The Potential of Using Grapefruit Peel as a Natural Support for Yeast Immobilization During Beer Fermentation

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The potential use of grapefruit peel as support material for yeast immobilization during beer fermentation was evaluated. After conditioning, FTIR analysis revealed a higher quantity of methoxy ($-OCH_3$) groups, suggesting that lignin is the major component of the support. Cell adhesion onto the conditioned support in 12°Plato laboratory malt wort was evaluated, observing a maximal cell adhesion ($2.25 \cdot 10^9$ cells/gram of dried support) at 20 h of cultivation, remaining almost constant in the subsequent time points. Evaluations of the fermentative behaviour of the biocatalyst at 15±0.5 °C in a 14 °Plato laboratory malt wort indicated good stability in terms of physical integrity (confirmed by SEM observation). The fermentation time was shortened to four days, and the rates of reducing sugar consumption and ethanol production were improved when compared to fermentations carried out with free suspended cells. These results show a promising potential of grapefruit peel as support material in beer fermentation.

Keywords:
grapefruit peel, natural supports, cell immobilization, alcoholic fermentation, FTIR

Introduction

Immobilization technology of cells has been used over the last years in biotechnological processes, including alcoholic fermentation. There are several immobilization methods listed in the literature, which are classified based on the way in which the cells interact among them and with the support. This includes natural or artificial flocculation, entrapment within a porous matrix, immobilization on the surface of a solid structure, and mechanical containment behind a barrier[1]. From the volumetric production point of view, beer production is the most important alcoholic fermentation process worldwide. It is commonly carried out with freely suspended yeast cells[2,3] cultured in cylindroconical fermenters. In the last decades, the immobilization of yeast has been investigated for beer fermentation with the purpose of obtaining some benefits, such as reducing the cost of the process and improving the sensory quality of the product[4,5]. For instance, it has been reported that immobilization of yeasts reduces the production of diacetyl, aldehyde, and higher alcohols, which strongly influences the sen-

sory quality of the beer[6,7]. It has also been shown that immobilization of yeast improves the fermentation kinetics: the fermentation time is diminished in comparison to fermentations with free cells, the ethanol productivity increases, and the fermentation profile at low temperature is improved[8]. The explored approaches in this field include immobilization by entrapment in polymeric matrixes and adsorption on solid materials. Immobilization by entrapment in polymeric matrix comprised the use of commercial polymers, such as alginate, chitosan, carrageenan, and PVA[9–11]. However, these materials are costly, and in some cases not available. The search for cheaper support materials derived from agricultural residues is considered a viable alternative in overcoming such limitations. These organic materials should be cheap, abundant in nature, non-toxic, and show good biocatalytic stability. There are few reports regarding the use of natural supports for yeast immobilization in beer fermentation, including the use of lignocellulosic materials, such as shavings and brewer's spent grains[8,12–17], gluten pellets[18], dried figs[19], corn cobs[15] and legume hulls[20]. In order to explain how the movement of cells is limited upon interaction with the support,

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the following mechanisms of yeast immobilization have been proposed: cell-support adhesion, cell-cell attachment, and cell adsorption (accumulation) inside natural shelters (support's surface roughness)[14].

Furthermore, agroindustrial residues are promising materials that could be used for immobilization of cells. In this sense, fruit peels are typically generated in large quantities in the fruit juice industry worldwide. Grapefruit has an agroindustrial importance because of the wide use of the fruits to produce concentrated juice and jams. Grapefruit is cultivated in all tropical and subtropical regions of the world. The global production of grapefruit in 2019/2020 recorded 6 970 million metric tons, the major producers being China (4 930 000 metric tons), USA (582 000 metric tons), Mexico (468 000 metric tons), South Africa (420 000 metric tons), Turkey (300 000 metric tons), Israel (155 000 metric tons), and EU (89 000 metric tons)[21,22]. On the other hand, the major consumers are China, EU, Mexico, and USA[22]. In addition, USA (285 000 metric tons), South Africa (124 000 metric tons), Mexico (95 000 metric tons), Israel (72 000 metric tons), and EU (15 000 metric tons)[22] are the most important countries where grapefruit is processed. Thus, large amounts of grapefruit peel are available throughout the world. Interestingly, grapefruit peel contains several water soluble and insoluble monomers and polymers[23–25]. The water-soluble fraction contains fructose, glucose, sucrose, and some xylose, while cellulose, hemicelluloses, lignin, and pectin constitute between 50 % and 70 % of the insoluble fraction. These polymers are rich in carboxyl and hydroxyl functional groups, which may potentially bind cells in aqueous solution[24,25]. Although a wide variety of agroindustrial residues exist in the world and can be proposed as supports for the immobilization of yeast, only a few of them could find application in fermentation processes on the industrial scale.

The present research evaluated the potential of grapefruit peel as a cheap and environment-friendly support material for yeast immobilization during beer fermentation. The study is also unique, since there is no existing report for the immobilization of yeast cells on grapefruit peel for beer fermentation.

