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Evaluating the potential of wine-making residues and corn cobs as support materials for cell immobilization for ethanol production

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ABSTRACT

Three wine-making residues (grape seeds, skins and stems), and corn cobs were evaluated as support material for immobilization of *Saccharomyces cerevisiae* and the ethanol production by the immobilized cells was assessed. The main objective of this study was to find an abundant and low cost material suitable for the cells immobilization and able to be used in a next step of wine production by immobilized yeast cells. The four natural materials were used as support in two different forms: untreated, and treated by a sequence of acid and basic reactions. Untreated grape skin and corn cobs provided the highest cell immobilization results (25.1 and 22.2 mg cells/g support, respectively). The maximum ethanol production yield (about 0.50 g/g) was also obtained when the cells were immobilized in these untreated materials. It was also found that the support materials released nutrients to the medium, which favored the yeast development and the ethanol production. The use of immobilized cells systems under agitated conditions gave ethanol yields similar to those obtained by the static fermentations, but the immobilized cell concentration was significantly lower. In brief, static fermentation with cells immobilized on grape skins or corn cobs appear to be an interesting alternative for use on wine-making. The use of grape skins, particularly, which is a by-product of the wine elaboration, could be of larger interest to obtain an integrated wine production process with by-product reuse.

1. Introduction

Increased interest has been observed in the last years on the use of fermentation systems with immobilized cells, due to the several advantages that these systems present when compared to the conventional free cell fermentation. Such advantages include a higher cell concentration in the fermentation medium and consequently, an improvement in the process efficiency and productivity. Moreover, immobilized cell systems make it possible for cells to be easily recovered for later use in repeated batch operations (Cohen, 2001; Mussatto et al., 2009a; Santos et al., 2005). Therefore, immobilized cell systems have been used in different fermentation processes to obtain a number of products, such as organic acids, edulcorants, oligosaccharides, beer, among others (Dragone et al., 2007; Meleigy and Khalaf, 2009; Mussatto et al., 2009b; Silva et al., 2007).

Considerable attention has also been given to the cell immobilization of yeasts in wine-making. Some authors consider that immobilized cell systems offer many prospects for enology, such as improved performance of alcoholic and malolactic fermentation, adaptation to continuous processes, and simplified systems for removing and reusing microbial cells in batch processes (Mallouchos et al., 2003). However, up till now the industrial application of this technology was not established due to some difficulties that have to be overcome. For example, as is well known, good performance of systems using immobilized cells mainly depends on the right selection of the immobilization support. For application in food and beverages industries, particularly, the support materials should be of food-grade purity, suitable for use under low-temperature fermentation and for long-term storage when necessary, and should contribute positively to the characteristics of the final product (Kourkoutas et al., 2006; Sipsas et al., 2009). As a whole, the supports to be used in immobilization belong to two major groups: natural organic and inorganic. Among these groups, several materials have been proposed for use in wine-making, including sodium alginate, Ca-alginate, kissiris, c-aloumina pellets, gluten pellets, DEAE-cellulose, delignified cellulosic materials, fruit pieces, and dried raisin berries (Sipsas et al., 2009). Some authors consider inorganic supports more advantageous than the organic materials; nevertheless such supports have been found undesirable for wine-making because of the high concentrations of mineral residues in the final product (Kourkoutas et al., 2004).

Although several immobilization supports have already been proposed for alcoholic fermentation, only few of them find application at the industrial level, and therefore the search for new

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materials is of great interest. According to Kourkoutas et al. (2004), efforts should be concentrated on low-cost, abundant, non-destructive and food-grade purity immobilization supports, which will improve quality and give a distinctive aroma profile and a fine taste to the final product. Considering that the cost of the support material is a factor of significant influence on the final price of the product, interest has been paid to the use of agro-industrial residues for the cells immobilization. Corn cobs and grape pomace (composed by the grape skins, seeds and stems) are agro-industrial residues proceeding from the maize production and wine-making, respectively, available in large amounts in Portugal as well as in several other countries worldwide. Therefore, it is of interest to find alternatives for the reuse of these residues. The present work evaluated the use of these residues for immobilization of the yeast *Saccharomyces cerevisiae*, aiming to find a material with suitable characteristics to promote the cells immobilization, as well as to obtain an efficient system for ethanol production by immobilized cells.

