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Original scientific paper

CONTINUOUS ETHANOL PRODUCTION FROM SUGAR BEET THICK JUICE BY *SACCHAROMYCES CEREVISIAE* IMMOBILIZED ONTO SUGAR BEET PULP

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The immobilization of Saccharomyces cerevisiae onto sugar beet pulp (SBP) by natural adhesion is an efficient and low-cost method for retaining high biocatalyst density in the ethanol fermentation system. In the present study, cells of S. cerevisiae 163, were immobilized by natural adhesion onto SBP. The retention of immobilized cells attained the level of about 1.7×10^{11} cells/gram of dry SBP. Continuous ethanol production from sugar beet thick juice (TJ) was performed in a cylinder glass bioreactor at a temperature of 30°C and pH 5 during a 27-day period. The stability of the fermentation process at dilution rate (D) of 0.025 h^{-1} and 0.05 h^{-1} was evaluated. The yeast-SBP system was shown to be stable for over a 15-day period at the dilution rate of 0.025 h^{-1}, while the dilution rate of 0.05 h^{-1} was found to be unsuitable due to the intensive yeast leaching from the support. At D of 0.025 h^{-1} the maximum sugar utilization (S_u), ethanol concentration (P), volumetric ethanol productivity (Q_p), ethanol yield ($Y_{p/s}$) and fermentation efficiency were 97.1%, 54.7 g/l, 2.3 g/lh, 0.498 g/g and 97.6%, respectively.

KEYWORDS: ethanol, continuous fermentation, immobilization, sugar beet pulp, sugar beet thick juice

INTRODUCTION

Ethanol is considered as an alternative to petroleum-based fuels, and much attention has been focused on the improvement of ethanol production using renewable raw materials as the feedstock. Therefore, the development of an efficient fermentation process using economical raw materials is essential for the biofuel ethanol production at a commercial scale (1). Continuous fermentation systems offer a number of advantages compared to batch processes which are generally resulting from enhanced volumetric productivity and, consequently, smaller bioreactor volumes and lower investment and operational costs. Other advantage of this operation is less non-productive idle time for cleaning and resterilization (2). An important aspect of continuous fermentation is the high volumetric efficiency, which is usually obtained by increased yeast cell concentrations in the bioreactor compared to traditional batch systems. Immobilizing yeast cells onto appropriate

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support types can provide high cell densities in the bioreactor, which, in combination with high flow rates in continuous processes, leads to short residence times, higher conversion rates, faster fermentation rates, improved product consistency, reduced product losses and environmental advantages (3). Currently, significant efforts are being made to explore the possibility of utilization of locally produced lignocellulosic fiber as immobilizing support for yeast cells in ethanol fermentation, owing to the operational easiness and the high ethanol productivity (4). Also, the possibility of recycling cells for inoculum in immobilized cell bioreactor permits the fermentation to be profitably carried out in repeated-batch or continuous mode (5). Thick juice (TJ), which is an abundant intermediate product of the sugar industry in Vojvodina province, Serbia, is at present one of the least expensive sources of sugar, and, in contrast to starch or cellulose raw materials, it does not require hydrolysis or special pretreatment. TJ was found to be an economically advantageous raw material for ethanol production, compared to molasses due to the reduced water usage, reduced wastewater purification costs, easier mixing, lower use of acids for pH buffering, and increased levels of nutrients (4). Besides, the concentration of pigmenting compounds (caramel substances, melanoidines, and iron–phenol compounds), which impair the metabolism of yeast in TJ, is quite low in comparison to conventionally used sugar beet molasses (6).

The present work reports results of a study of the cost-effective continuous ethanol production from TJ, without any nutrient supplementation, in a packed bioreactor with yeast immobilized onto very cheap, renewable and abundantly available sugar beet pulp (SBP). To the best of our knowledge, this is the first report concerning continuous ethanol production from TJ by *S. cerevisiae* immoiblized onto SBP, dealing with the effect of dilution rate on the main proces parameters.

