Synthesis and Characterisation of *Saccharomyces cerevisiae* Immobilised Cells from Cashew Apple Bagasse

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Cashew apple bagasse is biomass rich in little-exploited lignocellulosic material. This study used this biomass as a support for cell immobilisation of *Saccharomyces cerevisiae*. For this purpose, the immobilisation technique by attachment to a surface was applied. The bagasse used in this study contained 32.6% lignin. After delignification, the lignin content of the bagasse was 3.33%. The cell density was 1.21×10^8 cells g^{-1} for the immobilised cells prepared for 24 h. For the immobilised cells prepared for 48 h, the cell density was 1.71×10^8 cells g^{-1}. Microscopic observations showed that the adhesion of the yeast cells to the surface of the support occurred on all layers with the cells immobilised for 48 h. These results highlight the efficiency of cell immobilisation of *S. cerevisiae* on cashew apple bagasse.

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INTRODUCTION

The use of free cells for alcohol production aims to take advantage of the high production capacity of yeast. The selectivity and specificity of yeasts are studied to achieve good fermentation. However, yeasts have some disadvantages, such as limited efficiency due to substrate and product inhibition, yeast viability, and removal of excess yeast and CO_2 (Saha and Banerjee 2013).

The immobilised cell system is an effective solution, as it can increase productivity and minimise fermentation production costs (Tian *et al*. 2021; Dzionek *et al*. 2022). Cell immobilisation leads to higher cell densities, avoids losses of microorganisms, and facilitates cell/liquid separation (Vučurović *et al*. 2009; Krasňan *et al*. 2016). Thus, the choice of support and immobilisation technique is key to the immobilisation of *Saccharomyces cerevisiae*. The use of natural substrates, which are cheaper and more durable than synthetic substrates, can help to reduce costs and improve product quality (Santos *et al*. 2008; Karagoz *et al*. 2019).

Therefore, cashew apple bagasse (CAB) was used. It is composed of lignocellulosic residue and has good mechanical and physical properties (Santos *et al*. 2007; de França Serpa *et al*. 2020). The CAB was derived from the pressing of cashew juice. The material

is non-toxic, of no commercial value, and is discarded after pressing the juice. Indeed, in cashew cultivation in Côte d'Ivoire, only the nuts are exported, and the cashew apples are left at the harvesting site or processed into juice (Adou *et al*. 2011; Soro 2012; Gnagne *et al.* 2023). In 2021, cashew nut production in the world was estimated at 1,363,452.62 tonnes, and in Côte d'Ivoire at 837,850.12 tonnes (FAO 2023). The cashew apple represents 9 to 10 times the weight of the nut (Soro 2012). Côte d'Ivoire is therefore the leading cashew apple producing country with an estimated production of 8 million tonnes.

When the pseudofruit is processed into juice, bagasse is produced. To add value to bagasse and minimise its environmental impact, it would be ideal to use it as a support for cell immobilisation. Cellulosic materials are solid supports used for immobilisation by physical adsorption due to electrostatic forces or by covalent bonding between the cell membrane and the support (Santos *et al*. 2008; Kawaguchi *et al*. 2016).

The present study aims to use CAB as cell support for *Saccharomyces cerevisiae*, to investigate the effect of chemical treatment on the composition and structure of the CAB support.

EXPERIMENTAL

Biological Material

Cashew apples (*Anacardium occidentale* L.) (Fig. 1A) were collected in the Yamoussoukro area N 6°44'16.00944 W 5°22'42.29328 (Côte d'Ivoire). The apples were cleaned and washed by spraying them with running water. They were then disinfected in a 100 ppm active chloride solution for 30 min. After extraction of the juice, the bagasse (Fig. 1B) obtained was dried (Fig. 1C) and ground to obtain a powder (carrier) (Fig. 1D).

Fig. 1. *Anacardium occidentalis* L.: cashew apple (A); bagasse (B); dried bagasse (C); and powder (D)

Methods

Preparation of the substrate

The cashew apple bagasse (CAB) was processed according to the method of Pacheco (Pacheco *et al*. 2010). The CAB (100 g) was washed with distilled water and dried at 50 °C for 24 h. Then, the CAB was dissolved in a 3% HCl solution for 2 h 30 min with constant stirring in a thermostatic bath at 60 °C. It was then washed, dried at 50 °C, and delignified with 2% NaOH for 24 h. The bagasse obtained was then washed, dried, and ground in a mill (IKA MF 10 basic) to obtain a 0.5 and 0.2 mm particle size.

Microorganism and inoculum

The microorganism used was the commercial yeast *S. cerevisiae* (Saf-Instant LESAFFRE, France). A pure culture was isolated from the baker's yeast, inoculated onto Sabouraud Biolife agar, and incubated at 30 °C for 48 h. The inoculum was obtained in a 100 mL medium, composed of ($g.L^{-1}$): KH_2PO_4, 5; $(NH4)_2SO_4$, 2; $MgSO_4.7H_2O$, 0.4; yeast extract, 1; glucose, 10. The medium was sterilised at 110 °C for 10 min. The pH and temperature were maintained at 5.0 and 30 °C, respectively, for 24 h. Then, the cells were centrifuged (Hermle Z 207A, Germany) at 10,000 × g for 10 min to obtain the biomass for cell immobilisation.

