ethanol generation from non-sterilized beet molasses demonstrating maximum production of 53 g L^{-1} through fermentation of 250 g L^{-1} of sugars [46]. Moreover, Yu et al [47] reported ethanol production of 96.7 g L^{-1} from 200 g L^{-1} of sugars with the use of *S. cerevisiae* immobilized on natural sorghum bagasse. The results obtained here demonstrate that bioethanol produced from orange peel hydrolyzates through fermentations of *S. cerevisiae* immobilized on biochar-based materials could serve as a future sustainable fuel.

3.3.3. Repeated batch fermentations of *K. marxianus* for ethanol production

K. marxianus exhibits thermotolerant properties, low repression by glucose as well as the capacity to utilize hemicellulolytic hydrolyzates [48]. Based on these technological advantages a series of investigations have been conducted for ethanol production applying *K. marxianus* usually through supplementation of lactose, while delignified cellulose [49] and sodium alginate [50] constitute examples of carriers successfully used for bioprocess improvement. Herein, the yeast was immobilized on VPB, SGRB and NBC for production of the biofuel from citrus peel hydrolyzates in two repeated batch experiments, while a suspended culture was also conducted for comparison purposes (Fig. 5). During the first batch the performance of VPB- and SGRB-based biocatalysts were not evidently different as compared to suspended cells, both with respect to biofuel production and sugars consumption. However, *K. marxianus* immobilized on VPB significantly enhanced net ethanol production, which reached 73 g L^{-1} (52% higher compared to free cells) following 10 h of fermentation in the repeated batch experiment. Although the NBC-based biocatalyst generated net production of 56 g L^{-1} as compared to the 48 g L^{-1} formed by freely suspended cells at 10 h, the kinetics of the two experiments were not significantly different in the second batch. Net biofuel production using cells attached to SGRB remained at 43 g L^{-1}, demonstrating that similarly to the use of VPB for *S. cerevisiae* immobilization the specific biochar-based carrier could be efficiently applied for the development of advanced biocatalysts employing *K. marxianus*.

3.3.4. Repeated batch fermentations of *P. kudriavzevii* for ethanol production

P. kudriavzevii exhibits multiple types of tolerance against extreme conditions during alcoholic fermentation, including tolerance towards elevated temperatures and acidic conditions [51]. Thus, *P. kudriavzevii* KVMP10 was applied as a third technologically important yeast in alcoholic fermentations of orange peel hydrolyzates employing immobilized cells on NBC, VPB and SGRB, as well as suspended cultures in repeated batch experiments (Fig. 6). Nevertheless, the developed immobilized biocatalysts could not enhance ethanol formation as compared to free cells demonstrating that not all yeast strains could be efficient in the bioprocess proposed. Similarly to the present work, immobilized cells of *S. cerevisiae* and *P. kudriavzevii* into poly(vinyl alcohol) hydrogel lens-shaped particles have been previously compared