Materials and methods

Yeast strain and maintenance

The commercial lager beer yeast *Saccharomyces cerevisiae* SAFLAGER S-23 acquired as lyophilized powder from Fermentis was used in this study. After activation in sterile peptone solution (1 %w/v), the yeasts were inoculated in slant agar of malt extract and incubated at 30 °C for 48 hours.

Malt extract agar was prepared according to the instructions of the provider. After incubation, cells were maintained at 7 °C and subcultured every three months using the same agar medium.

Support material preparation

Grapefruit (*Citrus paradisi*) of the Ruby variety was used in the present study. The grapefruit peel was obtained after extraction of the juice, and used to prepare the support material. The peel (Fig. 1) was carefully separated from the flavedo (yellow part containing essential oils) using a knife. The resultant spongy pink part (albedo) was used to prepare the supports. The albedo was cut into small square-shaped pieces of about 10x10 mm in size, which were immersed into 10 %v/v ethanol for 24 h to remove sugars and other alcohol-soluble components. The material was subsequently washed several times until the bulk liquid became transparent. The treated pieces were dried at 70 °C until attaining constant weight and allowed to cool to room temperature in a desiccator. Subsequently, the treated material was sieved, and only the pieces that passed through a 4-mesh sieve (4.75 mm opening)
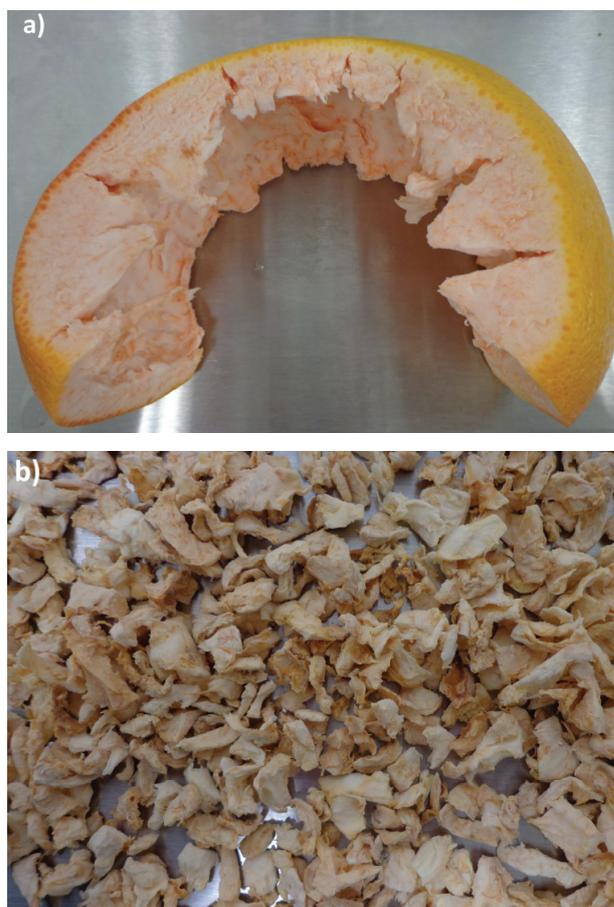
Fig. 1 – *Raw grapefruit peel (a) and support material (before treatment) prepared from the albedo of grapefruit peel (b)*

and were retained in a 6-mesh sieve (3.35 mm opening) were selected to be used as immobilization support. The dried supports were kept in a hermetic plastic bag at ambient temperature until their use.

FTIR analysis

A FTIR spectrometer (Perkin-Elmer, Frontier FTIR) was used for the characterization of the support material. Analysis was performed by the sampling technique of attenuated total reflectance. An amount of approximately 0.5 grams of dried support (treated or non-treated) was milled in a mortar until obtaining a powder, which was carefully placed in the sample holder (diamond crystal) of the equipment. IR spectra were acquired by averaging 16 scans in the range of 600–4000 cm^{-1}. The identification of functional groups and interpretation of spectra were carried out using the database of the IR-software provided by the manufacturer. To avoid any interference of the ambient humidity, a dehumidifier was installed at the laboratory. Analyses were carried out in duplicate at room temperature.

Yeast propagation for experiments

Propagation of yeast was carried out in 0.5 L Erlenmeyer flasks containing 0.2 L of laboratory malt wort. Samples of colonies were picked up from the slant agar with an inoculating loop, and inoculated into Erlenmeyer flasks containing the culture medium. The laboratory malt wort was prepared from a concentrated malt extract (Pilsner, Briess-CBW®), which was diluted with distilled water until reaching a concentration of 8°Plato. The pH of the medium was adjusted to 5.4 with NaOH before pasteurizing at 115 °C for 15 min. Propagation of yeast cells was carried out at 28 °C, 200 min^{-1} for 48 hours. After propagation, cells were collected by centrifugation (6000 min^{-1} for 8 min), and re-suspended in the same fermentation medium before used as inoculum.