2. Materials and methods

2.1. Yeast strain and inoculum preparation

A commercial *S. cerevisiae* strain (Lalvin QA23, Proenol) was used in the experiments. The inoculum was prepared by cultivation of the yeast in 500-mL Erlenmeyer flasks containing 200 mL of YPD medium with the following composition (%): yeast extract (1), peptone (2) and glucose (2). Cells were cultivated under static conditions, at 30 °C for 24 h, being subsequently recovered by centrifugation (5500 × *g*, 20 min), washed with distilled water and resuspended in the fermentation medium to obtain an initial concentration of 1 g/L (dry mass) at the beginning of fermentations.

2.2. Support materials for cell immobilization

Grape pomace (seeds, stems and skins), and corn cobs were used as support materials for the cells immobilization. The grape pomace was supplied by a local wine-making industry (Divisão de Vitivinicultura e Fruticultura da Direcção Regional de Agricultura de Entre Douro e Minho from Portugal), and the corn cobs were obtained from local farmers. Particles of seeds, stems and skins were separated from the grape pomace to be individually used in the experiments. The grape stems were cut in pieces of approximately 1 cm, while the grape skins were crushed in order to have an area close to 0.5 cm^2, and the grape seeds were used in their natural form. Corn cobs were ground and sieved, and only the particles with size between 0.45 mm and 2 mm were used as immobilization support.

All the support materials were used in the experiments in two different forms: untreated (U) and treated (T). The untreated supports were only washed with distilled water and dried at 60 °C until constant weight. Treated supports were prepared by mixing the materials with 3% (v/v) HCl solution in a solid:liquid ratio of 1:15 g:mL, and maintaining at 60 °C for 2.5 h. The remaining solids were separated, washed with distilled water until neutral pH and dried at 60 °C until constant weight. Subsequently, the acid-treated material was mixed with a 2% (w/v) NaOH solution in a solid:liquid ratio of 1:10 g:mL, at 120 rpm, 30 °C for 24 h. Finally, the solid residue was separated from the liquid fraction, washed with distilled water until neutral pH, and dried at 60 °C until constant weight.

2.3. Fermentation medium and conditions

Fermentation experiments were carried out in 500-mL Erlenmeyer flasks containing 2 g of support material and 200 mL of culture medium composed by (%): glucose (12), yeast extract (0.4), $(NH_4)_2SO_4$ (0.1), KH_2PO_4 (0.1), $MgSO_4$ (0.5). The flasks were inoculated with 1 g/L of cells and their immobilization occurred *in situ* by natural adsorption through the direct contact with the support materials. The fermentations were performed at 30 °C for 24 h, under static conditions. For comparison, assays under the same conditions described above but without the support addition, were also performed. Samples were taken periodically (at 12, 16, 20 and 24 h) for estimation of biomass concentration, glucose consumption and ethanol production.

To evaluate the nutritional effect of the support materials on the fermentation performance, the material was put in contact with the fermentation medium during 30 h, under static conditions at 30 °C. After this time, it was removed and the fermentation medium was inoculated with the yeast strain. Then, the ethanol production by free cells was performed under the same operational conditions described above. For comparison, fermentation runs with free cells were also carried out but without the previous stage of contact between the support and the fermentation medium.

The assays for evaluation of the influence of agitation on cells immobilization and ethanol production were performed under the same operational conditions described above, but in a rotary shaker at 150 rpm and 30 °C.

2.4. Analytical methods

Glucose and ethanol (*P*) concentrations were determined by high performance liquid chromatography (HPLC) in a Jasco chromatograph equipped with a refractive index detector (Jasco 830-RI) and a Varian Metacarb 67H column (300 × 6.5 mm) operated at 60 °C. A 0.01 N H_2SO_4 solution was used as eluent in a flow rate of 0.7 mL/min.

Immobilized cells concentration (X_i) was determined at the fermentations' end, according to Brányik et al. (2004a) with slight modifications. At the end of the fermentations, the biocatalyst (carrier with immobilized cells) was separated from the liquid medium, dried at 60 °C for 24 h and then, approximately 1 g was placed into an Erlenmeyer flask containing 20 mL of 3% (w/v) NaOH solution, and shaken at 30 °C, 120 rpm, during 24 h. After this time, the supernatant was recovered and used for counting cells on a Neubauer chamber. Correlation between the number of cells and the corresponding cell concentration were made using an analytical curve. The total removal of the immobilized cells from the support materials by means of the NaOH treatment was confirmed by scanning electron microscopy.

Free cells concentration in the fermentation medium was estimated by measuring the absorbance at 600 nm, which was correlated to an analytical curve (dry weight × optical density).