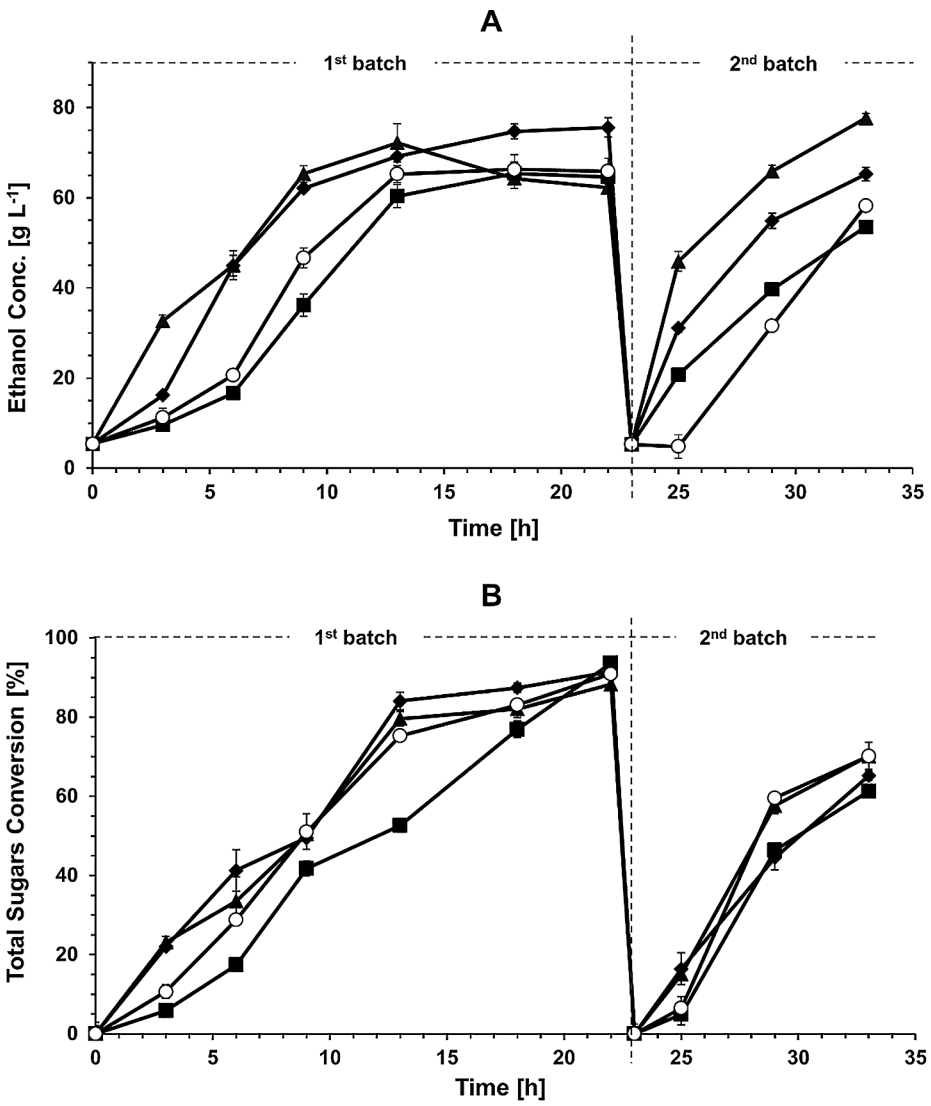

Fig. 4. Evaluation of *S. cerevisiae* immobilized biocatalysts for ethanol production. Symbols correspond to (A) ethanol concentration and (B) sugars' conversion in fermentations conducted at 37 °C employing: (i) —▲—: cells immobilized on VPB; (ii) —■—: cells immobilized on SGRB; (iii) —◆—: cells immobilized on NBC; (iv) —○—: freely suspended cells. Two repeated batch fermentations were conducted as described in Section 2.4.

for their capacity to increase bioethanol generation from waste paper, demonstrating that *S. cerevisiae* was better in biofuel production maintaining higher levels of metabolic activity in repeated batch experiments [52]. The reduced performance of *P. kudriavzevii* observed in the present work could be potentially attributed to various alterations that may occur in the physiology of immobilized cells as well as restricted mass transfer and reduced water activity, comprising significant stresses that cells often need to overcome during attachment on different carriers [53].

3.4. Critical aspects for the use of biochar in biofuel production

Traditional alcoholic fermentation systems utilize suspended cells in batch bioreactor operation. However, continuous ethanol fermentations provide, among other advantages, elevated conversion and fermentation rates as well as environmental merits. Immobilization of yeasts on different carriers offer recycling of the biocatalyst in continuous systems enabling high cell densities, enhanced productivity, improved stability of cells and economic benefits [52]. Herein, a novel biochar application was proposed constituting the first attempt to our knowledge for whole-cell immobilization in industrial biotechnology. *S. cerevisiae* immobilized on VPB exhibited the highest ethanol production as compared to the rest of the microorganisms tested, while the results obtained from the VPB-based biocatalyst using *K. marxianus* were also promising. Thus, considering the major industrial significance of *S. cerevisiae* and the applicability of *K. marxianus* as a lactose fermenting yeast [53] the technology proposed could offer an advanced technological solution to sustainable manufacturing of the biofuel.

