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Alcoholic fermentation by yeast immobilized on maize stem disks filled with Ca-alginate

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Abstract

The bioethanol production from sugar beet thick juice using whole cells of Saccharomyces cerevisiae immobilized by two new combined strategies of yeast natural adhesion onto the maize stem ground tissue (MSGT) disks and Ca-alginate entrapment was examined in order to improve process efficiency through use of mechanically resistant and physically stable biocatalyst. The first strategy involves the development of immobilized biocatalyst in the form of MSGT discs overlaid with Ca-alginate (IB_1), while in the second strategy the MSGT discs were filled with Ca-alginate (IB_2). The IB_1 was physically unstable and inconvenient for repeated batch or continuous processes. In contrast, the IB_2 showed high mechanical strength, high cells retention and fermentative activity during four successive cycles without any significant decrease in ethanol production. The average ethanol concentration, productivity and yield during repeated fermentation of sugar beet thick juice (initial sugar of 150 g/l) by IB_2 were 69.18 g/l, 1.44 g/lh and 0.473 g/g (92.6% of the theoretical), respectively. The repeated batch fermentation by IB_2 is useful for reducing the cost of bio-ethanol production due to the savings in time and energy for inoculum preparation, easier ethanol recovery, and lower need for the utilization of costly centrifuges for yeast separation.

Keywords: bioethanol; fermentation; *Saccharomyces cerevisiae*; immobilization; sugar beet thick juice

Introduction

During the recent years, fermentative production of ethanol from renewable resources has received attention due to the increasing petroleum shortage [1]. Generally, bioethanol is produced from sugar-based feedstock (sugar cane, sugar beet, molasses, cane juice, beet juice), starch-based feedstock (corn, potato, rice, triticale etc.) and cellulosic (bagasse and wood) resources. In particular, sugar-based feedstock contains readily available fermentable sugars and can be an ideal substrate for ethanol production by direct fermentation without need for previous hydrolysis [2]. Owing to the surplus of sugar on the World Market, the European Union (EU) decided to reduce economic support for refined sugar by about a third, in order to prevent export of excess sugar to non-EU markets. Because of this, many sugar producers in Europe are considering to adapt to a combined sugar-ethanol plant in order to achieve increased profitability and fuel efficiency in the sugar beet processing by using its by-products for ethanol production [3]. In this context, the overall ethanol production from different intermediate products (raw juice, thin juice, thick juice) and by-product (molasses) of the sugar beet processing is very important, especially the assessment of them as the most suitable substrate for ethanol production. Because of the large amount of fermentable sugars (approx. 55-65%) accompanied by profuse mineral elements and storability, thick juice was found to be an excellent feedstock for ethanol production [4].

Traditional ethanol industries produce ethanol by free or immobilized cells. The application of immobilized cells in the fermentation provides higher biomass concentrations, enhanced biological stability, and higher process productivity. The protective effect of support on immobilized cells against ethanol toxicity has been reported previously [5]. The yeast immobilization methods are usually based on the adsorption onto insoluble support or entrapment in the polymer matrices [6, 7]. The yeast entrapment in Ca-alginate polymer usually involve the problems of gel degradation and limitations of oxygen, nutrient and metabolite mass transfer. In contrast, natural adsorption is generally a simple and inexpensive procedure for cell immobilization without internal mass transfer limitations [7]. Many previous studies had proved efficient use of various low-cost plant materials as cells support such as: sorghum bagasse [5], thin-shell silk cocoons [7], sugarcane bagasse [8], corn cobs and grape pomace [9]. Maize stem ground tissue (MSGT) is light, chemically and mechanically stable lignocellulose biomaterial with porous honeycomb microstructure. The MSGT discs can be successfully used as support for *S. cerevisiae* immobilization by natural adhesion in ethanol production from sugar beet molasses and thick juice [10]. Also, the addition of MSGT meal in Ca-alginate can provide high surface areas for cell attachment and biofilm growth, and also increase alginate matrix porosity, enabling better mass transfer characteristics, physical strength and stability of Ca-beads [11]. However, the disadvantage of yeast immobilization by natural adhesion onto MSGT is the fact that yeast cells can be easily washed out from the support.

