Biocatalysis and Agricultural Biotechnology 22 (2019) 101392

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: http://www.elsevier.com/locate/bab

Use of discards of bovine bone, yeast and carrots for producing second generation bio-ethanol

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ARTICLE INFO

Keywords:
Valorization
Discards bovine bone
Yeast immobilization
Second generation bioethanol

ABSTRACT

The reutilization of agroindustrial wastes for the production of new products is an attractive option for reducing environmental pollution, generating value-added products and decreasing costs. Slaughterhouse discard bovine bone, brewery discard yeast and carrot discards from the Santa Fe province (Argentina) were used for producing ethanol. Bovine bone and yeast provided the support and active phase for fermentation biocatalysts while carrot discards were a sugar source. Discard bovine bone was collected directly from a slaughterhouse and then subjected to extraction with acetone for removing lipids and blood traces. Both uncalcined and heat-treated bovine bone (calcined for 2 h at 400, 600 and 800 °C) were studied. Uncalcined bovine bone had a suitable pore structure for yeast immobilization and a suitable mechanical resistance for being used in stirred tanks.

Two different strains of *Saccharomyces cerevisiae* were immobilized and tested. One was a brewery yeast discarded from a local beer brewery and the other one a commercial yeast. The biocatalysts thus obtained were subjected to a thermal treatment at 35 °C for 72 h in order to dehydrate cells and form adhesins. The activity of the biocatalysts was tested in the fermentation of carrot discards. The maximum concentration of the obtained ethanol was 11.98 g L^{-1}. It was possible to reuse the biocatalysts for 11 reaction cycles. The possibility of regenerating the biocatalyst bone support by ultrasound is an advantage of this kind of material in comparison to common gel supports.

1. Introduction

In the last years the use and valorization of the waste or by-products of diverse agricultural and industrial practices has been a topic of great interest in the scientific community. The accumulation of this type of waste in large quantities results not only in a deterioration of the environment but also in a loss of materials which can be processed to generate value-added products.

In Santa Fe, Argentina, bovine production is one of the most important agroindustrial activities. Two million ton of bovine cattle are slaughtered annually, generating 270 ton of bovine bone daily which are mainly used for producing bone meal for animal consumption (SENASA, 2016). The rest is disposed in open dumps or landfills causing an environmental impact.

Bone is a relatively cheap material that meets some of the desired characteristics of a good support for immobilizing cells and enzymes such as porosity and mechanical resistance (Clementz et al., 2016). Mechanical and stiffness properties of bone are due to its structure, being composed of about 65–70% hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$). The remaining 30–35% (on a dry basis) is attributed to organic compounds, mainly collagen which provides a site for the growth of HAP crystals and gives the bone tensile strength, elasticity and flexibility (Barakat et al., 2009).

The immobilization of cells or enzymes is one of the most effective tools used in the industry for an effective use of biocatalysts (Ismail et al., 2015). Compared to free biocatalysts systems, immobilization techniques offer many advantages (Moreno-García et al., 2018). A widely used immobilization method is based on the physical adsorption of cells or enzymes over solid carriers (Mohd Azhar et al., 2017). Many literature reports on the use of waste materials as supports for immobilization can be found, e.g. sorghum bagasse (Yu et al., 2007), orange peel (Plessas et al., 2007) and cassava tuber fibers (Kunthiphun et al., 2016). Physical properties of the support such as porosity and internal surface area have an important role in the immobilization process (Mohd Azhar et al., 2017).

In a previous paper, we analyzed the possibility of using discard

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https://doi.org/10.1016/j.bcab.2019.101392
Received 5 June 2019; Received in revised form 8 September 2019; Accepted 17 October 2019
Available online 19 October 2019
1878-8181/

bovine bone as a support for enzyme immobilization after removing the lipids and calcining at 600 °C. The new biocatalyst was active in the transesterification of olive oil (Clementz et al., 2016).

In this work the feasibility of using bovine bone discards as a support for yeast was studied. Two different strains were immobilized: *Saccharomyces cerevisiae (Scc)* commercial yeast and *S. cerevisiae CCUB (ScCCUB)* brewery yeast discarded from a local beer brewery. In Santa Fe city (Argentina) 1–1.5 ton of discards from yeast cells is generated daily by the beer manufacturing industry. Nowadays the elimination of these discard has not been solved due to its high oxygen biochemical demand (Aimaretti and Ybalo, 2012).

The activity of the biocatalyst was evaluated in a fermentative process that employed sugars from carrot discards as a substrate. In the same geographical area about 80–100 ton of this crop, about 30% of the total production, are discarded every day during the harvest because of carrots not meeting commercial standards with regard to size or shape. These discards are a good substrate for ethanol production because they can provide the necessary ions and the appropriate carbon-to-nitrogen ratio for the fermentation process (Aimaretti and Ybalo, 2012). Recent research work by our group reports the use of carrot discards as a raw material for fermentation, using free yeast and yeast immobilized on calcium alginate (Clementz et al., 2015).