The efficiency of different immobilized biocatalysts employing *S. cerevisiae* in bioethanol production is compared in Table 2. The productivity of ethanol achieved with the use of *S. cerevisiae* immobilized on NBC and VPB reached 6.0 $g\,L^{-1}\,h^{-1}$ and 7.2 $g\,L^{-1}\,h^{-1}$ respectively constituting substantially higher values compared to other carriers employed using similar feedstocks. Moreover, although initial sugar concentration was lower compared to other studies, substantially high ethanol concentrations were achieved (ranging between 60 and 72 $g\,L^{-1}$ for *S. cerevisiae* and *K. marxianus*) demonstrating the beneficial use of the novel approach. The literature studies presented on Table 2 incorporated fermentation temperatures that ranged between 30 and 33 °C. Thus, the elevated temperatures applied in the current work (37 °C and 42 °C for *S. cerevisiae* and *K. marxianus* respectively) constitute another advantage of the bioprocess enabling the reduction of operational costs due to decreased energy use for cooling and lower contamination risk. Although *K. marxianus* constitutes a promising yeast for efficient ethanol production at elevated temperatures (between 38 and 45 °C) [54], the strain includes lower tolerance in ethanol concentrations as compared to *S. cerevisiae*, which limits the production of the biofuel leading to high energy demand in the fuel-ethanol production plant [55]. Moreover, the prospect of yeast reusability enabled through immobilization of cells on biochar could significantly enhance