The overall objective of this study was to propose a new strategy for combined immobilization of *S. cerevisiae* by adsorption onto the MSGT disk and Ca-alginate incorporation in order to prepare physically stable immobilized biocatalyst for repeated batch ethanol fermentation. The innovation of the paper is to prepare physically stable biocatalyst in order to overcome the problem of easy yeast leakage from biological material, as well as problem of degradation of Ca-alginate beads due to the low physical strength. The first strategy involves the development of immobilized biocatalyst in the form of MSGT disc overlaid with Ca-alginate (IB_1), while in the second strategy the MSGT disc was filled with Ca-alginate (IB_2). The paper deals with speculation of the Ca^{2+} ions effect on the yeast cell penetration inside of disk and adsorption onto the surface of MSGT. Also, the present study was carried out in order to compare the potential of IB_1 and IB_2 in repeated batch ethanol fermentation of sugar beet thick juice. The efficient ethanol production from sugar beet thick juice by using a highly stable immobilized biocatalyst, prepared by chosen strategy was promoted in order to enhance the ethanol yield and productivity, and to lower high operating costs for inoculum preparation.

Materials and methods

Raw materials

Sugar beet thick juice from a domestic sugar factory was used as fermentation substrate, after the dilution with distilled water to give a initial sugar concentration (S_o) of 150 g/l. The pH of the fermentation substrate was adjusted to 5.5 by addition of 10% (v/v) H_2SO_4, and was sterilized by autoclaving at 121 ^oC for 30 min.

Microorganism and culture condition

Saccharomyces cerevisiae (strain DTN), was grown in 250 ml Erlenmeyer flasks containing 100 ml sterilized medium (glucose 20 g/l, peptone 10 g/l, yeast extract 10 g/l; pH 4.5). It was

cultured for 24 h at 30 °C in a rotary shaker (GFL, Germany, Type 3015) at 120 rpm. Yeast cells were separated by centrifugation (3000 rpm, 10 min), and suspended in sterilized 0.9% NaCl, again separated by centrifugation on centrifuge (Tehtnica Železniki, Slovenia).

Yeast cell immobilization and fermentation conditions

The stems of Gold Cup maize hybrid were collected from ready-to-harvest corn fields from Budisava site, the Vojvodina region, Serbia. The stalks were manually cleaned to separate the fibrous tissue and nodes from the ground tissue using knives. The maize stem ground tissue of the above ground internodes (9^{th} – 11^{th}) was cut into slices with a diameter 1.5–2 cm (width) and 0.5 cm long. The moisture content of the support material (12.45% m/m) was obtained after drying at 105°C for 3 h in oven (ST-06, Instumentaria, Croatia).

The preparation of the immobilized biocatalysts, inoculation and fermentation was carried out as folows. Five grams of the MSGT discs was placed into 1 l Erlenmeyer flask containing 500 ml of 0.9% NaCl solution, and was sterilized by autoclaving at 121°C for 30 min. After the sterilization, flasks were kept at room temperature for 24 h to facilitate hydration and deaeration of the support. After the hydration procedure, the liquid was decanted in order to remove dissolved and extracted compounds in NaCl solution. Then, 500 ml of sterile 0.1 M $CaCl_2$ solution (for IB_1 preparation) or 2% (m/v) Na-alginate (for IB_2 preparation) which contained 6 g of yeast dry matter was added into the flask with hydrated MSGT discs. In both cases, the penetration of yeast cells into the porous MSGT discs and the adsorption on the surface was carried out by shaking (120 rpm) in the thermostat (30°C) for 24 h. After 24 h, the discs were transferred under aseptic conditions into the sterile solution of 2% Na-alginate (IB_1) or 0.1 M $CaCl_2$ (IB_2) and left to harden in this solution for 15 min with stirring (300 rpm). The biocatalysts (IB_1 and IB_2) were washed with 500 ml of fermentation substrate (sugar beet thick juice), and then used for the batch fermentation of 500 ml of the fermentation substrate in 1 l Erlenmeyer flask. Fermentation media was inoculated with equal yeast concentration of 3 g/l on dry basis. Anaerobic batch fermentations (pH 5.5; 30°C; without shaking) were carried out in triplicate. At the end of the fermentation batch the fermented liquid was decanted and than another 500 ml of the fresh medium was added for the next fermentation batch.