The objectives of this work were to assess the properties of discard bovine bone as a support for biocatalysts and the possibility of its reuse and regeneration. Results are included on the physicochemical characterization of the support and the tests of activity, selectivity and stability of the new immobilized biocatalyst. An analysis of the immobilization process and the results of support reuse and regeneration are also presented.

2. Materials and methods

2.1. Microorganism

Two different strains were employed, *Saccharomyces cerevisiae CCUB (ScCCUB)* brewery yeast discarded from a local beer brewery and *S. cerevisiae (Scc)* commercial yeast provided by a local supplier (Calsa, Argentina). These two strains, available as pressed yeasts, were directly reactivated in carrot juice. Cells were kept in a sterile container at 4 °C.

2.2. Fermentation substrate

Carrot discards (Daucus carota) were collected from a packing shed of the Garay department area (31°25′S, 60°20′W, Argentina). As for handling and storage, the method described by Aimaretti and Ybalo was used (Aimaretti and Ybalo, 2012). Carrots were selected leaving aside those parts affected by microorganisms. Then their juice was extracted by continuous milling, compressing and filtering. Particulates in the carrot juice were separated by centrifugation at 3500 rpm for 10 min. The supernatant, carrot must, was labeled as CM. Its average sugar content was 89.8 ± 1.2 g L-1 and its pH was 6.4 ± 0.2. CM was not subject to sterilization.

2.3. Supports

Discard bovine bone was collected directly from the slaughterhouse and then subjected to a chemical treatment with acetone in order to remove lipids and blood traces. An autoclave reactor was used for treating the bone. The reactor had a volume of 7000 mL, temperature and pressure automatic controls and a stirrer. Extraction conditions were set at 50 °C, 7 bar, 300 rpm stirring rate, nitrogen atmosphere and 3 h treatment time. Finally the material was dried overnight at 50 °C to remove residual solvent. The sample thus obtained was called BB. A portion of BB was calcined in a muffle for 2 h at 400 °C, 600 °C and 800 °C. The samples thus obtained were called BBC400, BBC600 and BBC800, respectively. The calcination was carried out in order to remove organic components that remained occupying the bone pore space after the chemical treatment, thereby generating void volume. Supports were milled to an appropriate particle size (4 mm^2) using an Armfield blade grinder.

2.4. Support characterization

The nitrogen content in the bovine bone was determined by the Kjeldahl method AOAC 2001.11 (AOAC, 2000). The sample was digested in a Technicon II digester for 4 h and then finally distilled. The crude protein content was calculated as the nitrogen content multiplied by 6.25.

The lipid content was obtained by extraction with hexane (1:4 wt wt^{-1} ratio) in a Soxhlet extractor. The extraction was performed for 6 h until the extracted hexane was colourless. Hexane was finally removed in a rotary vacuum evaporator.

Supports were characterized by means of nitrogen adsorption at −194.6 °C. Before the adsorption, the bone samples were degassed under vacuum at 200 °C for 2 h. Adsorption isotherms were measured in an Automatic Micromeritics equipment. The specific area and pore size distribution were calculated with the BET (Brunauer, Emmett and Teller) and the BJH (Barrett-Joyner-Halenda) methods, respectively (Joyner et al., 1951). The method experimental error was 1%.

Infrared spectra were obtained with a Shimadzu 8101 M FTIR spectrometer. Samples were prepared in the form of pressed wafers (ca. 1% sample in KBr). All spectra involved the accumulation of 80 scans at 4 cm^{-1} resolution.

2.5. Immobilization process

3 g of pressed yeast were suspended in 40 mL of distilled water. Then 1 g of sucrose and 3.5 g of treated bovine bone were added. The mixture was gently stirred at 200 rpm and 30 °C for 1 h by means of a laboratory hot plate/stirrer. The immobilized biocatalyst was subjected to a thermal treatment at 35 °C for 24 and 72 h, following the technique of Rapoport et al. (2011). Finally it was washed with 1 L of distilled water in order to remove the cells that were not adsorbed.

2.6. Control of adsorption process

To assess the degree of immobilization of the cells the biocatalysts were subjected to a leaching test in an ultrasound water bath (TestlabTb02) for 2 min (White and Walker, 2011). The detached cells were measured using a Neubahuer counting chamber. The final result was expressed in number of cells per unit gram of support.

2.7. Fermentation conditions

CM was used as it was obtained, without any adjustment in sugar levels or initial pH, addition of nutrients, or sterilization treatment. The inoculum was adjusted to a value of 10^8 cell mL^{-1}. Batch fermentation was performed at 30 °C, and using 250 mL flasks. The flasks were placed on a hot plate/stirrer at 200 rpm and with controlled heating. The fermentation progress was monitored by measuring the CO_2 production with a gasometric test probe (Aimaretti et al., 2012). When fermentation was complete, samples were taken and centrifuged for 10 min at 5000 rpm. The supernatant was transferred to sterile tubes for storage and then kept at −18 °C for further use. All experiments were performed in triplicates.