Cell immobilization

The 30 g delignified media was sterilised at 110 °C for 10 min and then mixed with 150 mL of synthetic medium, consisting of ($g.L^{-1}$): glucose, 30; yeast extract, 5; $(NH_4)_2SO_4$, 10; KH_2PO_4, 4.5; $MgSO_4.7H_2O$, 1; $ZnSO_4$, 0.65. One percent (w/v) of the cells were inoculated into the medium containing the carrier and the mixture was fermented at 30 °C for 150 rpm for 24 and 48 h. The liquid was decanted and the cell-containing medium was washed with sterile distilled water to obtain the immobilised *S. cerevisiae* cells.

Characterisation of the Raw and Delignified Carrier

Extractives and lignocellulosic composition

Extractives were removed according to the procedure described by the author Poursat (Poursat 2015). The amount of sulphuric acid insoluble lignins was determined gravimetrically according to the laboratory analysis procedure (LAP) adopted by the National Renewable Energy Laboratory (NREL) (Sluiter *et al*. 2008). The holocellulose content was analysed *via* the chlorite method (Boudjema 2016). Cellulose was isolated from holocellulose after solubilisation of hemicelluloses in a dilute hydroxide solution (Yahiaoui 2018). The hemicellulose content was obtained by subtracting the cellulose content from the holocellulose content.

Scanning electron microscopy (SEM) coupled with energy dispersive spectroscopy (EDS)

The micrographic study of the CAB was performed using an SH-4000 M scanning electron microscope (Hirox, Tokyo, Japon) under the following conditions: 15 to 20 nm, 30x to 60,000x magnification, and 5 to 30 kV acceleration voltage in 5 steps. In addition, energy dispersive X-ray spectroscopy (EDS) was performed with an XFlash 6/30 detector (Brucker, Billerica, MA, USA) for the determination of chemical elements.

IR spectroscopy analysis

Spectroscopy was utilized to study the changes in the substrate. Fourier transform infrared spectroscopy (FT-IR) was performed in ATR (attenuated total reflectance) mode, using a Bruker Alpha Fourier Transform spectrometer, brand SHIMADZU (Kaduna, Federal Republic of Nigeria), equipped with a diamond crystal in the wavelength range of 400 cm^{-1} to 4000 cm^{-1}.

Characteristics of the Immobilised Support

Optical micrographs before and after immobilisation

To confirm the immobilisation of *S. cerevisiae* cells on bagasse, optical micrographs were taken before and after cell immobilisation using a BA310 Optical microscope (Motic, Barcelona, Spain).

Counting of cells adsorbed on the support

The mass of cells adsorbed on the support particles was quantified by counting on a Thoma slide (0.1 mm chamber, a central square of 400 small squares). Before counting, 0.5 g of support containing immobilised cells was added to 50 mL of 0.85% NaCl solution and mixed for 24 h under agitation at 150 rpm. Then, 0.1 mL of solution (containing released yeast cells) was placed on a Thoma slide for observation.

RESULTS AND DISCUSSION

Characterisation of the Raw and Delignified Substrate

Extractives and lignocellulosic composition

Figure 2 shows the changes in the lignocellulosic composition of the substrate as a result of the chemical treatment. This figure shows a reduction in lignin content, indicating that the treatment affected the degradation of the lignin structure. Similarly, an increase in the cellulosic fraction was observed at the expense of the hemicelluloses. This could be explained by the fact that in an alkaline environment, there is an increased dissolution of lignin, hemicelluloses, and extractives (Bensah *et al*. 2019; Ouattara *et al*. 2021; Bamba *et al.* 2023).

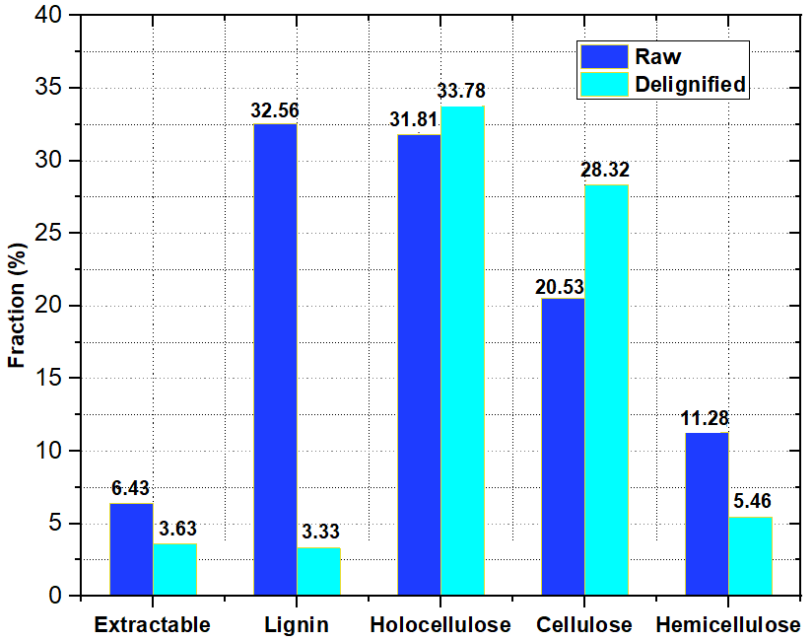

Fig. 2. Lignocellulosic and extractable composition of raw and delignified carrier