Immobilized biocatalyst characterization

The mass of cells immobilized onto the support was quantified as dry cell weight as follows. The support with immobilized cells was washed with sterilized distilled water and the dried at 105°C up to the constant weight. The support without cells recovered from the cell-free medium was used as a control to avoid any interference in weighing and optical density measurements. Weighing of dried discs was accomplished using an analytical balance. The dry weight of yeast cells in the liquid was calculated by measuring the optical density at 660 nm, using a spectrophotometer (Carl Zeiss, Jena, Germany). A previously constructed calibration curve was used to relate the optical density measurements to the dry cell concentration in the samples.

Evaluation of immobilization and fermentation parameters

The yeast immobilization parameters (cells retention R_i, concentration of immobilized cells X_i and immobilization efficiency Y_i) were calculated as proposed by Santos & al. [8] The fermentation parameters (sugar utilization S_u, volumetric ethanol productivity Q_p, ethanol yeild per utilized sugar $Y_{p/s}$, and fermentation efficiency $E_{p/s}$) were calculated as given previously [12].

Analytical methods

The fermentation kinetics was monitored by measuring the weight loss due to CO_2 release at various time intervals from the beginning of each fermentation batch. Samples of fermented liquids were analyzed for ethanol, sugar and yeast biomass. The samples were centrifuged at 3000 rpm for 15 min. The sample of supernatant was hydrolyzed in 33% HCl at 100 °C for 10 min and neutralized with NaOH solution and sugars were than determined using the 3,5-dinitrosalicylic acid [13]. The ethanol concentration was determined based on the density of the alcohol distillate at 20 ^{o}C, by pycnometer (AOAC method: 942.06). Dry mass, sucrose and total nitrogen, amino nitrogen and ash content of thick juice and molasses were estimated as per the standard AOAC Methods: 966.20, 968.28, 970.57, 969.37, 977.08, 969.36 [14].

Statistical analysis

Data were analyzed for statistical significance by a one-way analysis of variance (ANOVA). A 5% probability level (p=0.05) was used to accept or reject the null hypothesis. All statistical analyses were performed with the software Microsoft Office Excel 2003 for Windows.

Results and discussion

The novelty of this paper is the application of two strategies for yeast immobilization by combining adsorption on the MSGT disc and Ca-alginate entrapment, in order to prepare stable immobilized biocatalysts IB_1 and IB_2 . In the repeated batch or continuous fermentation process it is necessary that the applied supports have a high capacity of cells retention and stability over long fermentation time [15]. The MSGT also functions as a fortification against toxins and inhibitors, enabling efficient batch ethanol fermentation [10]. Also, in our other study, we have used MSGT meal for the preparation of combined alginate-MSGT beads (ABC) in order to overcome mass transfer limitations of Ca-alginate beads and improve ethanol productivity [11]. However, yeast cells can be easily washed out from the MSGT. Mechanical strength, geometric design of immobilizing support and the effect of Ca ions on the polymer cross-linking are very important phenomena in repeated batch or continuous fermentation based operations and is highly dependent on the volume of bioreactor [16, 17]. Therefore, in this work, the Ca-alginate was used to entrap the cells and prevent yeast cells leakage from the MSGT discs, and provide mechanically stable immobilized biocatalyst.