2.8. Regeneration of the biocatalyst

After the fermentation process the adsorbed yeasts were removed from the support by treatment in an ultrasound water bath (TestlabTb02) for 5 min. The effectiveness of treatment was evaluated measuring detached cells using a Neubahuer counting chamber. Then,

support was subject to a new immobilization process of yeasts.

In order to evaluate the effect of this regeneration technique on the properties of the bone structure, two samples were analyzed. Sample A was bone employed in five successive fermentation tests and then finally treated with ultrasound. Sample B was bone used in eight successive fermentation tests with ultrasound treatment after each fermentation.

2.9. Analytical methods

2.9.1. Sugar concentration

The concentration of total sugar was measured by the 3.5-dinitrosalicylic acid (DNS) method after acid hydrolysis (1.2 M HCl, at 65 °C for 15 min), neutralization with 1 M NaOH, and filtration (Yu et al., 2009).

2.9.2. Ethanol concentration

Ethanol concentration was measured by gas chromatography in a Shimadzu GC 2014 apparatus equipped with a ZB-Wax capillary column. Isopropanol was used as internal standard. The column temperature was 40 °C (isothermal), the injector and the detector were kept at 220 °C.

2.9.3. Fermentation parameters

The following fermentation parameters were calculated to compare the responses of different assays: (1) $Y_{p/s}$, ethanol yield per substrate, the ratio of total ethanol produced to the amount of consumed sugar, [g g^{-1}]; (2) $Y_{p/x}$, ethanol yield per cell, the ratio of total ethanol to the total biomass [g g^{-1}]; (3) Productivity, the ratio of total alcohol production to the total fermentation time [g L^{-1} h^{-1}]. In all experiences, the total time for fermentation was 6 h.

2.10. MATS method (microbial adhesion to solvents)

The MATS method evaluates the hydrophobic, hydrophilic, acid and basic properties of the surface of the microbial cells. The test compares the affinity of the cells for monopolar/non-polar solvent pairs of equal surface tension of the Lifshitz-van der Waals type. The solvent pairs used were (chloroform, Cicarelli, pro analysis, acid solvent/hexadecane, Cicarelli, pro analysis) and (ethyl acetate, Merck pro analysis, strongly basic solvent/decane, Merck pro analysis).

The electron-donor or electron-acceptor properties of the cellular surface can be determinate comparing the differences between the affinity of the yeast for the chloroform/hexadecane pair or the ethyl acetate/decane pairs. The hydrophobic characteristics correlate with the affinity for the non-polar solvents. Though the method was originally described for bacteria (Mortensen et al., 2005), have adapted it for measuring the affinity of yeasts for different solvents.

The MATS test was applied in this work to yeast suspensions of 4×10^7 cells mL^{-1} (corresponding to an optical density of 0.8 as measured at 400 nm) in a 150 mM NaCl solution (Cicarelli, pro analysis). Initially 2.4 mL of the cell suspension were transferred to an assay tube together with 0.4 mL of solvent. Then the mixture was subjected to vigorous stirring with vortex equipment for 1 min. Then it was left to rest for 15 min in order to get a suitable separation of the phases. Finally 1 mL of the aqueous phase was transferred to a cell for measuring the optical density at 400 nm. The percentage of cells taken by the solvent is measured according to the following formula:

$$\text{Affinity } \% = (1-(A_o\ A^{-1})) \text{ x } 100$$

Where

A_o is the optical density of the sample at 400 nm before adding the solvent.

A is the absorbance of the sample at 400 nm, after adding the solvent. The absorbance was measured in a Cary 50 UV–vis spectrophotometer.

Table 1
Surface area (Sg BET), pore volume (Vp) and mean pore diameter (Dp Wheeler) of the supports.

Support	Dp wheeler (Å)	Sg BET (m^2g^{-1})	Vg (mL g^{-1})
BB	106.70	4.90	0.013
BBC400	100.80	114.14	0.288
BBC600	135.70	64.90	0.220
BBC800	79.90	1.82	0.004

2.11. Determination of yeast diameter by optical microscopy

The yeast diameter was observed through the 40X objective of an optical Leica DME microscope coupled to a Leica DC 300 camera (Leica Microsystems Inc., Nussloch, Germany).

2.12. Scanning electron microscopy

The external surface of bovine bone and of the immobilized biocatalyst were coated with gold and examined in a JSM-35C JEOL scanning electron microscope (SEM).

3. Results and discussion

3.1. Physicochemical characterization of the supports

The amount of adsorbed cells is often limited by factors related to the structure of the support such as surface area, pore shape and size (Vucurovic et al., 2009). The pores must have an adequate size that allows the access of yeast and substrate as well as the elimination of fermentation products. The discard bone obtained directly from the slaughterhouse originally contained 22% of proteins and 26% of lipids. After the chemical treatment with acetone, the lipid content was reduced to <0.5%, whereas the protein content did not vary. These results would indicate that the chemical treatment is effective for the removal of lipids inside the pores of the bone but it is not effective for removing the collagen, the main protein component.