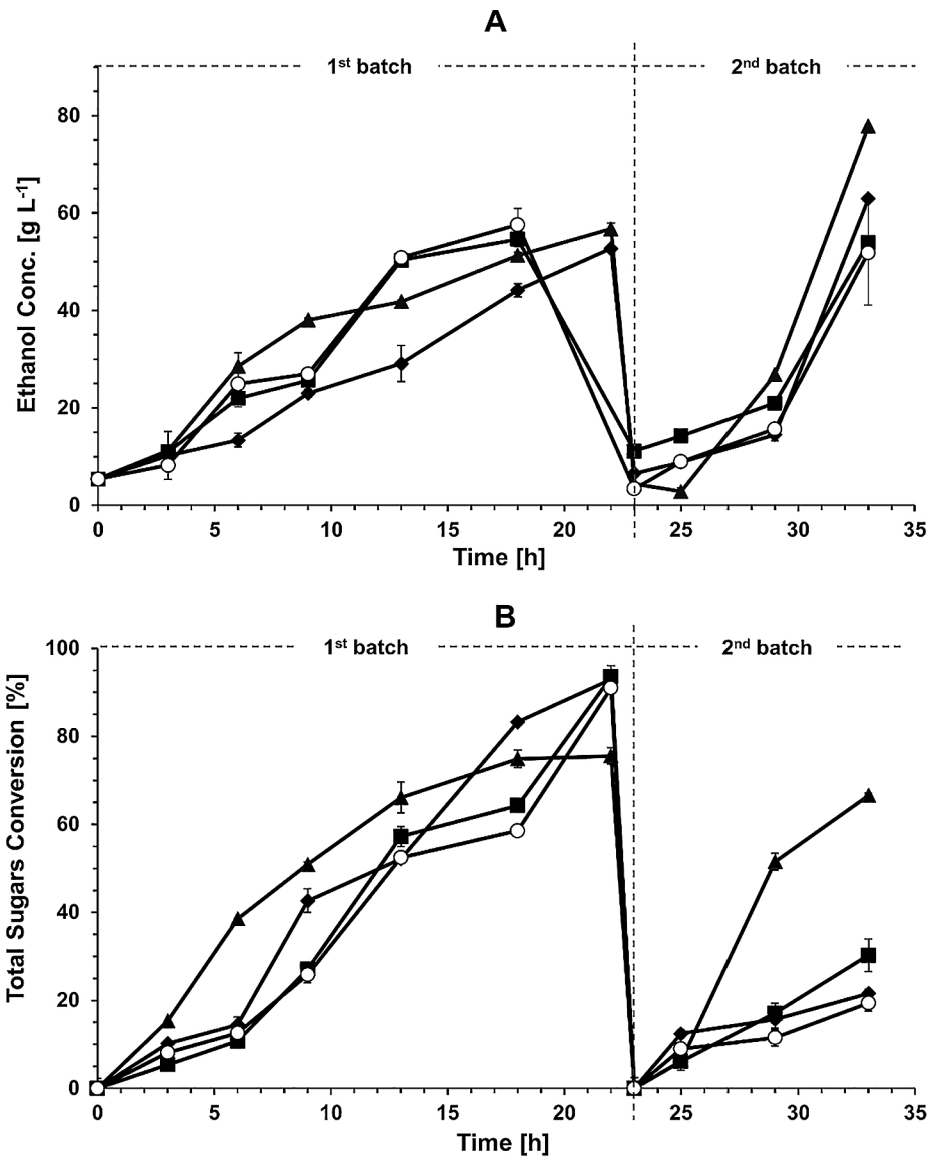

Fig. 5. Evaluation of *K. marxianus* immobilized biocatalysts for ethanol production. Symbols correspond to (A) ethanol concentration and (B) sugars' conversion in fermentations conducted at 42 °C employing: (i) —▲—: cells immobilized on VPB; (ii) —■—: cells immobilized on SGRB; (iii) —◆—: cells immobilized on NBC; (iv) —○—: freely suspended cells. Two repeated batch fermentations were conducted as described in Section 2.4.

the overall economics as well as the whole process offering new prospects for instrumentation and control [56]. Herein, high concentrations of ethanol were produced by *K. marxianus* cells immobilized on VPB (net ethanol production reached 73 g L^{-1}) and increased biofuel productivities were achieved at the second batch following the recycling of yeast cells. The specific results indicate that the technology proposed could be attractive for conducting high temperature fermentations and to test the stability of long-term bioprocess operation by biomass recycling of two major yeast strains. Therefore, future research is required towards the aforementioned directions.

The specific surface area of VPB (41.7 $m^2 g^{-1}$) was significantly higher than that of SGRB (5.3 $m^2 g^{-1}$) potentially enhancing yeast immobilization. However, although NBC involved higher specific surface area (73 $m^2 g^{-1}$), VPB was more efficient as cell carrier exemplifying that other parameters of the material could potentially affect the metabolic properties of the yeast. As previously stated in Section 3.1.2, the specific surface area of biochar is usually low and thus, different approaches have been proposed for biochar modification to improve the specific parameter including addition of magnesium and carbon activation [70]. Although activated carbon has been extensively applied as immobilization carrier for different applications, the cost related to carbon activation elevates the overall investment [71]. Nevertheless, the specific surface area of VPB was relatively high compared to other biochar materials, while activation was not employed in an attempt to reduce the cost required for biocatalyst development.