2.5. Scanning electron microscopy

Micrographs of the biocatalysts (after washing with deionized water and drying for 24 h at 60 °C) were obtained by scanning electron microscopy (SEM) using a Leica Cambridge S360 microscope. To be examined, the dried samples were fixed on a specimen holder with aluminum tape and then sputtered with gold in a sputter-coater under high vacuum condition. Each sample was examined at 700-fold magnification.

2.6. Fermentation parameters and statistical analysis

The ethanol yield ($Y_{P/S}$, g/g) was calculated by the ratio between ethanol produced (g/L) and glucose consumed (g/L). The ethanol productivity (Q_P, g/L h), was calculated by the ratio between the ethanol produced (g/L) and the fermentation time (h).

All the fermentation experiments were conducted in duplicate. The results were analyzed by analysis of variance (ANOVA). Significant differences ($p < 0.05$) among samples were detected by the Fisher's Least Significance Difference (LSD) multiple comparison test. Statgraphics Plus for Windows version 4.1 was the software used for data analysis.

3. Results and discussion

3.1. Influence of the support material and treatment stage on cells immobilization

Initially, the cells immobilization in the support materials as well as the effect of the treatment stage on the materials structure and cells adhesion was evaluated. Previous treatment of the materials by a sequence of acid and basic reactions promoted cleanness in the materials' structure with mass losses observed for all of them. The lowest mass loss occurred for the grape seeds, being recovered 75% of the original material mass after the treatment. For the other three evaluated materials (corn cobs, grape skins and grape stems), only 25% of the original mass was recovered after treatment. The recovered mass yield is strongly related to the original structure of the material. Grape seeds have a much harder structure than the particles of corn cobs, grape skins and grape stems, and probably, this structure hindered the acid and basic attack. When this sequence of treatment was applied for the brewer's spent grains, for example, a mass recovery yield of only 10% was obtained (Bránayik et al., 2001). However, the authors concluded that such methodology was efficient to provide high immobilized cells load in this material support.

The immobilized cell concentration in each untreated and treated support material at the end of the fermentation is shown in Table 1. It can be noted in this table that, for most of the cases, the cells were immobilized in larger amounts in untreated materials than in the treated ones. Among these, grape skins and corn cobs gave the highest immobilized cell concentration, which were not statistically different from each other at 95% confidence level. However, these results were different ($p < 0.05$) of those obtained for grape stems and seeds. Similar statistical differences were observed for the analysis of the treated supports, with the best results being also found for grape skins and corn cobs. It is worth mentioning that considerable values of immobilized cells were obtained in a short period (24 h), when using these two materials as support. Other authors obtained similar values of immobilized cells only after 75 h of fermentation (Bránayik et al., 2004b).

The yeast cells were immobilized on the materials surface by adhesion, a natural phenomenon that is preferred in the beverage production over the use of potentially harmful inducers. Scanning electron microscopy of the grape skins and corn cobs (in the untreated and treated forms), before and after the cells adhesion (Fig. 1), revealed that the immobilization did not occur in a homogeny form on the material structure, but it was more favored in specific regions, such as rough and porous structures. In fact, such structures allow microorganisms to attach more firmly to the materials surface than the smooth structures. This phenomenon has also been reported in other immobilization studies (Bránayik et al., 2004b; Kosaric and Blaszczyk, 1990; Yu et al., 2010).

Grape skins and corn cobs gave higher immobilized cells concentration than the other two evaluated materials, both when used in the untreated or treated forms (Table 1). However, when observing the structure of these two materials, it is evident the differences between them (Fig. 1). The untreated and treated corn cobs structures have many cavities (Fig. 1A and C), which provided a natural entrapment of the cells. In addition, due to the variable structure of the corn itself, a large range of corn cobs size (0.45 mm to 2 mm) was used, which was advantageous to the process since permitted to obtain bigger surfaces for the cells attachment. On the other hand, grape skins have not many cavities (Fig. 1E and G) but their rough structure was probably the responsible for the elevated cells adhesion, mainly in the untreated form. In this case, due to the few amounts of cavities in the material surface and the large amount of immobilized cells, it was observed the formation of a biofilm with multiple layers of cells (Fig. 1F). Similar behavior has been observed during the cells immobilization in treated brewer's spent grains (Bránayik et al., 2001). It is also important to observe in these figures that the grape skins structure was "cleaned" after the treatment, *i.e.*, most of its roughness appears to have been eliminated during this process (Fig. 1E and G). Such observation is in agreement with the lower immobilization results observed for treated grape skins when compared to the untreated material (Table 1), since the cells adhesion would have been hindered in this smoother surface.