Delignification removed a large part of the lignin, thus increasing the percentage of cellulose. This modification of the chemical composition improves the quality of the support, thus facilitating better access to the cellulosic groups during the cell immobilisation process (Bardi and Koutinas 1994; Correia *et al*. 2013; Kawaguchi *et al*. 2016).

IR spectroscopy analysis

The IR analysis was utilized to evaluate the structure of the raw and delignified substrate to identify the groups modified by the chemical treatment (Fig. 3). Thus, Fig. 3A corresponds to the IR of the raw substrate. The vibration of the aromatic and aliphatic hydroxyl stretching in the lignin is associated with the 3472 cm^{-1} band (Brazil *et al*. 2018). The 2922 cm^{-1} band has been attributed to the asymmetric and symmetric C-H stretching of the alkyl groups in the lignin structure (Li *et al*. 2017). The peaks observed at 1617, 1543, and 1438 cm^{-1} could be associated with the vibrations of the aromatic backbone of lignin (de Souza *et al*. 2016). The 1319 cm^{-1} peak corresponds to the bending of the C-H group in cellulose (Fig. 3A). The 1230 cm^{-1} peak could correspond to the C-O stretching of acetyl groups present in the hemicellulose molecular chain (Fig. 3A).

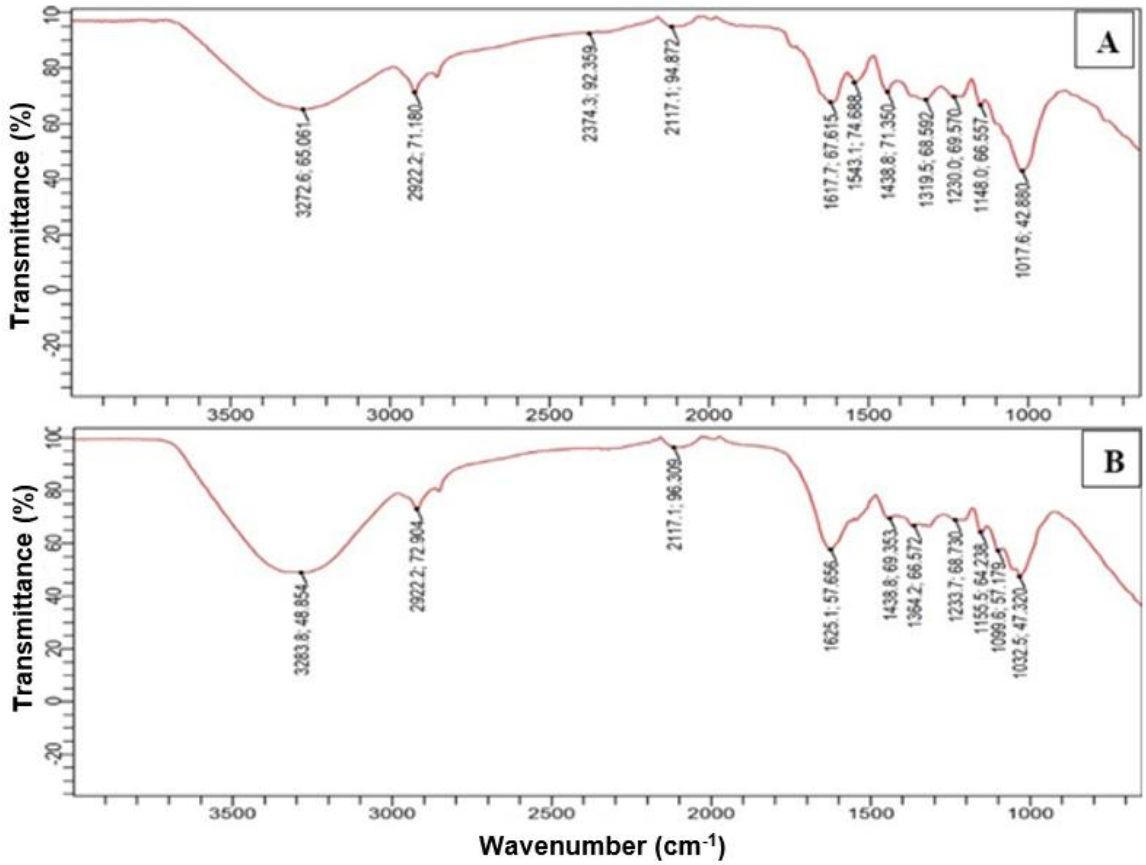

Fig. 3. IR spectra of the raw substrate (A) and raw delignified (B)