Strategies for mitigating global warming employ carbon sequestration, which includes forestation and reforestation, innovative technologies (such as underground geological and ocean CO_2 fixation) and use of carbonaceous materials for long term carbon storage [72]. Moreover, biochar constitutes a common renewable solid biofuel [73], while bioethanol from biomass (e.g. sugars, starch, lignocellulosics and algae) plays a crucial role as a supplement/substitute for petroleum fuels [37]. Thus, the use of biochar as a low-cost cell carrier in ethanol fermentations could upgrade biofuel production processes offering more stable performance [74] and biomass recycling [48], while eliminating yeast inhibition [58]. Considering that biochar is generated from biowaste, the current approach employing the material to enhance the productivity of ethanol bioprocesses enables integration of thermal and biological methods targeting the manufacture of commodities with increased added-value, lowering the environmental impact of industrial production [14]. Moreover, even though thermal methods often involve increased energy demand, no additional fuel is required for pyrolysis exhibiting energy self-efficiency [75]. Therefore, the combination of biofuel production with pyrolysis is expected to enhance energy gains constituting a highly novel approach for biowaste reduction [76]. Thus

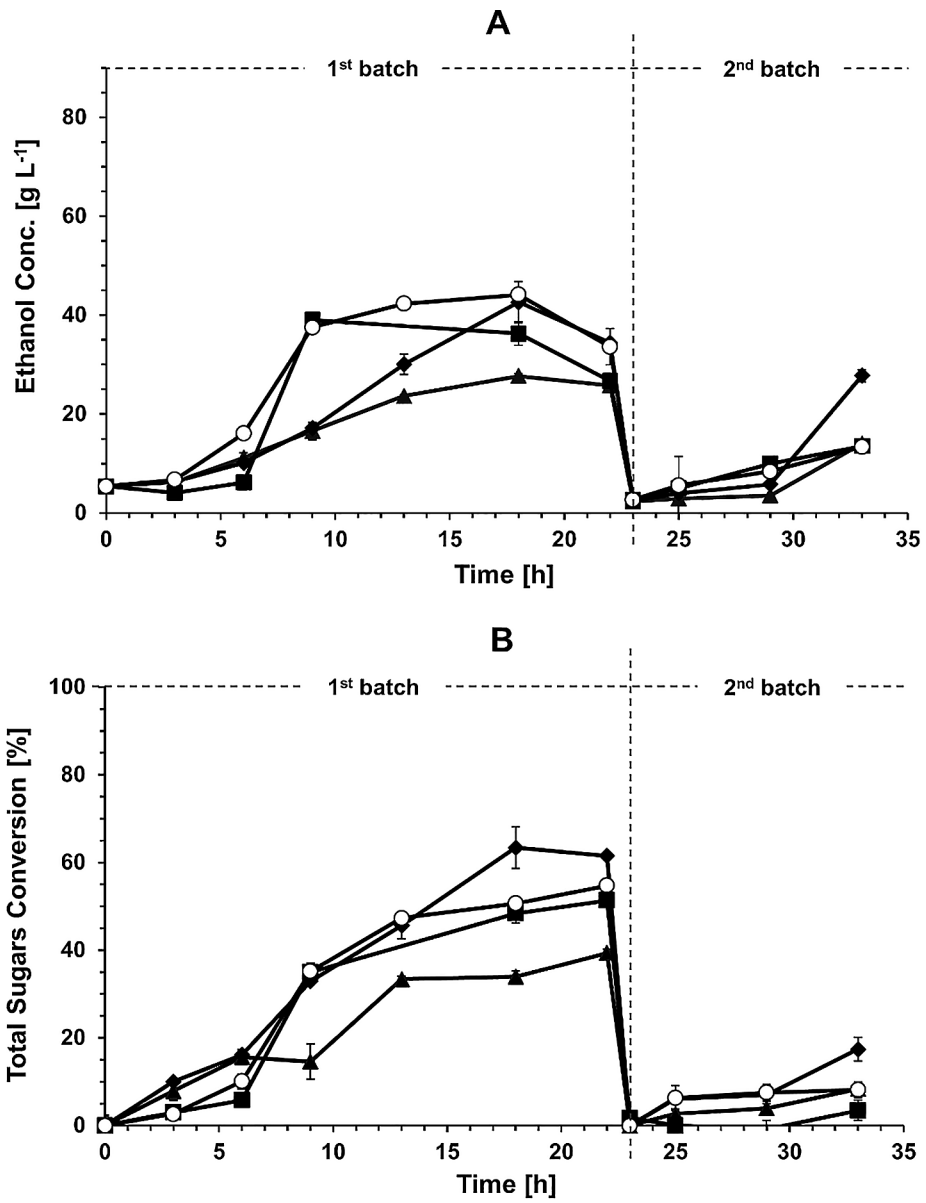

Fig. 6. Evaluation of *P. kudriavzevii* immobilized biocatalysts for ethanol production. Symbols correspond to (A) ethanol concentration and (B) sugars' conversion in fermentations conducted at 42 °C employing: (i) —▲—: cells immobilized on VPB; (ii) —■—: cells immobilized on SGRB; (iii) —◆—: cells immobilized on NBC; (iv) —○—: freely suspended cells. Two repeated batch fermentations were conducted as described in Section 2.4.

far, efforts to combine pyrolysis and fermentations have been attempted mainly in biorefineries using the thermal method either for biomass pretreatment or for the refinement of biorefinery residues [77]. The application of biochar as immobilization carrier for microbial cells constitutes a novel use of this stable by-product generated from biowaste.