The preparation of immobilized biocatalyst (IB_1), involves the penetration of yeast suspension in $CaCl_2$ into the pores of MSGT discs due to the high hydrophilic capacity of the support associated with the effect of capillary forces, followed by yeast adhesion. The process of yeast cell adhesion by biosorption consists at least of van der Waals forces and electrostatic interactions or covalent binding between the cell membrane and the support surface. As is well known, yeast cells possess a large number of negative charges on the cell wall, because of the presence of acidic (mainly carboxylic) groups that are negatively ionized under normal acidic conditions [8]. The basic phenomenon of interaction of ionic polysaccharide and cellulosic fibres of the MSGT in the presence of divalent cations and negatively charged cells is the key to understand the yeast adsorption onto the surface of the support. The divalent cations such as calcium are firstly adsorbed onto a negatively charged polysaccharide and cellulosic surface. The amount of calcium bound in cellulosic fibers indicates a remarkable affinity of Ca^{2+} ions in the cellulose fibres [17]. The calcium adsorbed on the cellulose surface is expected to attract the negatively charged yeast cells. Here, the function of calcium is to mediate the charge interactions between the negatively charged yeast and the anionic cellulose surface, so that the yeast cells can absorb to the fibre surface. After the adsorption

step, the discs soaked in the $CaCl_2$ were transferred in to the Na-alginate solution. The Ca-alginate thin layer (1-2 mm) is formed on the surface of the discs (IB_1). Due to the high viscosity, the Na-alginate solution does not diffuse through the newly formed Ca-alginate thin layer. Therefore, the entrapment of the yeast was performed only in the Ca-alginate thin layer on the outer side of discs, while it was not formed inside the discs pores. Besides, the presence of yeast probably affected the gel formation process by adsorption of Ca^{2+} ions to yeast cell walls and screening part of the cross-linking sites (i.e., carboxyl groups of gulucuronate residues) on alginate chains. In this way, Ca-alginate thin layer cannot permanently reduce the cells leakage from IB_1, and the cells located in the inside the porous disc are immobilized only by adhesion.

In the case of the preparation of immobilized biocatalyst (IB_2), after the entrance of yeast suspension in Na-alginate in the pores of MSGT and yeast adsorption, the soaked discs were transferred into $CaCl_2$ solution. In this way Ca-alginate was formed in the whole structure of porous MSGT discs and the most of yeast cells are entrapped in polymer. Aqueous solutions of alginates are known to form hydrogels in the presence of divalent cations such as Ca^{2+} ions, which act as intermolecular cross-linkers between the functional groups of alginate chains, i.e. the negatively charged carboxyl groups of G-blocks [17]. The gelation process which includes the alginate cross-linkage by Ca^{2+} ions diffusing from external solution inside the gel core is controlled by diffusion through the gel/liquid interface and the inner gel areas [18].

Based on visual observations, an original view of IB_1 and IB_2 cross section and mass transfer of sugar and CO_2 on the beginning and during the thick juice fermentation is presented in Figure 1. The obtained results of CO_2 production and sugar utilization during the first fermentation batch are presented in Figure 2.

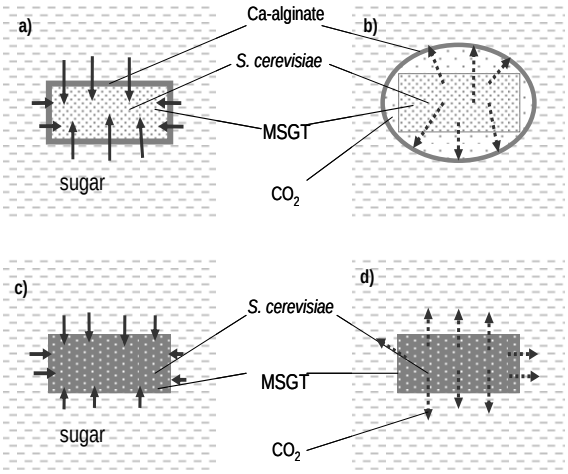

Figure 1. Mass transfer of sugar and CO_2 on the beginning and during thick juice fermentation by IB_1 (a, b) and IB_2 (c, d)