These results confirm the analysis of lignocellulosic characteristics obtained previously (Correia *et al*. 2013; de Souza *et al*. 2016). The lignin bands at 1625 to 1438 cm^{-1} show a decrease, demonstrating a lower lignin content compared to the content of the raw substrate (Fig. 3B). The characteristic cellulose bands in the region of 1233 to 1032 cm^{-1} can be mainly observed in the spectrum of delignified CAB, in which these bands are related to vibrations of the pyranosyl rings, showing an increase in the cellulosic fraction (Fig. 3B). The peak at 1032 cm^{-1} is attributed to absorptions from the hemicellulose, explicitly to the stretching of the C-O in the C-O-C bond (Fig. 3B). In contrast, the IR spectrum of the raw and delignified carrier analysed in this work did not change significantly.

Scanning electron microscopy (SEM) analysis

The scanning electron microscopy structure of the raw and delignified bagasse is shown in Figs. 4 and 5. The texture of the raw CAB shows an irregular structure covered with wax, generally found on lignocellulosic materials (Daud *et al*. 2013; Ouattara *et al*. 2021).

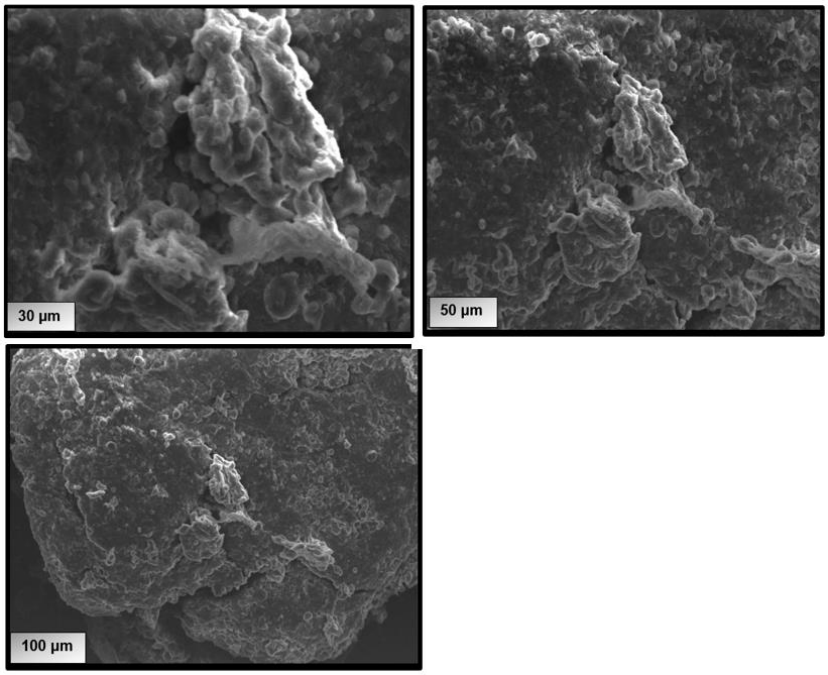

Fig. 4. Morphology of raw bagasse at different magnifications: 30 µm; 50 µm; and 100 µm

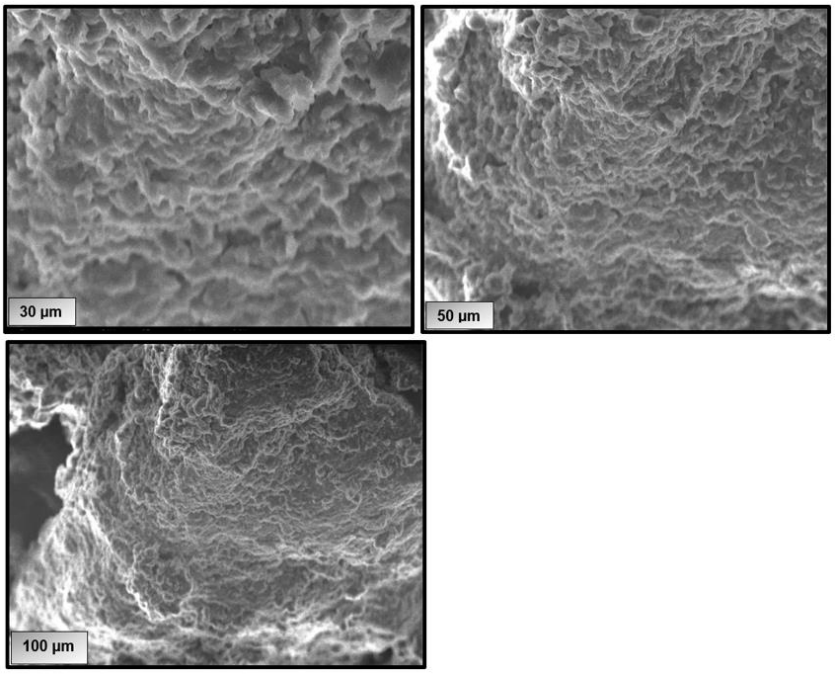

Fig. 5. Morphology of delignified bagasse at different magnifications: 30 µm; 50 µm; and 100 µm

After processing, wax residues are also removed from the delignified structure. The delignified bagasse structure has a smooth surface, which indicates that some of the lignin had been removed (de Souza *et al*. 2016).