In order to improve the efficiency of the immobilization process, a portion of BB was calcined at different temperatures. The obtained results of surface area, pore volume and mean pore diameter of all supports are shown in Table 1.

According to the results, the surface area and the pore volume increased 23 and 22 times, respectively, when the bovine bone was calcined at 400 °C. The heat treatment had one main effect, the degradation of the organic matter, which generated a greater porosity and increased the internal surface. However at calcination temperatures higher than 600 °C, both the porosity and the surface area were decreased. This could be caused by a sintering process happening at high temperatures that produced the collapse of the bone pores. Due to the poor textural properties the BBC800 sample was not used in subsequent assays.

3.2. Selection of immobilization supports

In order to select the immobilization support, two properties were evaluated, percentage of adsorbed cells and percentage of matter loss, both on a mass basis. The matter loss is related to the generation of powder during the incubation process as a result of the attrition of the

Table 2
Percentage of adsorbed cells and generated powder due to the attrition of the supports.

Support	Adsorbed cells (cells. g of support^{-1})	Generation of fines (%)
BB	3.57×10^8	10%
BBC400	5.78×10^8	23%
BBC600	6.12×10^8	30%

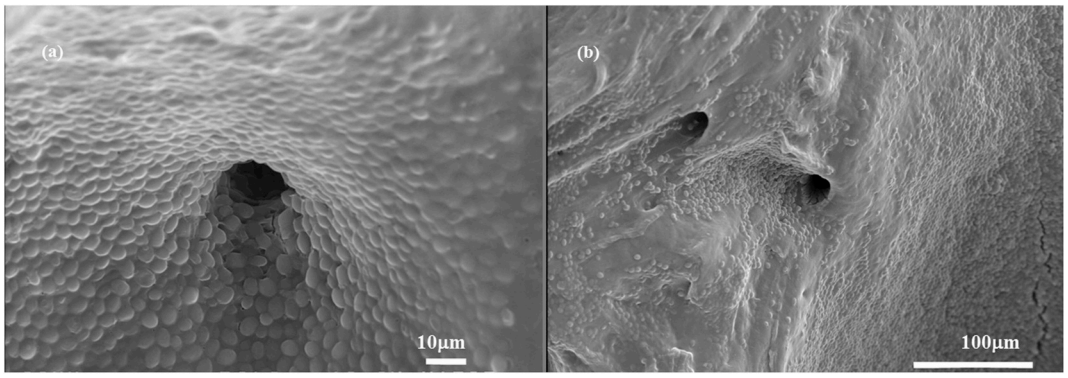

Fig. 1. Electronic micrographs of *S. cerevisiae* immobilized inside bovine bone: (a) yeast in a pore of BB, (b) yeast on the external surface of BB.

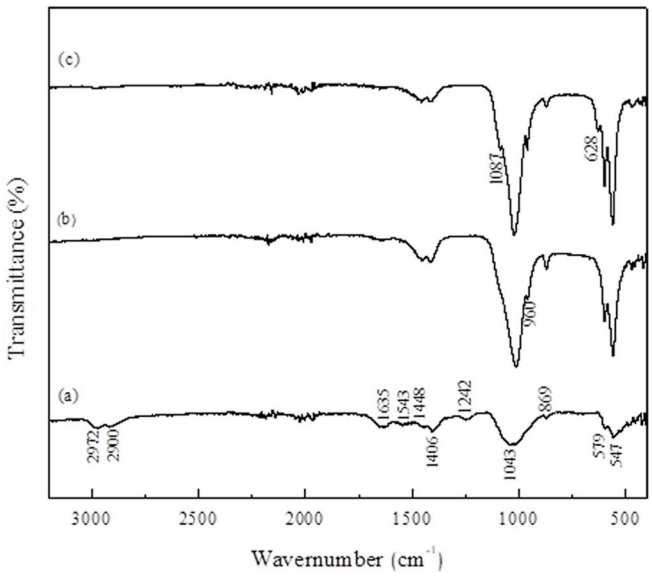

Fig. 2. FTIR spectra: (a) BB, (b) BBC400, (c) BBC600.

bone particles under stirring. The obtained results are detailed in Table 2.

In agreement with the exposed results the amount of adsorbed cells is roughly similar for the three supports. All have the same order of magnitude in spite of the supports having very different surface areas (Table 1). This phenomenon indicates that there is no direct relationship between the total surface area of the support and the amount of adsorbed yeast. Another property must therefore be influencing the immobilization process.