Attachment of yeast cells on support materials

Experiments of attachment of yeast cells on supports were carried out in 0.25 L Erlenmeyer flasks containing 0.1 L of 12°Plato laboratory malt wort prepared as previously stated. The pH of the medium was adjusted to 5.4 with NaOH. Each Erlenmeyer flask was inoculated with 5 %v/v inoculum prepared as described earlier, and after gentle mixing, was added approximately 1.0 g of dried support. During the experiment, the time of maximal cell attachment was evaluated. To determine the amount of cells attached onto the supports at different time points, eight cultures in Erlenmeyer flasks containing the same volume of medium and

cell concentration were established. The cultivation conditions were 25 °C and 120 min^{-1}. Every three hours, one Erlenmeyer flask was used to determine the amount of attached viable cells at the corresponding time point. Information of the time and conditions of maximal cell attachment was used to prepare supports for fermentation experiments.

The determination of attached cells was conducted by carefully separating the supports from the fermentation medium. The supports were gently rinsed with sterile distilled water to eliminate the weakly attached cells. The supports were subsequently placed in 0.25 L Erlenmeyer flasks containing 40 mL of Ringer solution (1/4 strength), and agitated at 120 min^{-1} for 1 hour in an orbital shaker, and then agitated vigorously at 300 min^{-1} for five minutes. After agitation, the supports were manually separated, and the liquid suspension was used to determine the viable cell concentration by the methylene blue staining method in a Neubauer chamber.

Fermentations with immobilized yeast cells

Fermentation experiments with yeasts immobilized on the supports were carried out in 0.5 L Erlenmeyer flasks containing 350 mL of pasteurized laboratory malt wort of 14°Plato prepared from a concentrated malt extract (Pilsner, Briess-CBW®). The pH of the medium was adjusted to 5.4 with NaOH. Approximately 2.0 grams of support containing attached yeast cells were aseptically inoculated into the Erlenmeyer flasks. Preparation of supports followed the methodology described previously, and after gentle rinsing with sterile water to separate the weakly attached cells, they were used as biocatalyst. Fermentation trials were performed under static conditions at 15±0.5 °C. Control experiment was implemented with suspended cells under similar conditions to evaluate the effect of immobilization in the fermentation kinetics. Initial cell concentration in this case was the equivalent amount of cells attached to two grams of dried support. Erlenmeyer flasks were stoppered with an air locker device to avoid contamination. Samples were taken every 24 h under aseptic conditions in a laminar flow cabinet, and frozen until analysis. Experiments were carried out in triplicate.

Scanning electron microscopy (SEM)

Pieces of the biocatalyst (supports plus attached cells) were gently rinsed with sterile distilled water over a sterile filter paper, and dried at 30 °C in a desiccator for two days. The supports containing immobilized yeast cells were coated with a thin gold layer by vacuum evaporation for two minutes to obtain an increase in electron conductivity. The prepared samples were studied with a scanning electron microscope (SEM).

Chemical analysis

Ethanol levels were determined by liquid chromatography (HPLC), reducing sugars by the DNS method[26], free amino nitrogen (FAN) by the ninhydrin method[27], viable cells by the method of methylene blue staining[28], and pH potentiometrically. Ethanol quantification was performed in a HPLC Agilent 1260 Infinity, provided with an autosampler, RID detector, and column type HC-75, Ca^{2+} form (305 x 7.8 mm). Samples were centrifuged (8 000 min^{-1} for 10 min), filtered, and diluted with H_2SO_4 10 mM before injection. The working conditions were column temperature, 28 °C; H_2SO_4 5 mM as mobile phase with a flowrate of 0.2 mL min^{-1}. Concentrations were determined using a calibration curve.

Statistical analysis

The data collected in triplicate served for calculating the mean value and the standard error by using Microsoft Excel.

Results and discussion

Grapefruit peel as support material

Citrus fruits such as grapefruit generate a large amount of lignocellulosic residues that could be used for some industrial applications. Grapefruit peel of the Ruby variety is a spongy material of thickness of about 2 – 3 cm, composed of flavedo and albedo (Fig. 1a), the latter being thicker than albedos of other citrus fruits such as orange and tangerine. To produce the supports, albedo must be separated carefully from flavedo, since the latter contains essential oils that could inhibit the microbial cells. The use of the whole grapefruit peel (flavedo plus albedo) was previously investigated by different researchers as adsorbent material for the decontamination of wastewaters containing heavy metals[29,30]. In this research, it is proposed for the first time as support material for immobilization of yeast cells. One advantage of using this material is its low cost in comparison to commercial polymers such as polyvinyl alcohol, κ-carrageenan, and sodium alginate, which are commonly used to immobilize cells. Grapefruit peel is mostly discarded as waste material with no practical use. Natural materials must be conditioned before using them as supports for cell immobilization in order to create micro regions allowing cell adhesion. This process is mostly carried out by partial delignification using mineral acids or alkali at different concentrations[8,14–17]. However, such treatments can be costly and time consuming. In this study, the soluble fraction was extracted by simple maceration during 24

h with 10 %v/v ethanol. After this process, the supports were dried at mild temperature, as indicated earlier. The appearance of the supports is shown in Fig. 1b.