The EDS chemical mapping (Figs. 6 and 7) reveals a carbon (C) content of 62.3% and an oxygen (O) content of 37% in the raw substrate (Table 1). After delignification, a lower C content (56.9%), and a higher O content (40%) were observed (Table 2). The increase in O content could be because of the oxidation of the lignin side chains, as part of the oxygen was involved in the lignin degradation reactions and was incorporated into the oxidised lignin products (Correia *et al*. 2013; de França Serpa *et al*. 2020). The chemical elements P, K, and Fe detected in EDS, would come from the chemical composition of the lignocellulosic biomass (Figs. 8 and 9). The removal of lignin by chemical processes tends to deform the rigid structure of lignocellulosic materials and improves the possibilities of immobilization of CAB cells on the structure of cellulose and hemicelluloses by increasing the porosity and roughness of the support material (Kopsahelis *et al*. 2007; Tran and Le 2014).

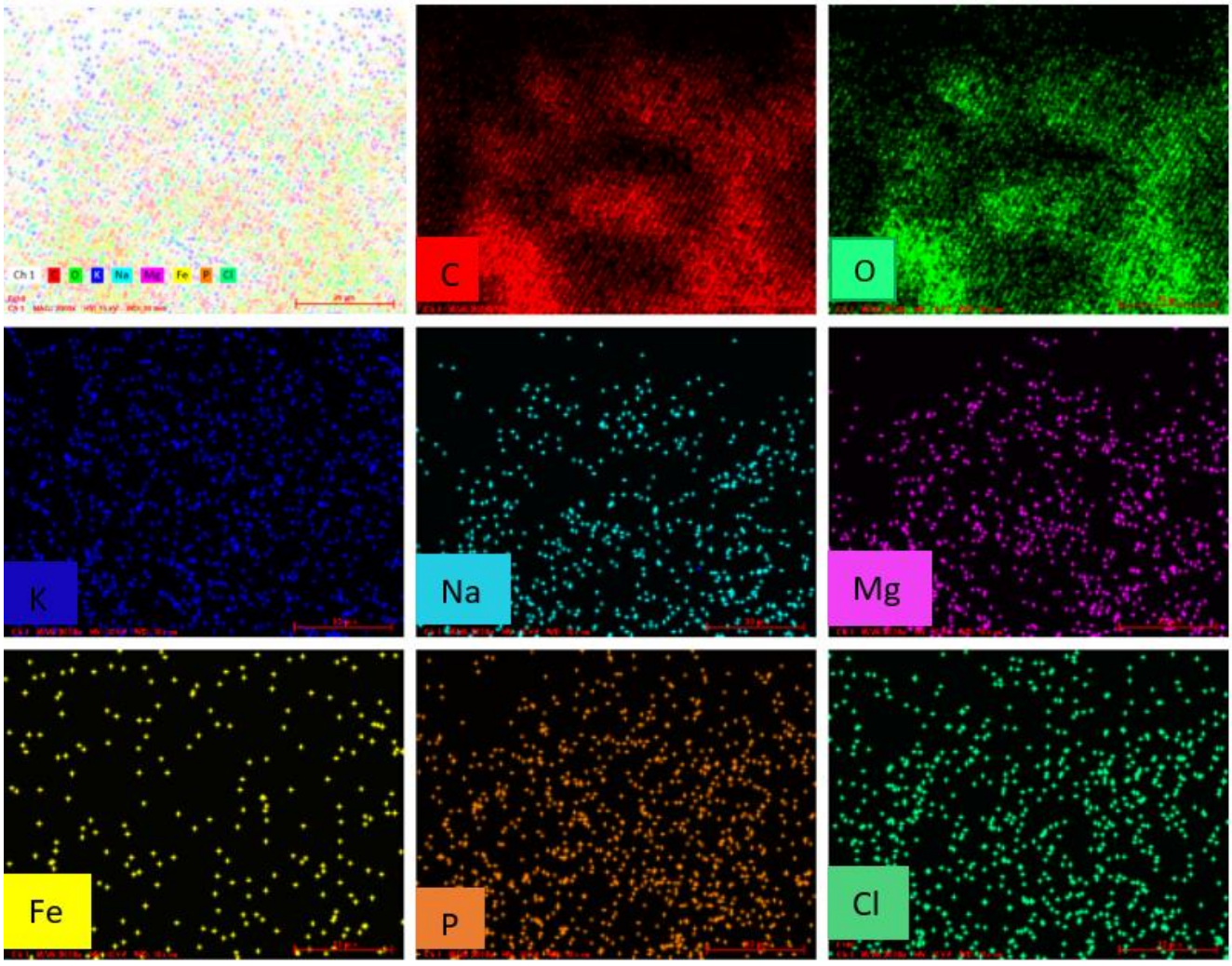

Fig. 6. Chemical element mapping of raw bagasse at 20 µm using the Brucker X-30 flash EDS