In brief, grape skins and corn cobs were the most suitable support materials for immobilization of *S. cerevisiae* cells during fermentation. Treatment of these materials by a sequence of acid and basic reactions did not improve the cells adhesion to the supports surface but on the contrary, a lower concentration of immobilized cells was obtained, probably due to some cleaning effect of the chemicals on the materials surface that reduced their roughness hindering the cells adhesion as a consequence.

3.2. Influence of the support material and treatment stage on fermentation performance

The time course of fermentations for ethanol production by immobilized or free cells is shown in Fig. 2. It is evident from Fig. 2A and B that the glucose consumption was faster in media containing immobilized cells than in the medium containing only free cells. In fact, not only the substrate consumption was faster but also the ethanol production (Fig. 2C and D) and the free biomass formation (Fig. 2E and F) was higher in the assays containing immobilized cells than in those using only free cells. For most of these assays (using untreated or treated supports) the maximum ethanol production occurred at 16 h fermentation, time in which the substrate has been almost totally exhausted from the media. For this same fermentation time, the glucose consumption, ethanol production and biomass formation in the free cells assays attained practically half of the values obtained for the immobilization experiments using untreated or treated support materials. This fact suggests that immobilized cells improved the fermentation rates and efficiency of bioconversion.

All the media containing immobilized cells gave similar maximum ethanol concentration (Fig. 2C and D) independently of the support material used, and if it was or not treated. This means that, even being obtained a significant higher amount of cells immobilized into grape skins and corn cobs, the product formation in these media was not more elevated than in the experiments with cells immobilized in grape stems or grape seeds. Such fact is probably related to the amount of free biomass in these media. For all the immobilization assays, it was observed a high formation of free biomass (Fig. 2E and F), which would have contributed with the substrate consumption and product formation. Nevertheless, considering that the immobilized cells may be easily recovered from the medium and reused in subsequent fermentation stages, the elevated ethanol production achieved when using grape skins and corn cobs as immobilization material (which yielded the highest immobilized cells concentration) is a very advantageous aspect for future applications in continuous or repeated batch fermentation systems.

Although the similar ethanol production and glucose consumption for all the experiments with immobilized cells, the exact calculation of the ethanol produced per consumed substrate ($Y_{P/S}$)

Table 1
Concentration of immobilized cells (X_i) and ethanol (P), ethanol yield ($Y_{P/S}$) and productivity (Q_P) obtained during the fermentations for ethanol production using treated (T) or untreated (U) materials as support for the cells immobilization.

Response	Support material							
	Corn cobs		Grape stems		Grape skins		Grape seeds	
	U	T	U	T	U	T	U	T
X_i (mg/g)	22.20 ^{b,2}	19.95 ^{a,2}	4.08 ^{a,1}	2.83 ^{a,1}	25.10 ^{b,2}	9.28 ^{a,2}	1.68 ^{a,1}	2.38 ^{b,1}
P (g/L)	53.48 ^{a,1}	52.24 ^{a,1}	53.89 ^{a,1}	53.30 ^{a,1}	54.46 ^{a,1}	51.37 ^{a,1}	54.05 ^{a,1}	48.26 ^{a,1}
$Y_{P/S}$ (g/g)	0.51 ^{b,2}	0.40 ^{a,2}	0.44 ^{a,1}	0.38 ^{a,1}	0.49 ^{a,2}	0.50 ^{a,2}	0.51 ^{b,2}	0.39 ^{a,2}
Q_P (g/L h)	3.35 ^{a,1}	3.27 ^{a,1}	3.37 ^{a,1}	3.33 ^{a,1}	3.41 ^{a,1}	3.21 ^{a,1}	3.38 ^{a,1}	3.02 ^{a,1}

Superscript letters: For the same support material and to each response individually, values with the same letter mean no significant difference at 95% confidence level, between U and T results.
Superscript numbers: To each response individually, considering only the treated or the untreated support materials, values with the same number mean no significant difference at 95% confidence level among the results for the four supports.

revealed a significant difference ($p < 0.05$) among the performance of the fermentation using cells immobilized in grape stems and the assays using the other 3 support materials, both in the treated and untreated forms (Table 1), with grape stems assays giving the lowest ethanol yield ($Y_{P/S}$) values. The use of treated supports did not favor the ethanol production for any of the evaluated cases. Ethanol production, yield and productivity were not different when using treated or untreated grape skins and grape stems. Moreover, the $Y_{P/S}$ value was worst when using treated corn cobs and grape seeds, instead of the untreated ones.