EXPERIMENTAL

Support and substrate preparation

SBP used as a yeast cell support, and TJ used as fermentation substrate, were kindly provided by the sugar factory near the city of Senta, Vojvodina, Serbia. Dry mass, sugar, total nitrogen, amino nitrogen and ash content of TJ were estimated by the standard AOAC methods (7). The total sugar content of TJ was 120 g/l, the pH was adjusted to 5.0 by the addition of 1M HCl, and TJ was sterilized by autoclaving at 121^{o}C for 30 min. The SBP hydration was carried out by placing an amount of 30 g of SBP on dry basis into 2 l Erlenmeyer flasks containing 1 l of synthetic culture medium consisting of glucose (120 g/l), $(NH_4)_2SO_4$ (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (5 g/l) and yeast extract (4 g/l). The mixture was sterilized by autoclaving at 121^{o}C for 30 min. After the sterilization, the flasks were kept at room temperature for 24 h.

Microorganism immobilization

Pure culture of *S. cerevisiae* strain 163 from the collection of the Faculty of Technology Novi Sad was maintained on sterilized slants consisting of malt extract (140 g/l), glucose (20 g/l), peptone (1 g/l), agar (20 g/l), pH 8. The cultures were stored at 4^{o}C

following the incubation at 30 °C for 48 h. Before starting the experiment, the microorganism was twice refreshed in the same medium in glass tubes, and incubated for 24 hours at a temperature of 30°C. The inoculum was grown by transferring a loopful of cells into 250-ml Erlenmeyer flasks containing 100 ml of sterilized synthetic culture medium (composition described above). It was cultured for 24 h at 30°C in a thermostat on the rotary shaker (GFL, Germany, Type 3015, shaking frequency 120 rpm, shaking diameter 30 mm). To immobilize cells, the flasks containing hydrated SBP were inoculated with 80 ml of yeast suspension (late exponential phase) and placed on a rotary shaker (120 rpm) in the thermostat at 30°C for 24 h. The mass of cells immobilized onto the support was quantified by placing 0.1 g of the support with immobilized cells on a filter paper to remove fluid. The cells immobilized onto the support, without extra medium, were pressed out using a glass stick and washed with 10 ml of sterilized 0.9% NaCl. The quantification of yeast cells in this suspension was made by counting using a Neübauer camera. To avoid interference in weighing measurements, the same amount of SBP recovered from the cell-free medium was firstly washed with distilled water and afterwards dried to measure dry matter content. Cell retention onto the support (R, number of yeast cells/g) was calculated as the ratio of the number of cells immobilized onto the support dry mass. The immobilization efficiency (Y_i, %) was calculated as the ratio of the immobilized (X_i) to the total (free plus immobilized) cells concentration (X_t) and multiplying by 100. A Carl Zeiss optical microscope connected to a Cannon S50 camera was used to capture yeast cells immobilized onto SBP.

Continuous fermentation

After the immobilization, the yeast suspension was decanted from the Erlenmeyer flask, and the SBP-supported yeast was aseptically transferred into the glass column bioreactor, which was previously sterilized by autoclaving (120°C, 30 min). The schematic diagram of the experimental set-up is shown in Fig. 1. Continuous ethanol fermentation was carried out in a vertical bioreactor in the form of water-jacked glass tapered column (the height 24 cm and internal diameter 5.5 cm), equipped with the perforated plates at the bottom and at the top to separate the particles from the liquid phase. The bioreactor temperature was maintained at 30±1°C by the recirculation of distilled water using a peristaltic pump. The TJ was fed through a silicon pipe system to the glass column bioreactor in a downstream gravitational flow from a feed tank (10 l) located above the column. A flow breaker was installed between the column and feed pump to prevent contamination of the feed line and the reservoir. The total volume of the bioreactor was 570 ml, and 480 ml (about 75% of the total volume) of TJ and SBP-supported biocatalyst were added to it. The effluent from the column was collected in a tapered 1 l sterilized gauge glass serving as product reservoir. The dilution rate (D) was set at 0.025 h^{-1} and 0.05 h^{-1} by changing the feed flow rate.