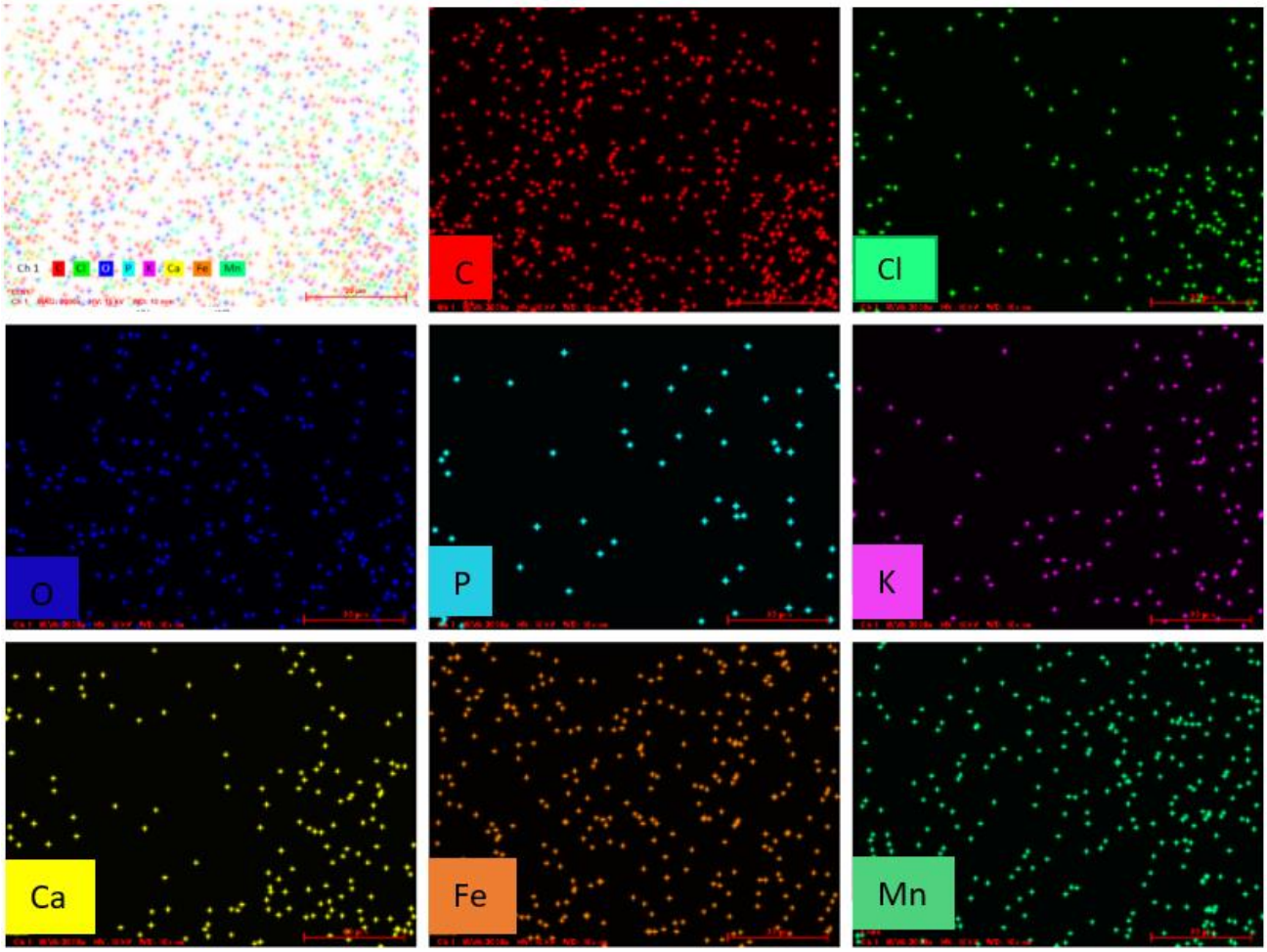

Fig. 7. Chemical element mapping of delignified bagasse at 20 µm using the Brucker X-30 flash EDS