The mean diameters of the two types of yeast were determined by optical microscopy. The results show that the diameter of *SsCCUB* was 8.73 μm while the diameter of *Scc* was 6.23 μm. As a consequence the meso and micropores of the supports should not be suitable for yeast immobilization. The immobilized biocatalysts were analyzed by SEM. The images obtained (Fig. 1) indicate the presence of macropores big enough to allow the yeast to be immobilized there. It seems obvious that for the immobilization process to be carried out efficiently an appropriate size of the pores is a necessary condition.

In addition to an adequate internal area and pore size, a good support must also have an adequate mechanical, thermal, chemical and biological resistance. Table 2 shows that the % of fine powder generated during stirring is increased at higher temperatures of calcination of the bone. The decrease of the mechanical strength of the supports upon calcination must be due to the removal of the collagen. This protein holds an important role on bone strength (Viguet-Carrin et al., 2006). In order to evaluate the loss of collagen in the supports after the calcination process, FTIR analysis of the samples was performed (Fig. 2).

Table 3
Biocatalysts incubated at 35 °C for different time lengths. Number of cells immobilized per gram of support and concentration of the obtained ethanol during three reaction cycles.

Yeast	Time of thermal treatment (h)	Number of cells immobilized (cells. g of support^{-1})	Ethanol concentration (g L^{-1})		
			Cycle 1	Cycle 2	Cycle 3
Scc	24	3.57×10^8	8.62	4.52	3.20
Scc	72	3.27×10^8	8.22	5.15	7.09
ScCCUB	72	1.34×10^7	0.35	-	-

The collagen is a protein that can be detected by FTIR because of infrared absorption in three spectral regions related to vibrations of the amide group. These vibrations are called “Amide I”, “Amide II”, and “Amide III” (Barakat et al., 2009). Bands related to Amide I can be found at 1620, 1636, and 1685 cm^{-1}. Amide II band has a related major peak at 1559 cm^{-1} and minor bands at 1521, 1533, and 1543 cm^{-1}. Amide III has characteristic peaks at 1231, 1248, and 1281 cm^{-1}. According to the results shown in Fig. 2, all these peaks are present in the spectra of BB and absent in the calcined samples. The results confirm that collagen was eliminated during calcination. Therefore the loss of the mechanical strength of the BBC400 and BBC600 supports and the consequent generation of fines can only be related to the removal of this protein. On the light of these results, BB was selected to continue with the different tests, because it has a higher mechanical strength, and its capacity to retain cells was similar to that of the other two supports.

3.3. Thermal treatment of immobilized biocatalyst

After incubation the biocatalyst was washed with water. Most of the yeasts were removed during this washing step, indicating that adsorption of the cells on BB was weak. The process of immobilization of the cells is determined by the surface properties of both the cells and the carrier (Kregiel et al., 2012). Therefore a change of the adsorption technique seemed necessary for improving the immobilization. Rapoport et al. (2011) have reported that the desiccation of cells at conditions similar to natural ones is accompanied by rather serious changes in cell surface structures which would not affect cell viability. These changes also include alterations of mannoproteins of the cell wall, changes in the cell surface charge, and the formation of big cell conglomerates. In order to analyze the effect of dehydration during the thermal treatment, the immobilized biocatalyst was incubated at 35 °C at different time spans between 24 and 72 h. Then it was employed in consecutive fermentative cycles. The results are shown in Table 3.

Aimaretti and Ybalo (2012) have reported that the ethanol

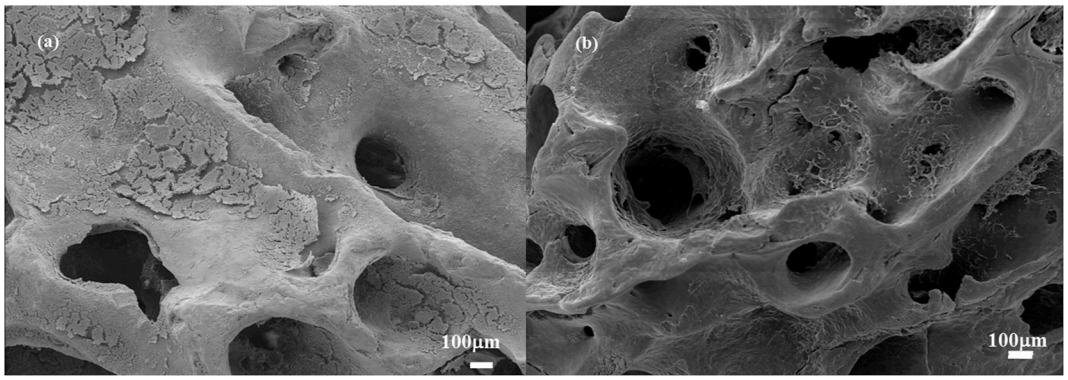

Fig. 3. SEM images of the immobilized biocatalyst and the generated biofilms. Magnification 48x. (a) After 24 h of thermal treatment. (b) After 72 h of thermal treatment.