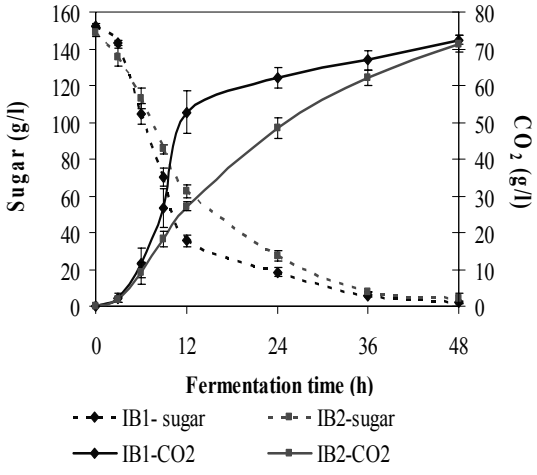

Figure 2. Sugar concentration and CO_2 production during the first fermentation batch of sugar beet thick juice (initial sugar of 150 g/l) by IB_1 and IB_2

In the beginning of fermentation, sugar solution easily diffuses through the Ca-alginate thin layer in IB_1 and cells ferments sugar to ethanol and CO_2 (Figure 1a). Due to the CO_2 production, and gas pressure, the Ca-alginate thin layer is gradually separated from the MSGT disc surface forming the bubble around the disc (Figure 1b). The formation of bubbles caused floating of the IB_1 on the surface of the substrate, and prevented the efficient contact of the

substrate with immobilized cells. The above described phenomena or mechanical changes are not observed during the fermentation by IB_2. The compact structure of the IB_2 is the result of the fulfilment of disk pores with Ca-alginate. This contributes to the gradual and relatively slow diffusion of sugar and CO_2 through the IB_2 during the fermentation (Figure 1 c, d).

When batch fermentation rate was considered, the CO_2 evolution rate could represent fermentation rate instead of ethanol production rate, since CO_2 is other product of ethanol fermentation from sugar by *S. cerevisiae*. The immobilized biocatalysts IB_1 and IB_2 were used to ferment sugar beet thick juice (150 g/l sugar). The thick juice analysis was the following (% m/m): 64.18 dry mass, 58.64 total sugars, 57.23 sucrose, 1.2 total nitrogen, 0.28 amino nitrogen, 2.2 ash. The results of fermentation kinetics (Figure 2) are in accordance to the above visual observations (Figure 1). Namely, in the period from the sixth to the twelfth hour of the fermentation by IB_1 the pressure of CO_2 on Ca-alginate thin layer lead to complete bursting of bubbles, which caused a rapid release of CO_2 from the fermentation system. Then, the substrate easily diffused into the pores of MSGT discs causing the leakage of the yeast cells immobilized by capillary forces in IB_1. The cells immobilized by adsorption were retained on the surface of the MSGT discs of IB_1. The efficient mass transfer of substrate through the IB_1, after the disruption of Ca-alginate layer, leads to the rapid sugar utilization and accordingly production of CO_2. In contrast to IB_1, in the first batch by IB_2 the sugar utilization and CO_2 production was gradual during 48 h. Thus, the alcoholic fermentation by IB_1 was faster compared to the fermentation by IB_2. The high concentration of CO_2 in the fermentation system can negatively affect microbial metabolism [19]. This could be reason for slower fermentation by IB_2. Due to the degradation of Ca-alginate layer during the first batch, IB_1 was not used in repeated fermentation.