Fourier Transform Infrared Spectroscopy (FTIR)

The pattern of cell adherence onto the treated grapefruit peel is attributable to the active groups and bonds present on its surface. The elucidation of active sites was performed by FTIR spectrophotometry. Peaks identified in the FTIR spectra of the support (Fig. 2) were assigned to various groups and bonds in accordance with their respective wavenumbers (cm^{-1}). The FTIR spectra revealed the complex nature of the support material. The absorption peak at 3328 cm^{-1} indicates the presence of free or hydrogen bonded O–H groups (represented by alcohols and carboxylic acids), which is associated to the presence of sugars, cellulose and lignin[31]. The broad mixed stretching vibration adsorption band was reduced considerably following the treatment with the ethanolic solution (Fig. 2b). This is due to the partial elimination of water-soluble compounds such as fructose, glucose, sucrose, xylose, or pectin. The peak observed at 2912 cm^{-1} is attributed to the C–H stretching vibrations of aliphatic acids[32]. The peak determined at 1735 cm^{-1} corresponds to the stretching vibration of C=O bond due to the non-ionic carboxyl groups (–COOH and –COOCH$_3$) and is assigned to carboxylic acids or their esters[33]. Asymmetric stretching vibration of ionic carboxylic groups appears at 1635/1605 cm^{-1} (Fig. 2a,b). In the FTIR spectrum, a weak peak is observed at 1365 cm^{-1}; according to other reports, it is assigned to symmetric stretching of –COO$^-$ of pectin[33]. The peak at 1260 cm^{-1} is indicative of the in-plane bending of cellulose O–H units. Additionally, the C–O band at 1014 cm^{-1} due to –OCH$_3$ group confirms the presence of the lignin structure in the support[33]. These findings indicate that the treatment with ethanolic solution had diminished the content of soluble compounds, while lignin remained undissolved. It is clear from the FTIR spectra that hydroxyl and –OCH$_3$ groups are present at high levels. The adherence of yeast cells onto the support may likely be due to the electrostatic attraction between groups of opposite charges.

Attachment of yeast cells on support materials

In aqueous solution, cells normally attach to a solid material according to its affinity to the functional groups present on its surface. Grapefruit peel is mainly composed of lignocellulose, pectin, and other minor compounds such as simple sugars. In this study, grapefruit peel was previously treated with 10 %v/v ethanol. This step was performed to
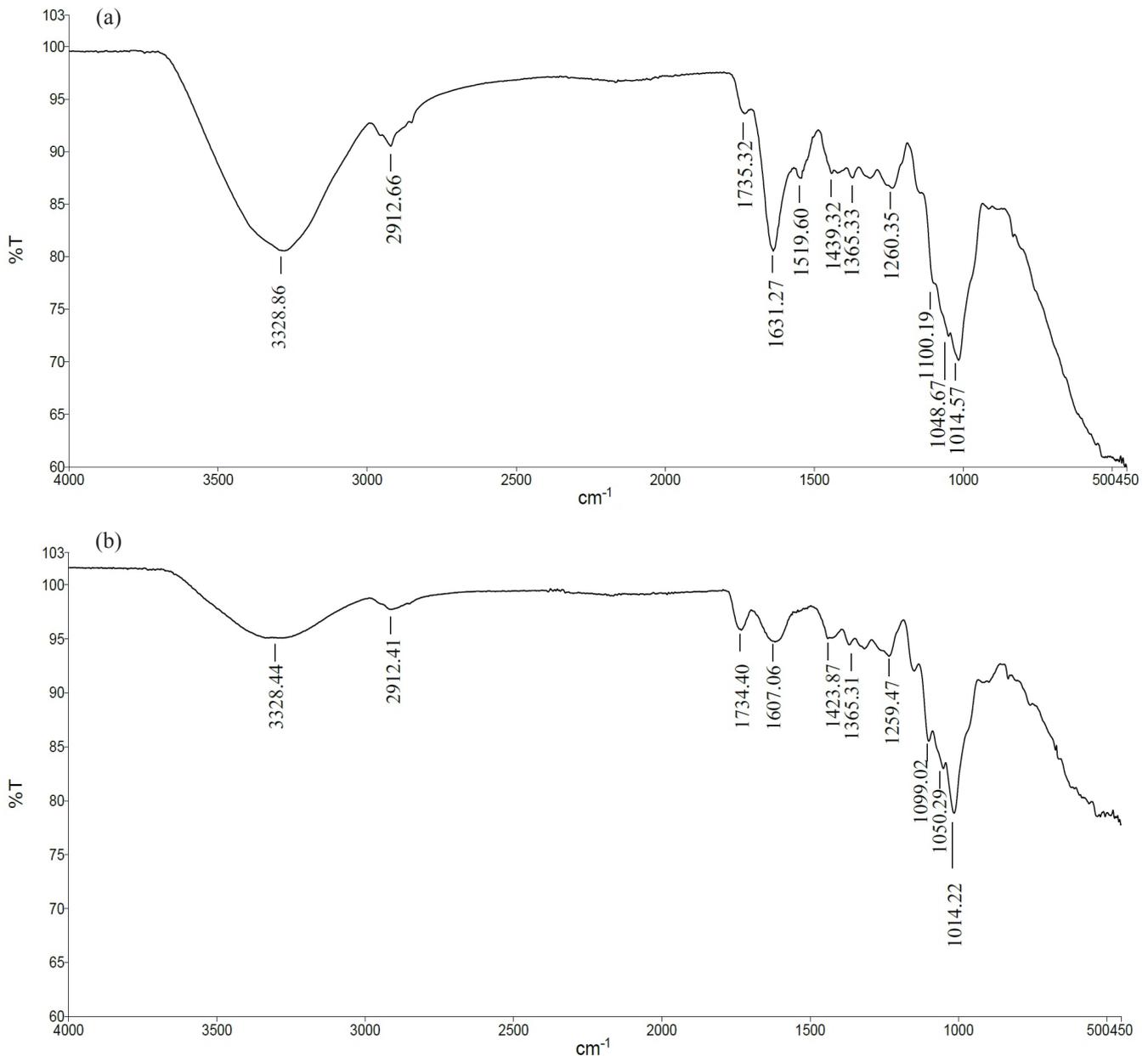
F i g . 2 – *FTIR spectra of the support material prepared from grapefruit peel before (a) and after (b) treatment with 10 %v/v ethanol*