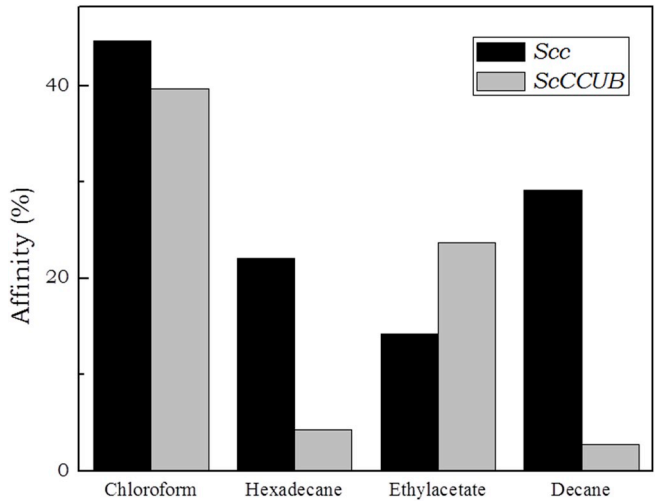

Fig. 4. Analysis of the affinity of *Scc* and *ScCCUB* strains for different solvents (chloroform, hexadecane, ethylacetate, decane) using the MATS method.

production rate is directly proportional to the biocatalyst concentration. Therefore for a fixed reaction time (4 h) the concentration of alcohol is related to the amount of cells in the biocatalyst. When the length of the thermal treatment was 24 h (Table 3) the obtained ethanol concentration decreased along the fermentation series, thus indicating the loss (desorption) of yeasts. When the time of incubation was 72 h there was a little cell desorption loss in the second fermentation cycle, but the ethanol concentration was recovered in the third cycle. This behavior indicates a stronger interaction between the cells and the support.

The cell adsorption process occurs in four stages (Berlowska and Kregiel, 2013). The first two involve interactions between the cell, the surface and the liquid phase (hydrophobic, electrostatic or van der Waals interactions). The third stage of the process is associated with the production of extracellular polymers: glycoproteins, adhesins, flocculins, hydrophobins or other proteins, in response to different external stresses. If the conditions are favorable, adsorbed cells grow, get divided and develop colonies which in turn build complex surface communities called biofilms (last stage) (Mathew et al., 2013). The four stages can be present or not in the adsorption process, depending on the microorganism being immobilized and the kind of material used as support. The increment of the ethanol production in cycle 3 (Table 3, second row) may be due to the growth of biomass on the surface. Probably the yeast generated adhesins in this step in response to the temperature treatment. SEM images were taken from the biocatalyst after 24 and 72 h of thermal treatment (Fig. 3). The images show that after 72 h a biofilm multi-layered structure was developed. The biofilm attached the cells to the support thus allowing their proliferation. Moreover 72 h of treatment seemed necessary to prevent desorption of the yeast.

Despite the thermal treatment, *ScCCUB* did not adsorb on the support. It can be seen in Table 2 that a small amount of yeast is retained on the support after washing. This may be due to a low electrostatic or hydrophobic affinity between the surface of BB and the cells.

3.4. MATS results

The MATS method was used in order to elucidate the reason for the difference in adsorption of the different strains. The obtained results are plotted in Fig. 4.

The results indicate that the two strains have different hydrophobicity and donor-acceptor electronic properties. The electron-donor and electron-acceptor properties of the surface cells are correlated with the affinities for the chloroform:hexadecane and ethyl acetate:decane pairs. In the case of *ScCCUB,* the affinity difference between the first solvent pair is greater than ethyl acetate:decane pair. This would point to an electron-donor behavior. This property was found to be present in both strains but it was stronger in *ScCCUB*. Electron-donating activity could be attributed to the presence of different groups such as carboxyls, phosphoryls and hydroxyls in the surface cell (Kregiel et al., 2012). According to the MATS method, the most hydrophobic strain is that has a high affinity for non-polar solvents. According to the results (Fig. 4), this would be the case of the *Scc* strain.

The incubation process was performed at pH 4.5, under this value the bone has a positive surface charge (data not shown), and the strain-support interactions should be attractive. Under these conditions, the strain which has electron-donor properties should be easily adsorbed, however *ScCCUB* is the less adsorbed. This result could be indicating that another mechanism, different to electrostatic attraction, would be the responsible of immobilization process.

According to literature reports (Liu et al., 2004; Kang and Choi, 2005) an increase of the hydrophobicity of the cell surface allows an easier approach of the cells to the support, and in consequence, activate the forces responsible for adsorption. On the basis of the results obtained, the main force responsible for adsorption of the strain in bone is controlled by the hydrophobicity/hydrophilicity forces and not by the electrostatic interactions.