Analytical methods

Effluent samples were withdrawn aseptically and were analyzed for sugar, ethanol and suspended biomass concentrations every day. The fermented liquid was centrifuged

at 3000 rpm for 15 min. The supernatant sample was hydrolyzed in 33% HCl at 100°C for 10 min and neutralized with NaOH solution, and sugars were then determined using the 3,5-dinitrosalicylic acid (DNS) method (8). The ethanol concentration in the distillate was determined based on the density of the alcohol distillate at 20°C with pycnometer by the AOAC method 942.06 (7). Yeast cell viability was qualitatively evaluated by routine methylene blue stain technique.

Fermentation parameters

Sugar conversion (S_u, %) was calculated as the ratio of the utilized sugar to the initial amount, multiplied by 100. The ethanol yield ($Y_{p/s}$, g/g) was calculated as grams of ethanol produced per gram of utilized sugar. Also, the fermentation efficiency was calculated as the percentage of the maximal theoretical ethanol yield ($E_{p/s}$, %). The volumetric ethanol productivity (Q_p, g/lh) was calculated as grams of ethanol produced per liter per hour.

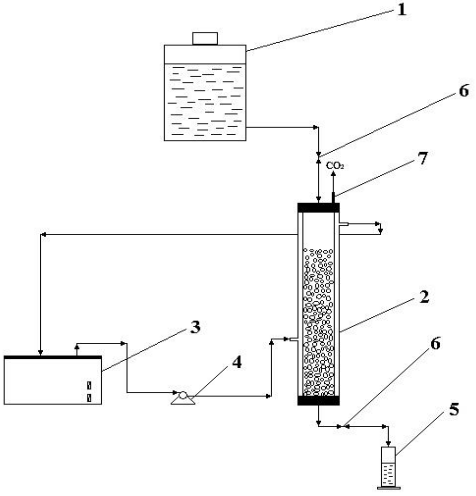

Figure 1. Schematic of the experimental set-up: 1 - feed tank, 2 - immobilized cells bioreactor, 3 - thermostat, 4 - peristaltic pump, 5 - glass gauge, 6 - air vent with flow controller, 7 - fermentation bung for the release of CO_2

RESULTS AND DISCUSSION

The process of continuous ethanol fermentation of TJ was carried out to investigate the operational stability and suitability of the immobilized *S. cerevisiae* strain 163 on SBP. For the fermentation system, two different D of 0.025 h^{-1} and 0.05 h^{-1} were selected based on preliminary batch experiments. The TJ analysis gave the following results (%, w/w): 62.66 dry mass, 60.51 total sugars, 0.329 total nitrogen, 0.084 amino nitrogen, and 0.18 ash. Based on the obtained results of the content of main components in TJ it can be concluded that this raw material can provide basic nutrients for the yeast during the fermentation.

The *S. cerevisiae* was previously found to be effectively immobilized by natural adhesion onto SBP. The adhesion forces and the surface energy of the immobilization support were not affected by the TJ composition during the fermentation (6). In the present work, yeast immobilization within the SBP tissues was confirmed by the results of the initial cells retention (R=1.7·10^{11} cells/g) and immobilization efficiency (Y_i =18.61 %), as well as by optical microscopy (Figure 2). As expected, yeast cells were bound in the volume and not only on the surface of SBP after the immobilization step, but also at the end of the fermentation, at both examined D.