It is well known, that the mechanical stability of Ca-alginate beads during fermentation is dependent on the CO_2 development. The gel strength and deformability depend on the Ca^{2+} concentration [17]. In order to achieve a better gel strength around and in the disk is necessary to optimize the initial Ca^{2+} concentration and the contact time with alginate. Therefore, the method of yeast immobilization has a significant impact on the stability of immobilized biocatalyst, which still has repercussions on the efficiency of fermentation. According to above observations, we believe that the IB_1 and IB_2 preparation occur as a complex of specific interactions between Ca^{2+} and yeast cells on one side and cellulosic structure of disk and alginate on the other. Much more knowledge is necessary to understand complex multi-stage interactions that occur between yeast cells, MSGT and alginate. These interactions may be physical and/or chemical nature and may affect the stress sensitivity of cells and their composition, and thus the cells metabolism.

The repeated-batch fermentation process is considered to be a promising method for cost effective ethanol production, due to the reduction in the time and costs associated with inoculum preparation [20]. The sugar beet thick juice was found to be very promising industrial substrate for repeated batch ethanol fermentation and can be used without any nutrient supplementation [12]. The final ethanol concentration obtained at the end of each fermentation batch by IB_1 and IB_2 is shown in Figure 3.

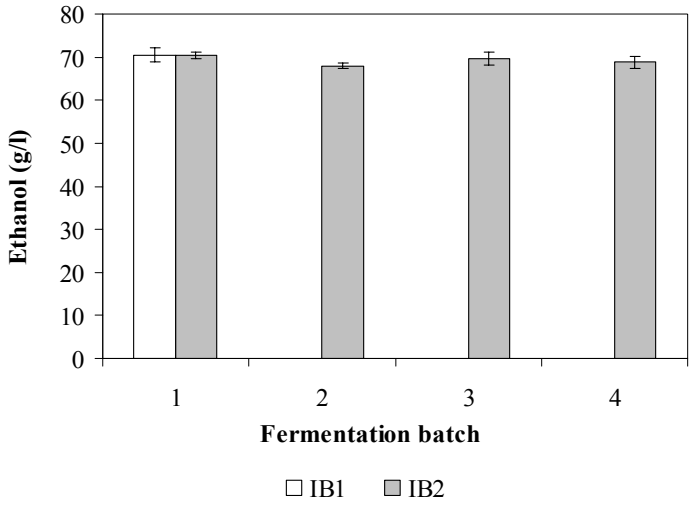

Figure 3. Ethanol concentration at the end of the each batch fermentation of thick juice (initial sugar of 150 g/l) by IB_1 and IB_2

After the first batch, ethanol concentration obtained by IB_1 was 70.50 ± 1.62 g/l. The ethanol concentration was maintained on the same level ($p>0.05$) during the four repeated batches by IB_2. The obtained ethanol concentration were 70.38 ± 0.78, 67.93 ± 0.67, 69.55 ± 1.51, and 68.84 ± 1.39 g/l in the first, second, third and fourth fermentation by IB_2, respectively. The present results imply that the cells survived and were physiologically active during four cycles of fermentation by IB_2. These findings are also confirmed by other fermentation parameters presented in Table 1.

Table 1. Fermentation parameters obtained at the end of each batch of thick juice by IB_2.

Biocatalyst	Batch	Sugar utilization S_u (%)	Ethanol productivity Q_p (g/lh)	Ethanol yield per utilized sugar $Y_{p/s}$ (g/g)	Fermentation efficiency $E_{p/s}$ (%)
IB_1	1	98.59 ± 0.34	1.469 ± 0.034	0.477 ± 0.013	93.29 ± 2.46
IB_2	1	97.21 ± 1.97	1.466 ± 0.016	0.483 ± 0.015	94.48 ± 2.96
IB_2	2	95.68 ± 1.77	1.415 ± 0.014	0.473 ± 0.013	92.66 ± 2.63
IB_2	3	98.95 ± 0.49	1.449 ± 0.031	0.469 ± 0.008	91.69 ± 1.53
IB_2	4	98.19 ± 1.38	1.434 ± 0.029	0.468 ± 0.016	91.49 ± 3.14