3.5. Optimization of the immobilization over HB

The effect of increasing the cell load in the initial immobilization solution (0.0125 g mL^{-1} – 0.1 g mL^{-1}) was studied employing approximate 3.5 g of support. The biocatalysts were incubated for 1 h and thermally treated at 35 °C for 72 h. The results are shown in Fig. 5. It can be seen that when the yeast concentration in the initial water solution was increased, the amount of cells adsorbed increased to 0.075 g mL^{-1}

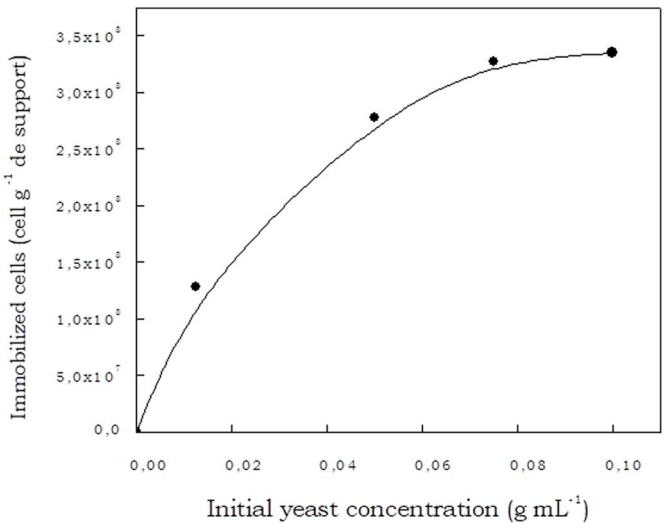

Fig. 5. Effect of initial yeast concentration on the amount of immobilized cells.

Table 4
Stability of the immobilized biocatalyst in successive cycles of fermentation.

Cycle	Ethanol (g L-1)	Productivity (g L-1 h-1)
1	9.32	1.55
2	8.97	1.50
3	9.71	1.62
4	9.90	1.65
5	10.08	1.68
6	11.14	1.86
7	11.98	1.99
8	11.93	1.99
9	11.29	1.88
10	8.60	1.43
11	8.64	1.44

and then remained constant, probably because of surface saturation. This value was thus selected as optimal.

3.6. Stability of the immobilized biocatalyst in successive cycles of fermentation

Successive batch fermentations employing fresh must were carried out to determine the stability of cells immobilized in bovine bone. Values of ethanol production and productivity are included in Table 4. The results shown in Table 4 indicate that both the ethanol production and the productivity increase in successive cycles. Then, between cycles 6 and 9, these parameters are constant and finally decrease. The increment of both the ethanol production and the productivity after cycle 5 is explained by the growth of biomass on the support. The SEM image of the biocatalyst shows a high microbial load, with formation of biofilms after several reuses (Fig. 6). The presence of this complex structure probably negatively affects the mass transfer of the nutrients (Mathew et al., 2013), causing the fall of the fermentation production and productivity after cycle 9.

Table 5 contains reported values of the total ethanol production and productivity for different biocatalysts comprising *Scc* unsupported (free cells) and on a different supports using carrots discards as fermentation substrate, in order to compare the performance of the developed biocatalysts with that of other reported by our group. The values of ethanol concentration and productivity reported in a recent report using yeast immobilized in calcium alginate and using carrots discards as fermentation substrate (Clementz et al., 2015), were 29.9 g L^{-1} and 7,5 g L^{-1} h^{-1}, respectively. Similar results were reported in a previous paper (Aimaretti and Ybalo, 2012/ce:cross-ref>) using free cells. Although these values are higher than those obtained using bovine bone as support, it was possible to use the calcium alginate beads for only 2 cycles and then they were destroyed due to the loss of the mechanical resistance of the gel (Razmovski and Vucurovic, 2012). In this sense, discard bovine bone not only allows the reuse for a greater number of times, but also allows for successive regeneration and re incubation (see section 3.6). Similar results were reported by Rakin et al. (2009) where the strain *S. cerevisiae var. Ellipsoideus* was immobilized onto two types of materials: beads of calcium alginate and polyvinyl alcohol (PVA). Using the first support they obtained the highest ethanol concentration. The alginate beads were however reused only in two fermentation tests, while yeasts adsorbed on PVA were reused in five fermentation cycles.

3.7. Regeneration of the biocatalyst

The possibility of regenerating the support for subsequent reincubation and use, is an important advantage of the studied materials over

Table 5
Comparison of the performance of different biocatalyst.

Reference	Biocatalysis	Ethanol production (g L^{-1})	Productivity (g L^{-1} h^{-1})	Reuse
Aimaretti (2012)	Free *Scc*	37.1	7.2	None
Clementz (2015)	*Scc* immobilized in calcium alginate	29.9	7.35	Twice
This paper	*Scc* supported on treated bone	11.98[a]	1.99[a]	Eleven times

[a] Maximum value corresponding to a reuse of seven times.