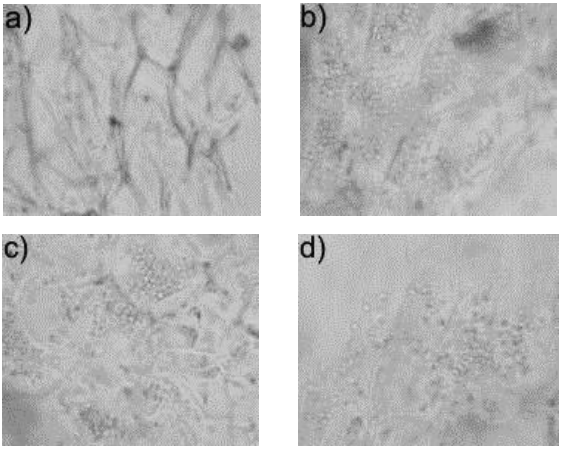

Figure 2. Optical microphotograph (400×) of: a) SBP, b) *S. cerevisiae* 163 immobilized onto the SBP after the immobilization, c) *S. cerevisiae* 163 immobilized onto the SBP after 27 days of continuous fermentation at D=0.025 h^{-1}, d)) *S. cerevisiae* 163 immobilized onto the SBP after 12 days of continuous fermentation at D=0.05 h^{-1}

Figure 3 illustrates the time course of the residual sugar (S_r), sugar utilization (S_u), ethanol concentration (P), yield ($Y_{p/s}$) and fermentation efficiency ($E_{p/s}$) during the continuous ethanol fermentation at D=0.025 h^{-1}, and D=0.05 h^{-1}. As can be seen from Figure 3a, the continuous ethanol production at D of 0.025 h^{-1} was stable and highly efficient during 15 days, without significant decline of main fermentation parameters. In this period, the immobilized yeast cells were highly active, utilizing 89.1-97.1% of sugar from the substrate, producing 51.0-54.7 g/l of ethanol, achieving ethanol productivity of 2.1-2.3 g/lh. Thus, the process of continuous fermentation was equally efficient as the other processes involving immobilized biocatalysts (2, 9, 10). These results indicated that SBP-supported yeast utilized almost all available sugar from TJ, suggesting efficient exploitation of this raw material for continuous ethanol production. From 16th to 27th day, a gradual decrease of sugar utilization (from 83.3 to 50.8%) and, consequently, of ethanol production (48.0-29.0 g/l) was observed. Neverthless, the results of ethanol yield per consummed sugar (0.452-0.507 g/g) and fermentation efficieny (88.5-99.4%) suggest that the fermentation activity of the immobilized yeast cells remained at a very high level until the end of fermentation. Hence, there was no obvious ethanol inhibition of cells owning

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to the continuous removal of ethanol from the column, thus the cell activity was maintained at a stable level during the fermentation. In addition, the slice increase and sustainable periodical (4 days) oscillations of the $Y_{p/s}$ and, consequently $E_{p/s}$, were observed after the 14th day of the fermentation. Similar oscillations during the continuous fermentation by *S. cerevisiae* were reported previously. The mechanisms provoking these phenomena were believed to be quite different, such as asymmetric budding cycle of *S. cerevisiae* or change of the microbial profile of yeast involved in their size, viability loss, cell death and, perhaps. the intracellular material storage (9).