As expected, after the first batch by IB_1 the cells almost completely utilized the present sugar (S_u of 98.59% in average). The values of S_u in thick juice repeated fermentations by IB_2 were in the range 95.68–98.95%, without significant decline from the first to the fourth batch, suggesting that yeast maintained high sugar utilization ability. The ethanol volumetric productivity (Q_p) obtained in the first cycle was similar for both biocatalysts (1.469 g/l h for IB_1 and 1.466 g/l h for IB_2, respectively) and it maintained on the same level up to the fourth cycle by IB_2 (1.434 g/lh). The obtained $E_{p/s}$ values were in accordance with the $Y_{p/s}$. The ethanol yield per utilized sugar ($Y_{p/s}$) obtained in the first batch by IB_1 was 0.477 g/g, equal to 93.29% of its theoretical value. The $Y_{p/s}$ and consequently $E_{p/s}$ were on the same level ($p>0.05$) during four repeated batches by IB_2. The values of $Y_{p/s}$ obtained from the first to the fourth batch were in the range 0.483-0.468%, corresponding to $E_{p/s}$ of 94.48-91.49%, implying that yeast immobilized in IB_2 retained a very high fermentative activity. Although, the results of fermentation in the first batch were in favour of IB_1 application, this biocatalyst was mechanically unstable and cannot be recommended for repeated batch or continuous

fermentation. On the other hand, the IB_2 was stable and metabolically active during four cycles of the fermentation, and therefore is a convenient biocatalyst for potential application in the repeated batch and continuous processes. As is shown in Table 2, the IB_2 was equally efficient for repeated batch ethanol fermentation, with other immobilized biocatalysts that have been previously proposed in literature [5, 6, 7].

Table 2. Fermentation parameters (average values) obtained in repeated batch fermentations by *Saccharomyces cerevisiae* cells, immobilized on various supports, at 30 °C

Support	Medium	Batch	Initial sugar S_0 (g/l)	Ferm. Time t (h)	Ethanol P (g/l)	Ethanol productivity Q_p (g/lh)	Ethanol yield $Y_{p/s}$ (g/g)	Fermentation efficiency $E_{p/s}$ (%)
Sorghum bagasse [5]	Sorgum sucrose	13	200	16	96.0	5.72	0.48	98.7
Agar-agar [6]	Mahula flowers	3	70	96	25.2	0.262	0.47	95.07
Ca-alginate [6]	Mahula flowers	3	70	96	25.75	0.268	0.48	96.75
Thin-shell silk cocoons [7]	Blackstrap molasses	4	240	48	81.90	1.708	0.46	89.8
IB_2 [This work]	Sugar beet thick juice	4	150	48	69.18	1.44	0.47	92.6

In the order to evaluate the immobilization capacity of biocatalysts (IB_1 and IB_2) and examine the possibility of its reuse in the repeated batch, immobilization parameters as yeast retention onto the support (R), immobilized cells concentration (X_i), free cells concentration (X_s), total cell concentration (X_t), and immobilization efficiency (Y_i) at the end of the fermentation batch were determined and summarized in Table 3.

Table 3. The yeast immobilization parameters onto the IB_1 and IB_2

Yeast	Batch	Yeast cells retention R_i (g/g)	Immobilized cells concentration X_i (g/l)	Free cells concentration X_s (g/l)	Immobilization efficiency Y_i (%)
IB_1	1	0.296 ± 0.036	2.96 ± 0.36	3.27 ± 0.16	48.9 ± 3.9
IB_2	1	0.335 ± 0.013	3.35 ± 0.13	1.52 ± 0.66	71.7 ± 6.9
IB_2	4	0.347 ± 0.003	3.47 ± 0.03	0.97 ± 0.03	78.2 ± 0.7