In brief, the previous treatment of the support did not improve the ethanol production neither favored the cells adhesion, being thus proved to be an unnecessary step for the ethanol production process. The no need of the support material treatment previous its

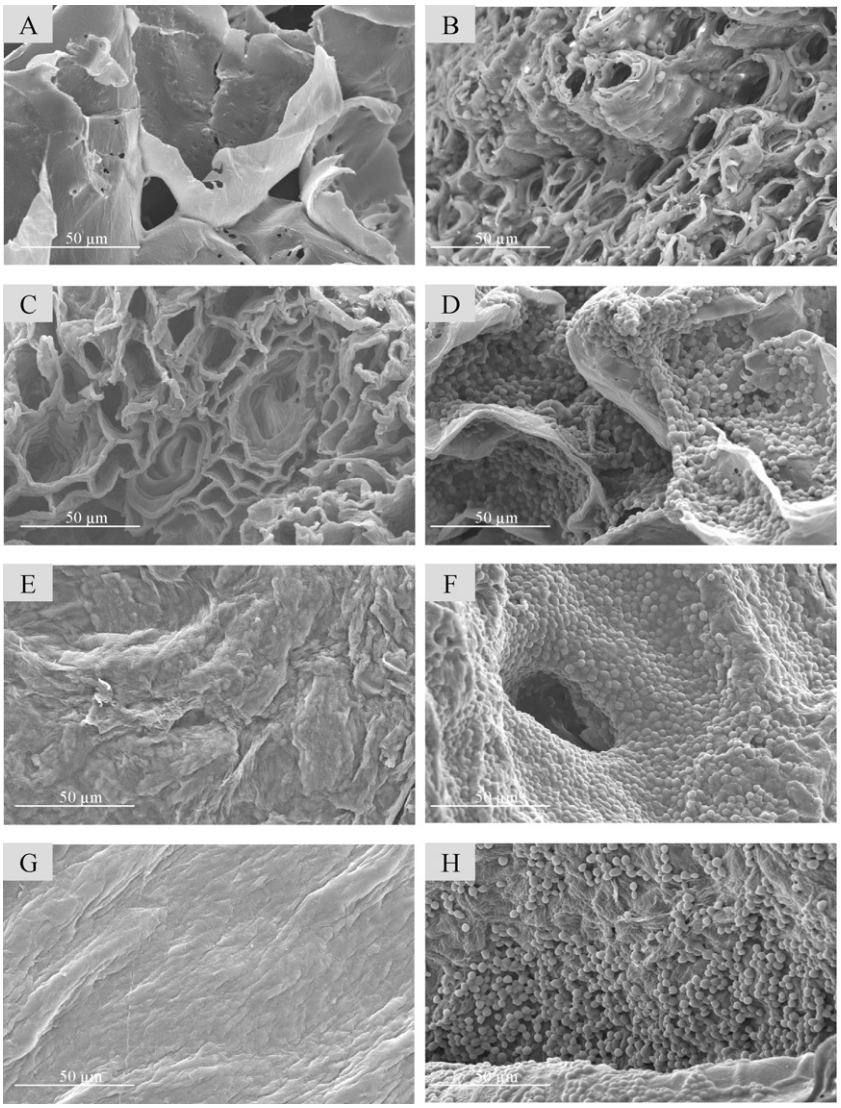

Fig. 1. Micrographs by scanning electron microscopy (SEM) of the support materials used for cells immobilization. Untreated corn cobs before (A) and after (B) the cells immobilization; treated corn cobs before (C) and after (D) the cells immobilization. Untreated grape skins before (E) and after (F) the cells immobilization; treated grape skins before (G) and after (H) the cells immobilization. Magnification: 700-fold.