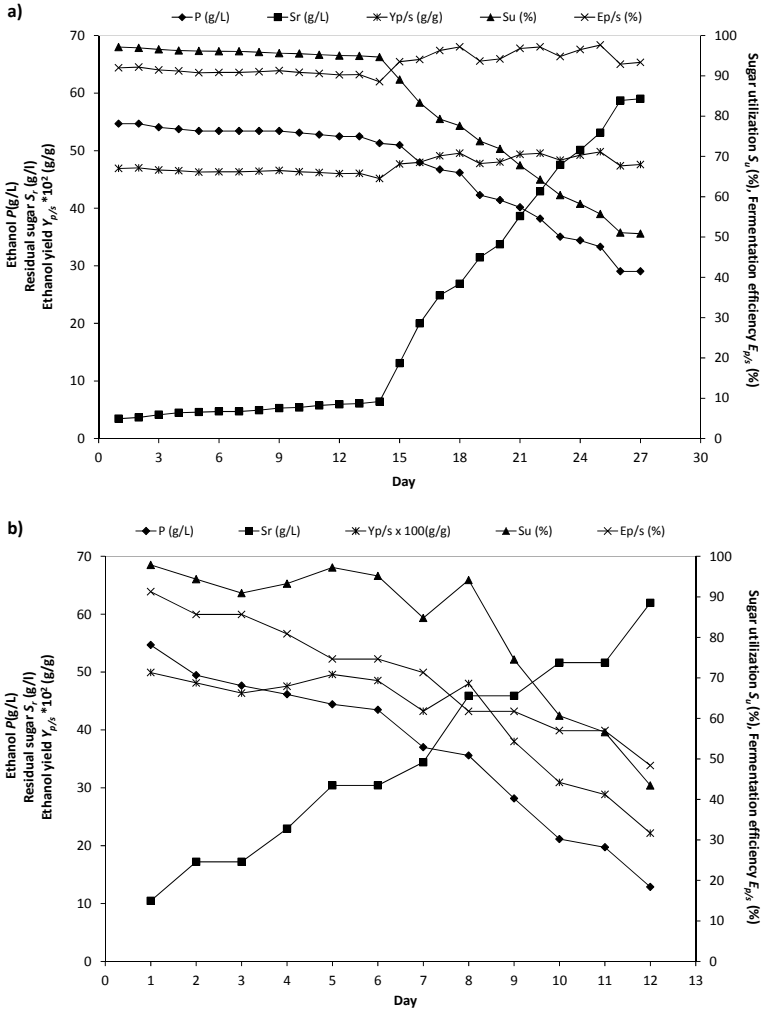

Figure 3. Fermentation parameters vs. time during continuous fermentation at a) D=0.025 h^{-1}, b) D=0.05 h^{-1}

As it is evident from Figure 3b, during 12 days of the continuous fermentation at D of 0.05 h^{-1} the significant gradual decreases of fermentation parameters such as sugar utilization (98.8-43.4%), ethanol concentration (54.7-12.9 g/l), ethanol yield (0.499-0.222 g/g), fermentation efficiency (91.3-48.4%) and ethanol productivity (2.2-0.5 g/lh) were observed. Therefore, this fermentation was not carried out further. As the amount of CO_2 produced during ethanol fermentation inside the column was small and could not suspend effectively the yeast cells, the deposition of yeast cells took place. The lack of liquid mixing and/or microporous mechanical barriere above the outflow resulted in the lost of the floating biocatalyst, which was thus dragged, together with the medium, through the outflow. The yeast biomass concentrations in the fermented medium and cells viability are presented in Figure 4. As can be seen, in both experiments, a significant yeast cell loss from the fermentation system was observed.

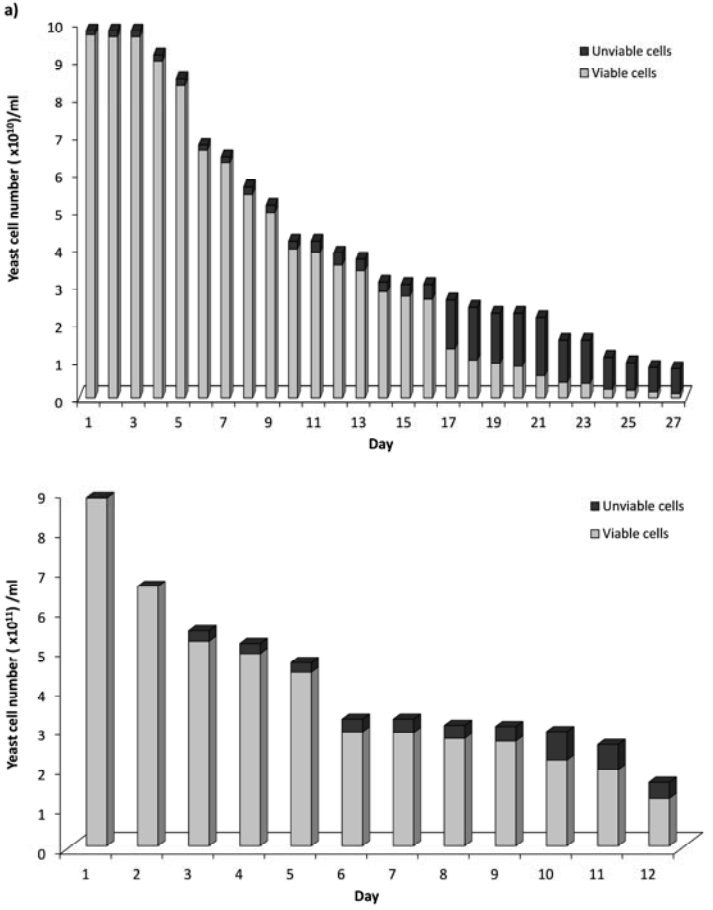

Figure 4. *S. cerevisiae* biomass concentration and cell viability in the effluent vs. fermentation time at a) D=0.025 h^{-1}, b) D=0.05 h^{-1}