The initial values of yeast retention (R_i) in IB_1 (0.310 g/g) and IB_2 (0.303 g/g), obtained after the immobilization procedure, were very similar ($p > 0.05$), suggesting that both immobilization strategies are equally successful. By comparing the initial and final R_i values of IB_1 (0.296 g/g) it can be noted that there is no statistically significant difference ($p > 0.05$), and that the amount of immobilized yeast cells on the IB_1 at the end of fermentation is practically the same as at the beginning. At the end of fermentation by IB_1 the concentration of immobilized cells (2.96 g/l) was significantly ($p < 0.05$) lower than free cells concentration (3.27 g/l). Therefore, the efficiency of immobilization (Y_i) was approximately 48.9%, which confirms that the yeast cell grow during the fermentation, as well as intense leakage of immobilized yeast cells from the support. These results are in accordance to the visual observation, which confirmed the Ca-alginate thin layer disruption in IB_1. On the basis of present results it can be concluded that MSGT discs overlaid with Ca-alginate did not meet expectations as the efficient immobilization support. In contrast to IB_1, there was a significant

($p < 0.05$) increase of cells retention in IB_2 during the fermentation from 0.303 g/g to 0.335 g/g. This is the result of growth of immobilized cells, which in this case are retained in the structure of the support. Accordingly, at the end of fermentation the concentration of immobilized cells in IB_2 (3.35 g/l) was significantly higher ($p < 0.05$) than the concentration of free cells (1.52 g/l), which results in immobilization efficiency of about 71.7%. On the basis of these findings it can be concluded that the yeast cells leakage is not completely prevented but most of the cells immobilized in IB_2 remain immobilized on to the support. In our previous study [19], the ethanol concentration obtained at the end of fermentation of sugar beet thick juice (150 g/l sugar) by *S. cerevisae* immobilized by natural adhesion on MSGT discs was 68.25 g/l, after 48 h of fermentation, which is quite efficient. However, at the end of the fermentation batch only 30.3% of yeast in the system remained immobilized by passive adhesion into the MSGT discs [19]. Namely, the achieved immobilization efficiency at the end of the first batch by IB_2 was 2.2 times higher than that previously obtained by MSGT supported yeast [19], leading to the conclusion that the filling of the pores of MSGT discs with Ca-alginate is an effective method for improving the cells retention onto the support. In addition, by analyzing the immobilization parameters obtained after the fourth batch by IB_2 it can be observed that both cells retention (0.347 g/g) and immobilization efficiency (78.2%) were increased in comparison to the first batch, which is probably caused by the yeast cell growth in inner pores of discs. The major problem that arises during fermentation by yeast immobilized in Ca-alginate beads by standard method is their relatively low physical strength and stability, gel degradation due to the release of CO_2 and severe mass transfer limitation of substrate and products [11, 17]. In the present work MSGT discs filled with Ca-alginate (IB_2) showed good physical strength and operational stability during four fermentation cycles. Besides, the advantage of the yeast immobilization by this method is less demand in terms of necessary equipment in comparison to Ca-alginate beads preparation, resulting in fewer investments. In addition, yeast immobilization in IB_2 could be carried out in the same bioreactor before the fermentation, which reduces the risk of microbial contamination, especially on industrial scale.

Conclusion

In the present work, the repeated batch fermentation of sugar beet thick juice using immobilized *S. cerevisiae* in porous maize stem ground tissue discs overlaid (IB_1) and filled (IB_2) with Ca-alginate was investigated. The IB_2 showed an increase of yeast cell stability and ethanol production during four successive batch fermentations, while the IB_1 were mechanically unstable and unsuitable for repeated batch or continuous fermentation. Ffilling of porous MSGT discs with Ca-alginate (IB_2), provided high surface areas for yeast attachment and growth, and prevented leakage of the cells from discs. The IB_2 showed good mass transfer characteristic and better physical strength and stability in comparison to yeast immobilized in standard Ca-beads beads. Therefore, IB_2 can be recommended as novel physically improved biocatalyst for long-term use in repeated fermentations in order to save considerable time and energy for inoculum preparation. Bioethanol production from sugar beet thick juice by IB_2 would be advantageous both from a technical and economical point of view due to savings in inoculum preparation, easier ethanol recovery, and lower need for the utilization of costly centrifuges for yeast separation.

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