The redox properties of biochar have been shown to promote biological activity and the formation of biofilms acting as significant electron acceptors or donors [78]. However, the addition of external electron acceptors in alcoholic fermentations is known to substantially boost enzymatic activity [79] and ethanol yield, which was increased from 0.62 mol ethanol/mol xylose to 1.35 mol ethanol/mol xylose following acetoin addition, doubling at the same time the specific ATP production without any increase in *S. cerevisiae* biomass content [80]. Thus, a potential cause for the elevated ethanol productivity achieved in the present study could be attributed to the quantity and type of electron accepting and donating units within biochar.

3.4.1. Engineering implications

The annual production of solid residues from citrus processing industries constitutes 25×10^6 t of waste [81]. Given a production of 1 L of hydrolyzate generated from 252 g of dried citrus peel waste (CPW) [82], as well as the moisture content in citrus peels, about 9.92×10^6 m^3 of the fermentation feedstock could be potentially available worldwide. In this work, 72 g L^{-1} of ethanol was produced from the CPW hydrolysate through use of the VPB-based *S. cerevisiae* biocatalyst. Thus, 252 t of dry CPW can be converted to 1000 m^3 of hydrolysate, suggesting that a total 0.71×10^6 t (0.91×10^6 m^3) of biofuel could be produced from the residue globally.

In the case of ethanol production from corn, 400 L of ethanol can be generated using 1 t of the crop [83] demonstrating that a total of 2.26×10^6 t of corn would be required to produce 0.91×10^6 m^3 of the biofuel. Taking into account that land productivity for the specific crop constitutes 8.53 t of corn $hectare^{-1}$ as well as a production cost of \$106.3 t^{-1} of corn [83], the land required for generating the same amount of ethanol from corn would account for 265×103 ha (slightly more than the area of Luxembourg) at a cost of $\$241 \times 10^6$. The revenue produced from biofuel manufacturing could reach $\$624 \times 10^6$ given a price of \$873 t^{-1} ethanol [1]. Thus, employing CPW for ethanol generation could substantially reduce the land use for cultivation of the grain often applied for biofuel manufacturing along with generation of additional revenue. Moreover, considering that apart from the

Table 2
Bioethanol production with the use of immobilized yeast cells.