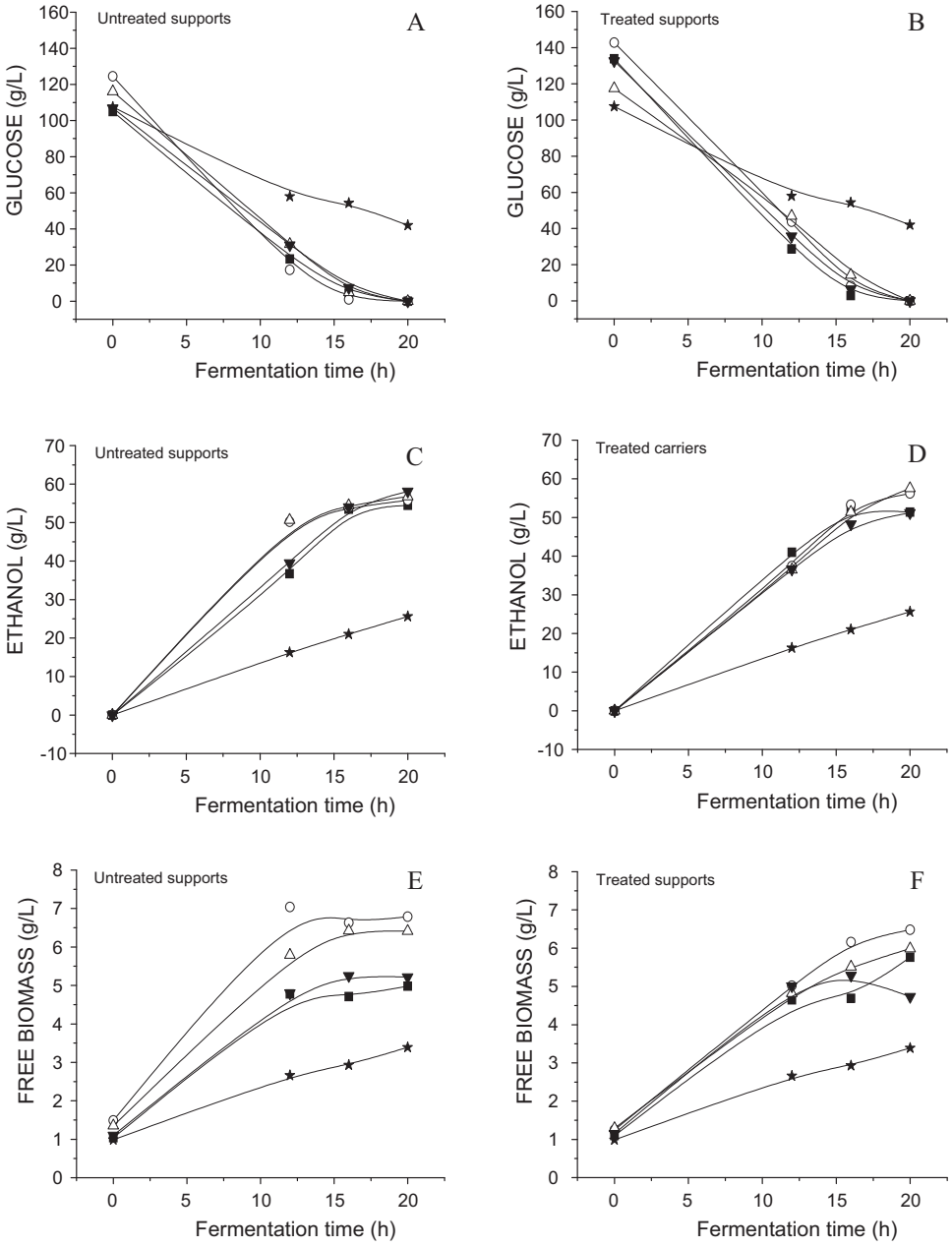

Fig. 2. Time course of glucose consumption (A and B), ethanol production (C and D) and free cells formation (E and F) during the fermentation with cells immobilized in the different support materials (untreated and treated) and from the medium containing only free cells. Corn cobs (■), grape stems (○), grape skin (△), grape seeds (▼), and free cells medium (⋆). The standard deviation to each point represented in the curves is lesser than 10%.

use for the cells immobilization is an important advantage considering the simplicity of preparation, and mainly the economy of the process, since eliminates one stage from the global process, reducing the energy and chemicals consumption and also avoiding the mass losses that the treatment causes on the material structure.

3.3. Evaluation of the nutritional effect of the support materials on fermentation performance

A curious fact observed during the fermentation runs was the elevated formation of free biomass in the media containing the support materials (treated or not) when compared to the medium containing only free cells (Fig. 2E and F). Based on these results, experimental assays were performed aiming to evaluate if the support materials have contributed with nutritional sources to the medium, which would have favored the microorganism growth. In these assays, the cultivation medium was or not put in contact with the support material during 30 h previous the use in the fermentation runs, which were only performed with free cells in suspension. Fig. 3 clearly shows that the previous contact between the support materials and the fermentation medium favored the glucose consumption and ethanol production by the yeast, as well as the biomass formation. Probably, some mineral or protein present in the materials' composition were solubilized to the fermentation medium and contributed for a better performance of the microorganism.