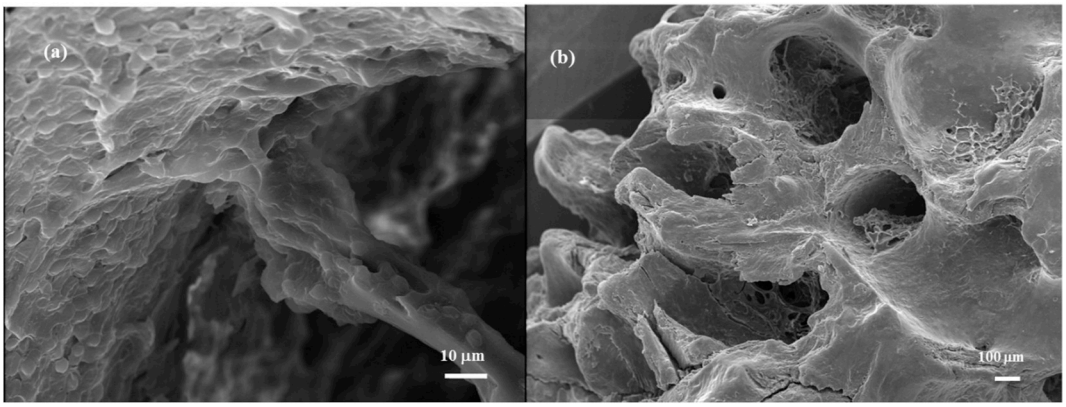

Fig. 6. SEM images of the biocatalyst after 11 cycles of reaction. (a) Magnification x1000. (b) Magnification x48.

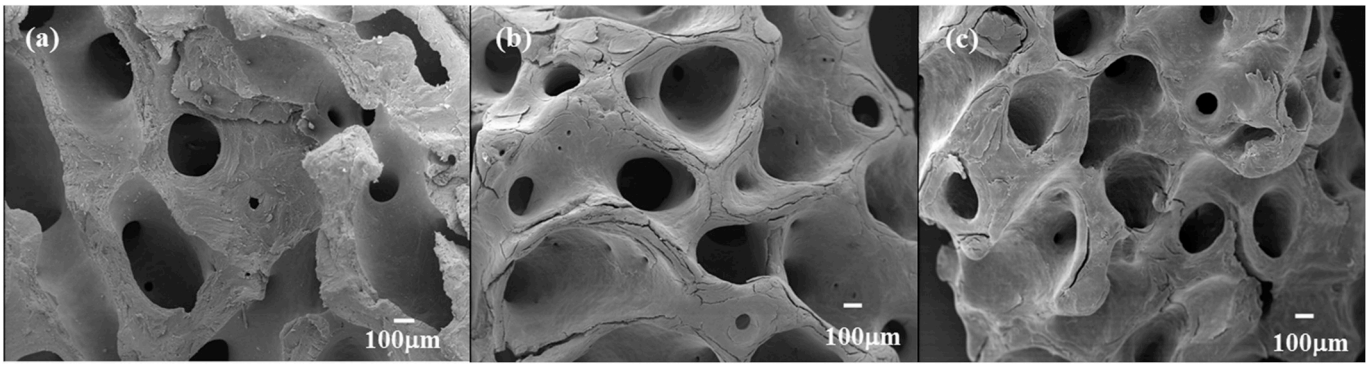

Fig. 7. Regeneration of BB: (a) BB before incubation, (b) Sample A, (c) Sample B. Magnification x48.

some other supports. After the fermentation process the adsorbed yeasts were removed from the support by treatment with ultrasound for 5 min. In order to evaluate the effect of this regeneration technique on the properties of the bone structure, two samples were analyzed. Sample A was bone employed in five successive fermentation tests and then finally treated with ultrasound. Sample B was bone used in eight successive fermentation tests with ultrasound treatment after each fermentation. SEM images of samples A and B are shown in Fig. 7 along with the image of sample BB before incubation. It can be seen that the surface of the bones was not affected by the regeneration with ultrasound.

4. Conclusions

The removal of lipids from discard bovine bone (BB) allows obtaining an adequate support for the immobilization of *S. cerevisiae* cells. BB has the suitable characteristics of a good support for cells adsorption: an adequate internal area, an appropriate size of pores and a high mechanical strength due to the presence of collagen. Thermal treatment at 35 °C for 72 h is necessary to improve the adsorption of *Scc*. Despite the thermal treatment, *ScCCUB* did not adsorb on the support. This could be due to a low hydrophobic affinity between the surface of BB and the cells.

Under the conditions obtained the immobilized biocatalyst was active in the fermentative process using carrots discards as substrate. The ethanol concentration maximum and the productivity were 11.98 g $L^{-1}$and 1.99 g L^{-1} h^{-1}, respectively. Although these values were low with respect to those reported in the bibliography, the use of bovine bone discards as support offers two great advantages: i) its reuse: the immobilized biocatalyst was used in 11 cycles of reaction; ii) its regeneration by ultrasound, allowing its use in a subsequent fermentative processes.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

The authors wish to thank Latin American Educational Centre University (UCEL) for their valuable cooperation. This work was carried out with the financial support of Project PICT Start Up 2015–3857 by the FONCyT (ANPCyT) Argentina.

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