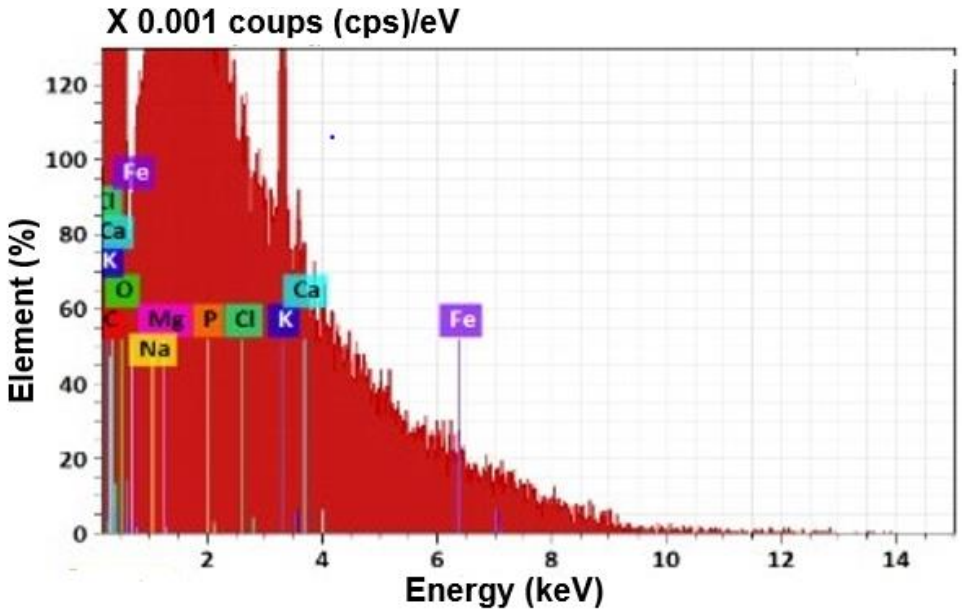

Fig. 8. Microanalysis spectrum of raw bagasse at 20 µm

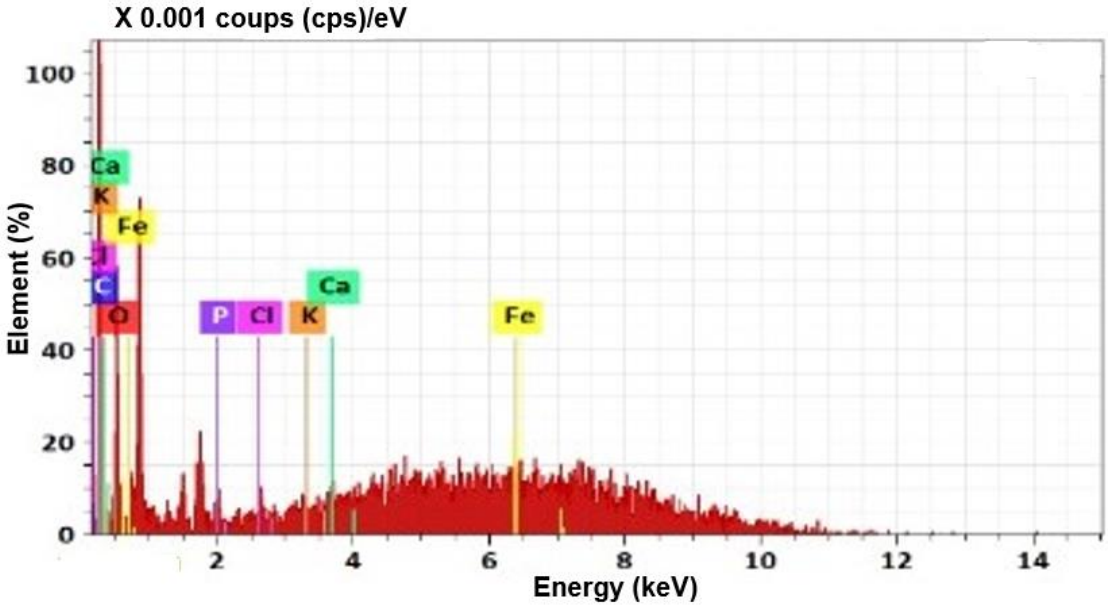

Fig. 9. Microanalysis Spectrum of delignified bagasse at 20 µm

Table 1. Quantification of Chemical Elements in Raw Bagasse

Element	A	lign	Net	Mass (%)	Mass.norm (%)	Atom (%)
C	6	K-series	83133	62.36	62.36	68.85
O	8	K-series	33866	37.53	37.53	31.11
K	19	K-series	1141	0.08	0.08	0.03
P	15	K-series	523	0.02	0.02	0.01
Fe	26	K-series	26	0.01	0.01	0.00
			Total:	**100.00**	**100.00**	**100.00**

Table 2. Quantification of Chemical Elements in Delignified Bagasse

Element	A	lign	Net	Mass (%)	Mass.norm (%)	Atom (%)
O	8	K-series	332	40.94	40.94	34.82
C	6	K-series	584	56.99	56.99	64.57
Cl	17	K-series	0	0.00	0.00	0.00
Fe	26	K-series	62	1.05	1.05	0.26
K	19	K-series	14	0.23	0.24	0.08
Ca	20	K-series	38	0.79	0.79	0.27
P	15	K-series	0	0.00	0.00	0.00
			Total:	**100.00**	**100.00**	**100.00**

Characteristics of the Immobilised Support

Optical micrographs before and after immobilisation

Figure 10 shows the microscopic observation of the support with and without cells. It can be seen in Fig. 10A that the cell-free supports were irregularly shaped. Microscopic

observations revealed that the adhesion of the yeast cells to the surface of the support occurred on all layers with cells immobilised for 48 h (Fig. 10C). By contrast, for cells immobilised for 24 h (Fig. 10B), the adhesion was slight on the surface of the support. The authors observed an intensive accumulation of yeast that could increase the immobilisation rate of yeast on the surface of the support immobilised for 48 h (Fig. 10C).