It is worth mentioning that the ethanol production was faster in the samples with previous contact with the support (Fig. 3B), but it was slower than in the fermentations with immobilized cells (Fig. 2 C and D). Similarly, glucose was consumed faster by immobilized cells (Fig. 2A and B) than by the free cells cultivated in the medium previously maintained in contact with the support

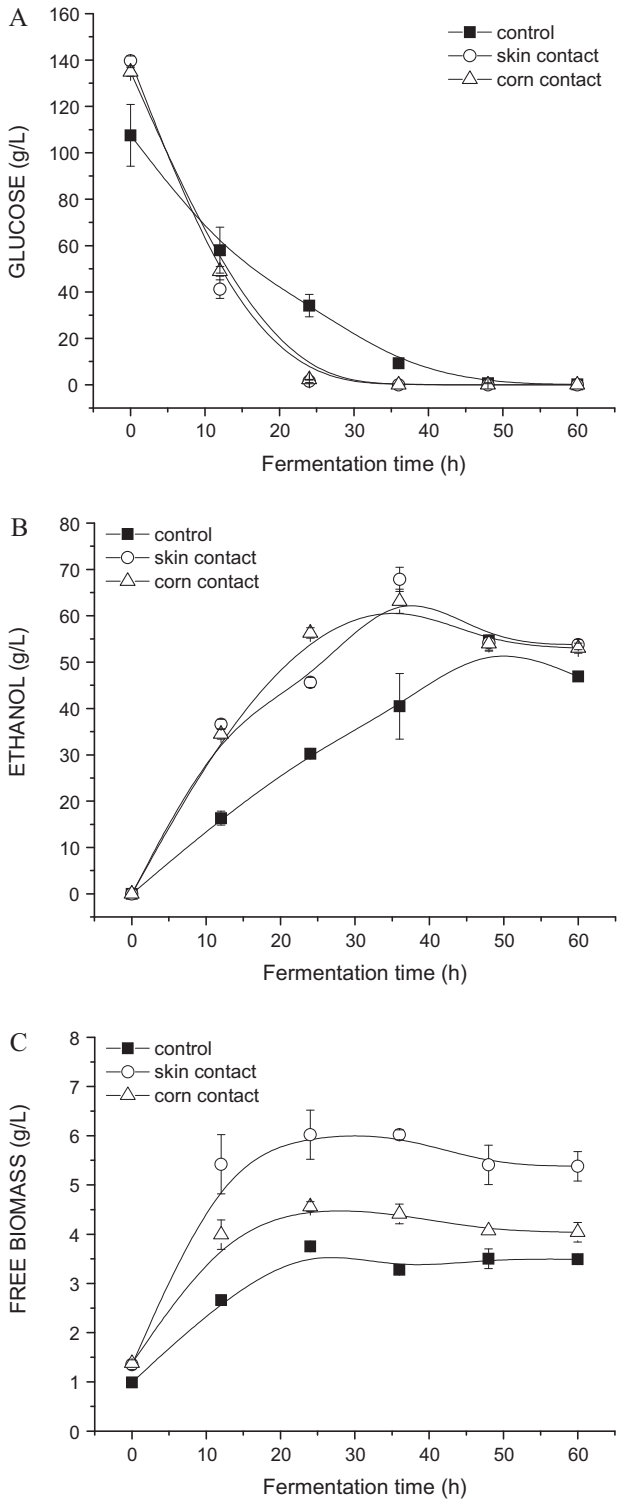

Fig. 3. Time course of glucose consumption (A), ethanol production (B), and free biomass formation (C) during the fermentation runs using the medium with or without (control) previous contact with the support materials. Fermentation assays only with free cells in suspension.

materials (Fig. 3A). Such results allow concluding that the use of the natural materials, mainly grape skins and corn cobs, for the cells immobilization during the fermentation for ethanol production is advantageous for two main reasons: (1) the materials allow the immobilization of high cell loads, which could be reused in other fermentation systems, and (2) the materials also provide nutrients to the medium, improving the yeast bioconversion performance.

3.4. Use of immobilized cell system under agitated conditions

In a last stage, the influence of the agitation on cells immobilization and ethanol production was evaluated. Use of agitated systems did not affect the ethanol production, which was similar to that obtained for the static systems. However, a strong negative influence of the agitation was observed on the cells adhesion to the supports. Under agitation, untreated corn cobs and grape skins were able to immobilize only 13.9 mg cells/g support, and 10.3 mg cells/g support, respectively; values that correspond to approximately half of those obtained under static conditions. As a consequence of this lower immobilization results and the higher aeration of the medium, the free cells concentration in these media was about two times higher than those observed under static conditions. Therefore, the ethanol production was not affected, but if it is desired the cells reuse in other fermentation operations, the use of immobilized cell systems under static conditions would be a better alternative.