Yeast strain	Feedstock	Method	Carrier	Sugar conc. ($g L^{-1}$)	Ethanol conc. ($g L^{-1}$)	Ethanol productivity ($g L^{-1} h^{-1}$)	Specific surface area ($m^2 g^{-1}$)	Reference
S. cerevisiae	Sugar molasses	Cross-linking and covalent binding	Alginate-based MCM-41 mesoporous zeolite composite	170	78.6	6.55	–	[57]
S. cerevisiae M30	Cane molasses	Cross-linking	Bacterial cellulose-alginate sponge	220	92	1.92	–	[58]
Mutant baker's yeast 3013	Glucose and sucrose	Adsorption	Sorghum bagasse	200	92.7	5.72	3.0–5.0 [59]	[44]
S. cerevisiae MTCC 174	Sugarcane bagasse	Adsorption	Sugarcane bagasse	50	15.4	0.43	3.0–12.7 [60]	[61]
S. cerevisiae M30	Blackstrap molasses	Adsorption	Thin-shell silk cocoon	240	80.6	1.85	–	[62]
S. cerevisiae DTN	Sugar beet thick juice	Adsorption	Sugar beet pulp	120	52.3	1.09	3.0–16.5 [60]	[63]
S. cerevisiae NP 01	Sorghum juice	Adsorption	Sweet sorghum stalks	230	98.5	1.37	3.0–5.0 [59]	[64]
S. cerevisiae CTCRI	Mahula flower	Entrapment	Calcium alginate	350	25.8	0.27	–	[65]
S. cerevisiae var. *ellipsoideus*	Corn meal	Entrapment	Calcium alginate	150	88.9	2.34	–	[65]
S. cerevisiae T0936	Wheat straw	Entrapment	Calcium alginate	51.4	37.1	0.38	–	[66]
S. cerevisiae	Glucose	Cross-linking and covalent binding	Mineral Kissiris	113	48	3.06	2.2 [67]	[44]
S. cerevisiae	Molasses	Cross-linking and covalent binding	Orange peel	128	58.9	4.17	0.4 [68]	[69]
S. cerevisiae	Orange peel waste hydrolysate	Cross-linking and covalent binding	NBC	90	60	6.0	73.0	Current Study
S. cerevisiae	Orange peel waste hydrolysate	Cross-linking and covalent binding	VPB	90	72	7.2	41.7	Current Study
K. marxianus	Orange peel waste hydrolysate	Cross-linking and covalent binding	NBC	90	56	5.6	73.0	Current Study
K. marxianus	Orange peel waste hydrolysate	Cross-linking and covalent binding	VPB	90	73	7.3	41.7	Current Study

hydrolysate applied for bioethanol production a solid fraction would still remain as residue following CPW pretreatment, the specific material would not be expected to be landfilled given that it could be suitable for more sustainable practices (e.g. anaerobic digestion, animal feed). Anaerobic digestion has been previously successfully integrated with ethanol fermentations further enhancing the energy balance through the production of biogas from remaining solid fractions [84]. Thus, future optimization of the proposed technology will indicate the energy-effectiveness of ethanol production from CPW using biochar-based biocatalysts.

4. Conclusions

Herein, an innovative technology was developed for the production of renewable energy, which mitigates the environmental effects from food waste disposal and improves the sustainability of energy systems. Specifically, an advanced use of biochar was explored evaluating applicability of the material for immobilization of whole-cells in a major industrial biotechnology process. Vineyard prunings, seagrass residues and sewage sludge were applied in pyrolysis without activation, while process temperature and the type of feedstock strongly affected the physicochemical properties of the biochar produced. The increase in temperature resulted in higher specific surface area and porosity, whereas both crystalline and amorphous structures were formed incorporating varying elemental composition and carbon content. Three major yeast strains were immobilized on vineyard prunings and seagrass residues biochar generated at 500 °C as well as on a non-renewable form of non-biological char to evaluate bioethanol production using a Valencia orange peel hydrolyzate. *S. cerevisiae* and *K. marxianus* demonstrated significant potential for the development of biochar-based biocatalysts yielding elevated biofuel production in repeated batch experiments as compared to the relevant literature. The technology was effective in terms of ethanol productivity facilitating biocatalyst reusability, while the process could be further enhanced through optimization of fermentation parameters.

Declarations of interest

None.

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