According to Yu *et al*. (2007), the lignocellulosic materials used as support allow the yeast cells to be adsorbed on the surface of the supports. Only a few of these yeast cells are firmly embedded in the inner side of the supports.

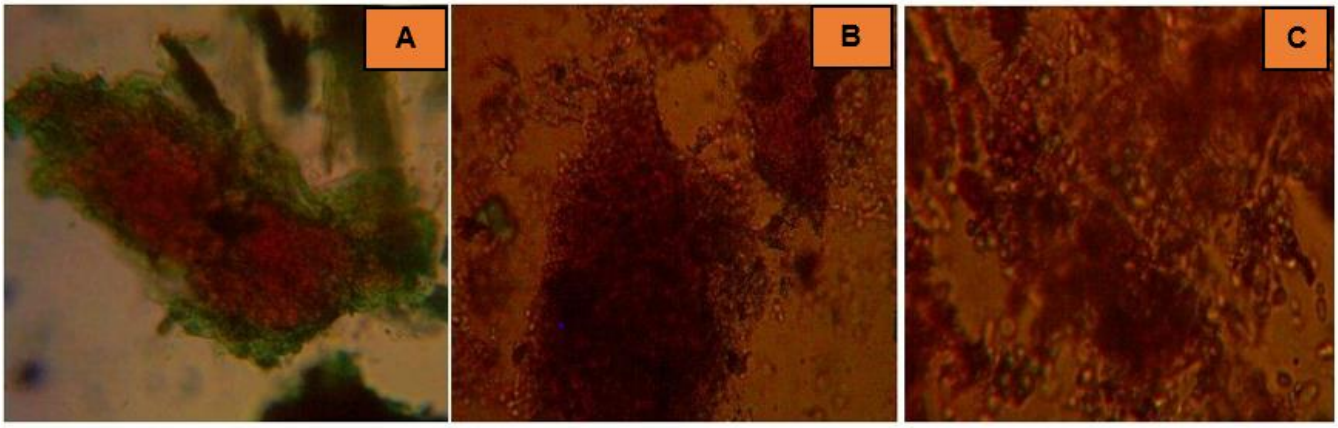

Fig. 10. Optical microscopy of CAB (×400): without cells (A), with cells immobilised 24 h (B), and with cells immobilised 48 h (C)

It has been reported that the adhesion of yeast on delignified agricultural waste depends on electrostatic interactions between the support and the negatively charged cell surface by physical adsorption (Santos *et al*. 2008; Ahmadi *et al*. 2016).

Counting of cells adsorbed on the support

During the cell immobilisation performed, the cell density was taken to know the initial cell load of yeast firmly attached to the surface of the support. The cell density was 1.21×10^8 cells g^{-1} for cells immobilised for 24 h. While for cells immobilised for 48 h, the cell density was 1.71×10^8 cells g^{-1}. These results are in agreement with the work of the authors (Żur *et al*. 2016; Wang *et al*. 2018), who observed that cell immobilisation is a time-dependent process and they attribute this dependence to two main factors: cell multiplication and the formation of a strong and irreversible adhesion. The high cell load presented in this work can be attributed to the average size of supports and the immobilisation time. According to Branyik *et al*. (2001), when the support is small, it allows for a high cell load. These results confirm and suggest cell immobilisation on lignocellulosic materials. Thus, experiments on fermentation conditions with immobilised *Saccharomyces cerevisiae* cells should be studied.

CONCLUSIONS

1. The characteristics of the bagasse proved to be important in highlighting these properties. Bagasse contained 32.6% lignin, and after delignification with sodium hydroxide (NaOH 2%) it contained 3.33%. Delignification disrupted the composition of the biomass by removing the lignin, which prevents enzymatic or chemical access to the cellulose.
2. The structure of the delignified carrier, as observed by scanning electron microscopy (SEM), had a smooth appearance compared to the structure of the raw carrier. This demonstrates that sodium hydroxide (NaOH 2%) had broken bonds within the carrier components.
3. Infrared (IR) analysis showed peaks at 1617, 1543, and 1438 cm^{-1} that characterise the lignin in the raw substrate. After delignification, the lignin bands showed a decrease, demonstrating a lower lignin content compared to the content of the raw support.
4. Preparation of the cells immobilised for 48 h resulted in higher cell loads of 1.71×10^8 cells g^{-1} and showed that *Saccharomyces cerevisiae* uniformly colonised cashew bagasse.

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