4. Conclusions

Based on all the findings of this study it can be concluded that static fermentations using cells immobilized in untreated grape skins or corn cobs appear to be interesting alternatives to obtain an efficient ethanol production and high immobilized cells concentration. Such systems have potential to be successfully used in wine-making, since the support materials are of low cost, available in large amounts and have food-grade purity. The use of grape skins, particularly, which is a by-product of the wine elaboration, could be of larger interest to obtain an integrated wine production process with by-product reuse. The performance of the fermentation for wine-making using cells immobilized in these two selected support materials, as well as the sensorial analysis of the obtained products will be the focus of our future studies.

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References

Brányik, T., Vicente, A.A., Machado Cruz, J.M., Teixeira, J.A., 2001. Spent grains – a new support for brewing yeast immobilization. Biotechnol. Lett. 23, 1073–1078.
Brányik, T., Vicente, A.A., Machado Cruz, J.M., Teixeira, J.A., 2004a. Continuous primary fermentation of beer with yeast immobilized on spent grains the effect of operational conditions. J. Am. Soc. Brew. Chem. 62, 29–34.
Brányik, T., Vicente, A.A., Oliveira, R., Teixeira, J.A., 2004b. Physicochemical surface properties of brewing yeast influencing their immobilization onto spent grains in a continuous reactor. Biotechnol. Bioeng. 88, 84–93.
Cohen, Y., 2001. Biofiltration – the treatment of fluids by microorganisms immobilized into the filter bedding material: a review. Bioresour. Technol. 77, 257–274.
Dragone, G., Mussatto, S.I., Almeida e Silva, J.B., 2007. High gravity brewing by continuous process using immobilised yeast: effect of wort original gravity on fermentation performance. J. Inst. Brew. 113, 391–398.
Kosaric, N., Blaszczyk, R., 1990. The morphology and electron microscopy of microbial aggregates. In: Tyagi, R.D., Vembo, K. (Eds.), Wastewater Treatment by Immobilized Cells. CRC Press, Boca Raton, FL, pp. 79–100.
Kourkoutas, Y., Bekatorou, A., Banat, I.M., Marchant, R., Koutinas, A.A., 2004. Immobilization technologies and support materials suitable in alcohol beverages production: a review. Food Microbiol. 21, 377–397.
Kourkoutas, Y., Kanellaki, M., Koutinas, A.A., 2006. Apple pieces as immobilization support of various microorganisms. Lebensm-Wiss Technol. 39, 980–986.
Mallouchos, A., Komaitis, M., Koutinas, A., Kanellaki, M., 2003. Evolution of volatile byproducts during wine fermentations using immobilized cells on grape skins. J. Agric. Food Chem. 51, 2402–2408.
Meleigy, S.A., Khalaf, M.A., 2009. Biosynthesis of gibberelic acid from milk permeate in repeated batch operation by a mutant *Fusarium moliniforme* cells immobilized on loofa sponge. Bioresour. Technol. 100, 374–379.

Mussatto, S.I., Rodrigues, L.R., Teixeira, J.A., 2009a. β-fructofuranosidase production by repeated batch fermentation with immobilized *Aspergillus japonicus*. J. Ind. Microbiol. Biot. 36, 923–928.

Mussatto, S.I., Aguilar, C.N., Rodrigues, L.R., Teixeira, J.A., 2009b. Fructooligosaccharides and β-fructofuranosidase production by *Aspergillus japonicus* immobilized on lignocellulosic materials. J. Mol. Catal. B: Enzym. 59, 76–81.

Santos, J.C., Mussatto, S.I., Dragone, G., Converti, A., Silva, S.S., 2005. Evaluation of porous glass and zeolite as cells carriers for xylitol production from sugarcane bagasse hydrolysate. Biochem. Eng. J. 23, 1–9.

Silva, S.S., Mussatto, S.I., Santos, J.C., Santos, D.T., Polizel, J., 2007. Cell immobilization and xylitol production using sugarcane bagasse as raw material. Appl. Biochem. Biotechnol. 141, 215–227.

Sipsas, V., Kolokythas, G., Kourkoutas, Y., Plessas, S., Nedovic, V.A., Kanellaki, M., 2009. Comparative study of batch and continuous multi-stage fixed-bed tower (MFBT) bioreactor during wine-making using freeze-dried immobilized cells. J. Food Eng. 90, 495–503.

Yu, J., Yue, G., Zhong, J., Zhang, X., Tan, T., 2010. Immobilization of *Saccharomyces cerevisiae* to modified bagasse for ethanol production. Renew. Energy 35, 1130–1134.