Although yeast wash out ($9.7\text{-}2.7\times10^{10}$) was observed during the 15 days of continuous fermentation at D of 0.025 h^{-1}, this was of little importance since the cells viability remained very high (99.0-91.7%). Up to the end of this fermentation (27th day) the cells leakage showed a decrease ($2.6\text{-}1.2\times10^{10}$). At the same time, a significant decrease of the yeast cells viability was observed. The loss of yeast cells viability was in accordance with the decrease of sugar utilization and ethanol production. However, at D of 0.05 h^{-1} much more intense yeast cell wash out ($8.8\text{-}1.2\times10^{11}$) occurred, along with the loss of cells viability (99.9-75.0%). This is not surprising since natural cell adhesion and adsorption onto SBP is sesitive to mechanical stress and changes in the bioreactor environment. At high dilution rates in continuous fermentation the biomass growth and the protective effect of support are reduced. As a concequence of this, yeast cell viability was low and conversion of sugar to ethanol is not complete. To avoid the biocatalyst loss from the fermentation system the application of upstream flow along with yeast cells recirculation, or occasional air supply, could be adventageous.

CONCLUSION

The process of continuous TJ fermentation at D of 0.025 h^{-1} ensured a favorable growth enviroment for the yeast cells and high cells retention onto SBP and inside the packed bioreactor. This significantly improved their ethanol production performance, which resulted in lower residual sugar and higher ethanol concentrations. The SBP-supported biocatalyst proved to be mechanically and chemically stable during 27 days of continuous ethanol production. Further, it met the requirements of high cell load, stability, food grade, and the posibilities of regeneration and sterilization. Although long-term operational stability of the system was not studied, the continuous ethanol fermentation of TJ operated reliably for almost one month, without any nutrient supplementation. The process could be improved by the application of upstream flow along with yeast cells recirculation or occasional air supply.

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КОНТИНУАЛНА ПРОИЗВОДЊА ЕТАНОЛА ИЗ ГУСТОГ СОКА ШЕЋЕРНЕ РЕПЕ ПОМОЋУ *SACCHAROMYCES CEREVISIAE* ИМОБИЛИСАНИХ НА РЕЗАНЦИМА ШЕЋЕРНЕ РЕПЕ

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Имобилизација *Saccharomyces cerevisiae* на резанцима шећерне репе (РШР) природном адхезијом је ефикасан и јефтин метод за постизање високе концентрације биокатализатора у ферментационом систему. У овом раду ћелије *S. Cerevisiae 163*, су имобилисане на РШР. Постигнут је степен имобилизације од 1.7×10^{11} ћелија/грам РШР. Континуална производња биоетанола из густог сока шећерне репе је испитана у стакленој колони, при 30°C и pH=5 током 27 дана. Испитана је стабилност ферментационог процеса при брзини разблажења (D) од 0.025 h^{-1} и 0.05 h^{-1}. Ферментација је била стабилна током 15 дана при D=0.025 h^{-1}, док је при D=0.05 h^{-1} утврђено интензивно испирање ћелија из система. Максимално искоришћење шећера од 97.1%, концентрација етанола од 54.7 g/l, волуметријска продуктивност етанола од 2.3 g/lh, принос етанола од 0.498 g/g и ефикасност ферментације од 97.6% су остварени при D=0.025 h^{-1}.

Кључне речи: биоетанол, континуална ферментација, имобилизација, резанци шећерне репе, густи сок шећерне репе

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