avoid the release of most soluble compounds during alcoholic fermentation, which could affect the sensory quality of the product. After the extraction of soluble solids and conditioning, the support material was used for the immobilization of yeasts, and fermentation experiments were performed with such biocatalyst. The binding capacity of cells onto the supports depends on many factors, including medium composition, pH and temperature, the physiological state of cells, and the net charge of its surface. The dynamics of cell attachment during the fermentation process is very complex, since the cultivation conditions are continuously changing. For instance, the pH diminishes due to the production of organic acids, the ethanol content increases due to sugar fermentation and several compounds are gen-erated. Fig. 3 shows the amount of cells attached onto the support material after 22 h of cultivation. The maximal cell attachment ($2.25 \cdot 10^9$ cells/g of dried support) was reached at approximately 20 h of cultivation. After this time, the cell concentration remained almost constant.

Both the physical structure of the support material (pores and their distribution) and physiological state of cells are important factors that may influence the attachment of cells onto the material[34,35]. Thus, the growth phase may have a strong impact on the adsorption properties. It has been reported that the stationary phase is the preferred phase of growth for the attachment of *S. cerevisiae* onto support materials[36]. It is known that cells from the lag phase are usually characterized by greater sensitivi-
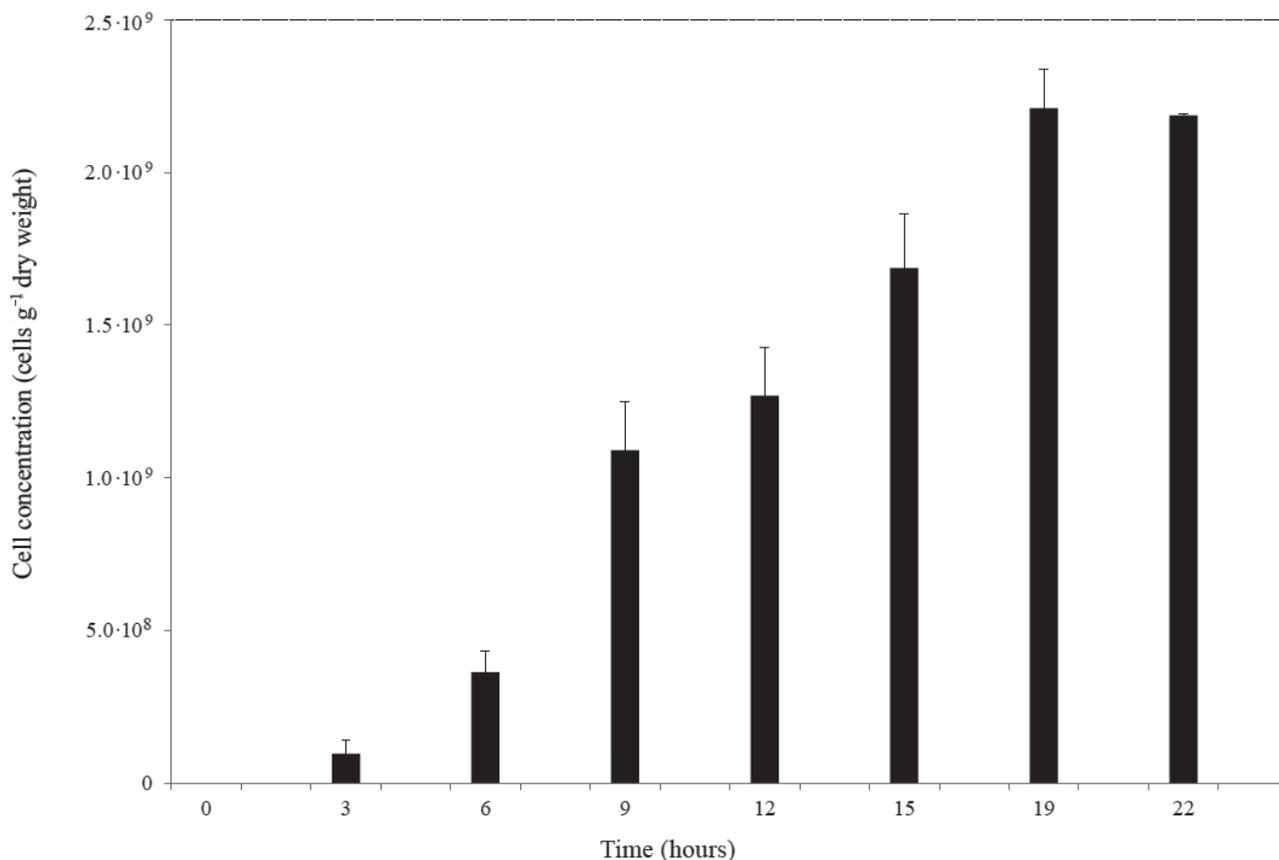
F i g . 3 – *Concentration of yeast cells attached to the support material during cultivation in 12°Plato laboratory malt wort at 25 °C*

ty to changing environmental factors, and lower resistance to stress. In our experiments, the immobilization of brewing yeasts onto the support particles was characterized by an initial slow yeast accumulation rate (lag phase), followed by an exponential-like stage of biomass accumulation.

Fermentations with immobilized yeast cells

Beer fermentation is normally carried out with free suspended cells. This fermentation practice has some disadvantages, such as the loss of cell viability along successive fermentations, a diminished yeast fermentative capability at low fermentation temperatures, the need for continuous propagation, and the successive centrifugations of the product to separate the cells. Due to these limitations, the use of immobilization techniques has been investigated. Several studies have shown that the immobilization of cells in polymeric matrixes, such as calcium alginate, improves their fermentative behaviour in terms of ethanol production and sugar consumption rate. Additionally, the cell viability is kept high and cells are easily recovered from the fermentation broth. Current investigations are focused on finding a practical use of natural supports in beer fermentation technology. Our study underpins the use of grapefruit peel as support material for immobiliza-

tion of yeast and its application in beer fermentation. The fermentation kinetics obtained with immobilized and free suspended cells are shown in Fig. 4. The analysis performed on days 1 and 2 of fermentation revealed that reducing sugar consumption and ethanol production rates were higher when immobilized cells were used (16.8 ± 0.92 g d^{-1} and 8.41 ± 0.58 g d^{-1}, respectively), compared to fermentations carried out with free suspended cells (12.6 g d^{-1} and 6.28 g d^{-1}, respectively) (Fig. 4a). Maximum ethanol productivity was attained with immobilized cells (average 6.72 g L^{-1} d^{-1}), showing the efficiency of the biocatalyst for alcoholic fermentation. Studies carried out by other authors with the same yeast strain immobilized in alginate beads have shown a higher extract consumption and ethanol production compared to fermentations carried out with suspended cells[37]. This indicates that immobilization of *S. cerevisiae* SAFLAGER S-23 had improved the yield of ethanol as a result of a higher sugar consumption. In experiments carried out with immobilized cells, the fermentation time was 4 days in comparison to fermentations with suspended cells that lasted more than 6 days (gas production had also stopped). Regarding pH changes, a higher rate of pH decrease was observed during the first two days in fermentations performed with free suspended cells (Fig. 4b). After this time, the pH pro-
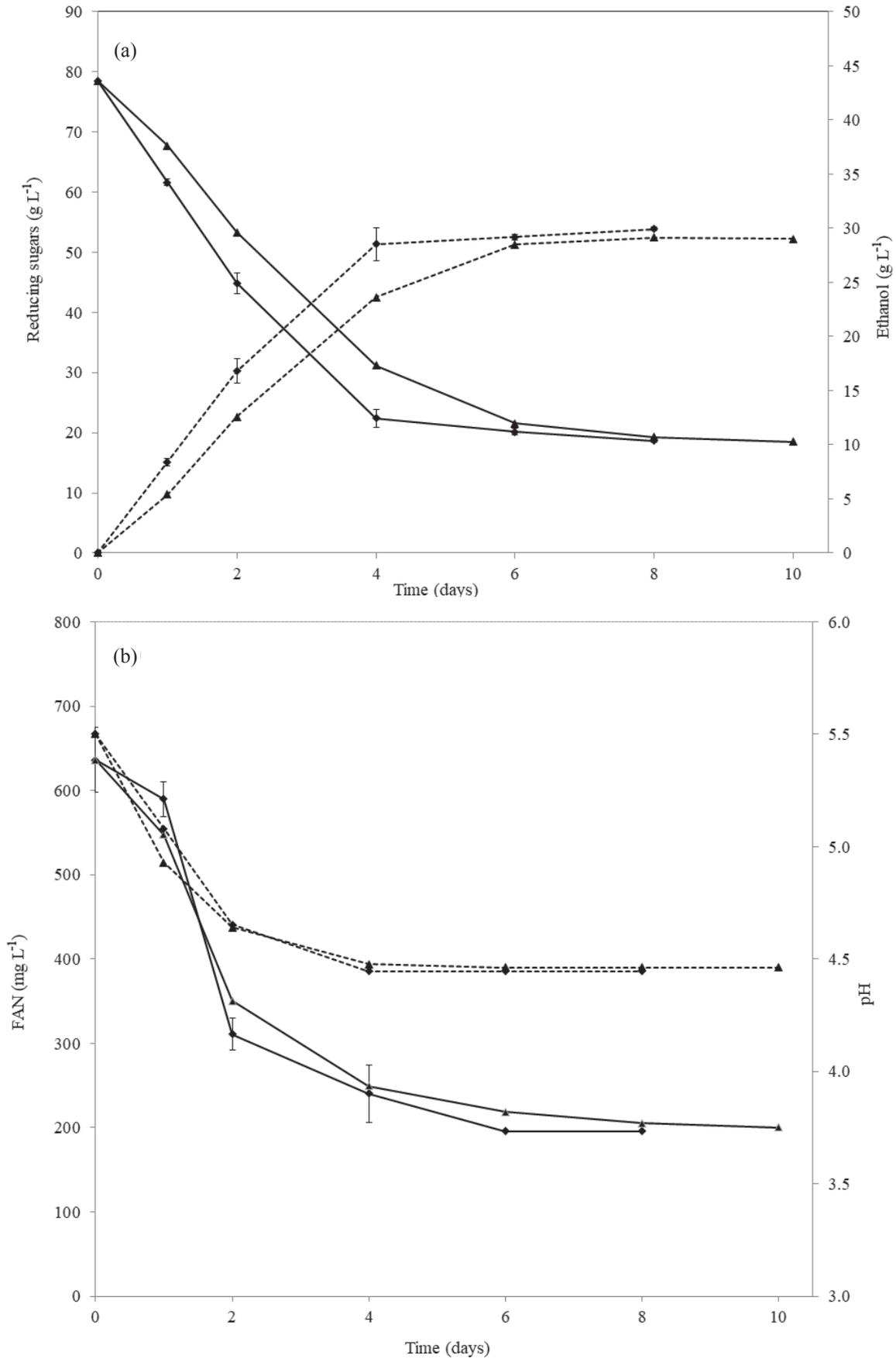
F i g . 4 – *Fermentation kinetics carried out with free (▲) and immobilized cells (♦) cultivated in 14°Brix laboratory malt wort at 15 °C. Continuous lines (reducing sugars and FAN), dotted lines (ethanol and pH). Reducing sugar consumption and ethanol production (a), FAN consumption and pH variation (b).*
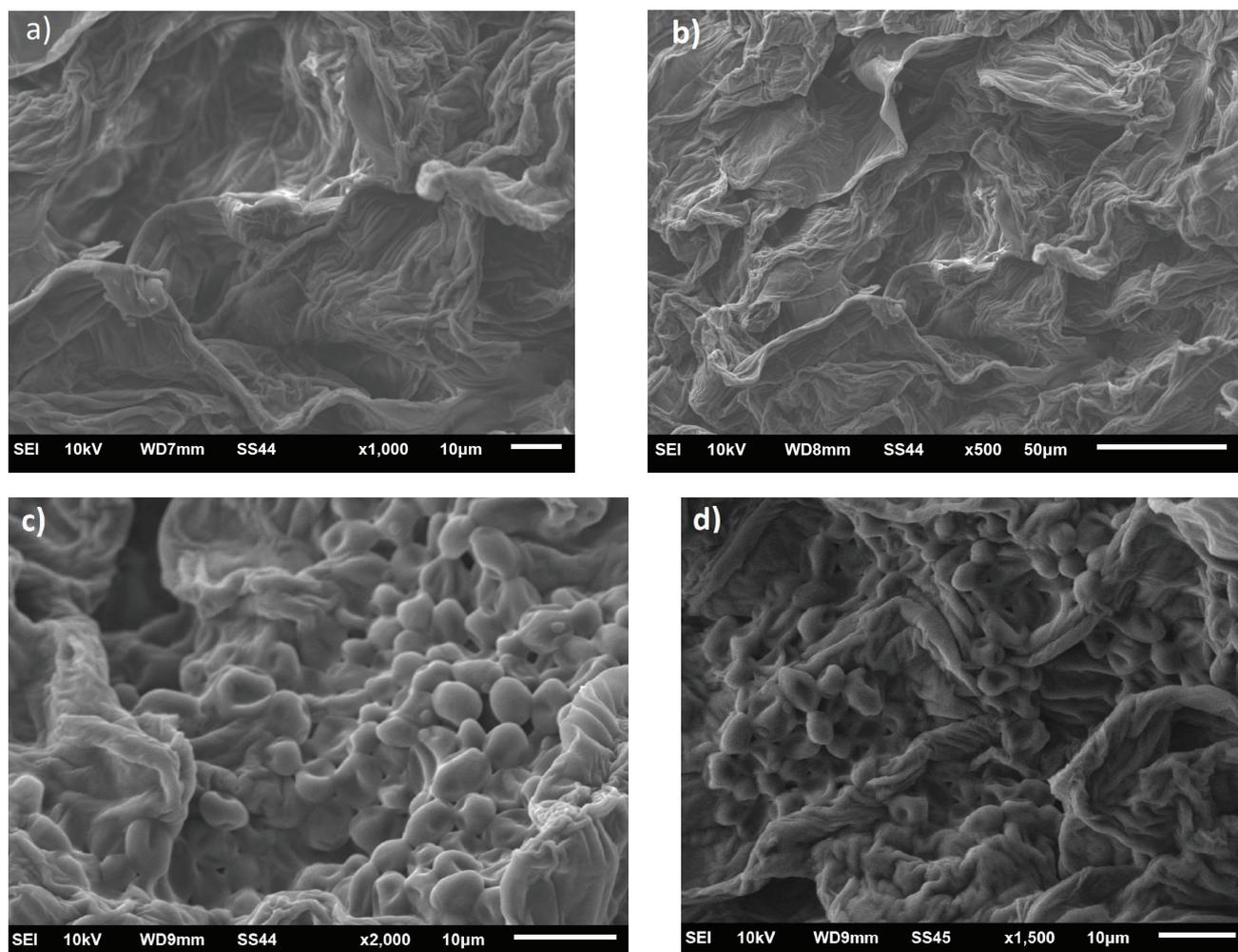
F i g . 5 – *Scanning electron microscopy of supports without use (a,b) and after three successive usages in batch experiments of beer fermentation (c,d)*

file at the end of the fermentation was similar in both cases. With respect to FAN change, a higher FAN consumption was observed during the first day in fermentations implemented with free suspended cells. However, at the end of experiments, the FAN consumption in fermentations conducted with immobilized cells was higher compared to fermentations carried out with free suspended cells (Fig. 4b).

Successive fermentation experiments performed with the same biocatalyst showed good results in terms of usage of the support and fermentative behaviour. SEM analysis of the support (after three continuous batch usages) revealed important information on the surface morphology (Fig. 5). It was observed that the immobilization had not occurred homogeneously on the support structure (Fig. 5c,d). Adhesion of yeast cells to a support material depends on complex physicochemical interactions between the cell surface, the support, and the liquid phase, and on the charge on the yeast cell surface determined by the presence of functional groups. According to literature, yeast cells are predominantly charged negatively due to the presence

of carboxyl, phosphoryl, and hydroxyl groups[38]. The existence of localized positive charges on the yeast cell surface and increased cell-surface hydrophobicity also participates in the cell adhesion process[39–41]. As seen in Figs. 5a and 5b, the support material has cavities in its structure, and the cells can be found attached inside the cavities. As mentioned earlier, yeast cells adhere to the surface because of either natural entrapment into the porous cellulosic material or due to physical adsorption by electrostatic forces. An effective immobilization of yeast cells was established by the ability of the biocatalyst (after gently washing to remove the weakly attached cells) to perform repeated batch fermentations efficiently (three continuous repetitions) using fermentation medium of the same composition (14°Plato laboratory malt wort) at 15 °C. Fermentation times were short, indicating that the biocatalyst required no adaptation time in the fermentation medium.

The main risk in fermentations carried out with yeasts immobilized onto natural supports is the detachment of some cells due to the relatively weak

interaction of the cell with the support in a changing environment in terms of ionic strength, pH, medium composition, and mechanical stress[42]. During fermentation, the formation of free biomass (data not reported) was observed, which could have contributed to substrate consumption. Nevertheless, the fact that immobilized cells can be recovered easily from the medium and be reused in subsequent fermentations generates an attractive advantage that should be validated through future evaluations comprising a repeated batch fermentation approach.

Conclusions

The results of FTIR data indicate that the conditioned support material has a high amount of hydroxyl (–OH) and methoxy (–OCH$_3$) groups, which are related to the presence of lignin and cellulose. The conditioning of grapefruit peel with a 10 %v/v ethanolic solution is an important step in eliminating soluble compounds released during fermentation that have a negative impact on the sensory characteristics of the final product. Under the tested conditions, the maximal cell adhesion ($2.25 \cdot 10^9$ viable cells/g dried support) was reached after 20 h of cultivation. At further time points, the concentration of adhered cells remained constant, suggesting that pseudo-equilibrium between cell adhesion and detachment had been established.

The grapefruit peel keeps its physical integrity along successive fermentations, as confirmed by SEM observation and a high adhesion capability of viable cells. From the fermentation kinetics point of view, immobilization improved the fermentative behaviour of the yeast strain tested in terms of ethanol production and reducing sugar consumption rates, as compared to fermentations conducted with free suspended cells. Therefore, grapefruit peel is proposed as an attractive biocatalyst support for beer fermentation. From the sensory point of view, more studies are needed to evaluate the impact of the immobilization of yeasts in the production of compounds related to the sensory quality of the product.

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