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Bioconversion of crude glycerol to 1,3-Propanediol by immobilized cells of *Citrobacter freundii*

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ABSTRACT

1,3-Propanediol (1,3-PD) was produced from crude glycerol through the fermentation by immobilized *Citrobacter freundii* cells. Microorganisms were immobilized on keramsite. and transferred to fermentation broth "M" with 50g/L glycerol. In parallel, conversion of glycerol was led in two bioreactors: column bioreactor for continuous production and standard bioreactor for static culture at the same conditions. The obtained results have shown that the use of immobilized cells of *C. freundii* reduced concentration of crude glycerol about 60%. The results have also demonstrated that immobilization was appropriate process for production of 1,3-PD in both continuous and static (bioreactor) conditions.

***Keywords*:** immobilization, bioconversion, keramsite, crude glycerol, 1,3-PD, *Citrobacter freundii*

1. INTRODUCTION

Shortage of oil resources, environmental and economic aspects, enforces the need to seek new sources of energy. Biodiesel is most common biofuels, which is based on the production process of esterification of vegetable oils and animal fats. Increase of biodiesel production, increases the amount of main by-product: waste glycerin phase comprising glycerol, methanol, mono-and diacylglycerols, free fatty acids and soaps [1]. In Europe, biodiesel is produced mainly from rapeseed oil (e.g. Germany, Poland) and sunflower (e.g.

Spain). Biodiesel production generates about 200-300 thousand tons of glycerin per year, which partly contributes to its high price. The ideal solution seems to be the use of crude glycerol or glycerin phase as a carbon source for the bacteria in biotechnological processes such as the production of 1,3-Propanediol, which has attracted much interest [2,3] 1,3-Propanediol (1,3-PD) is one of the commonly known fermentation products. It was identified in 1881 by August Freund, in a glycerol fermentation mixed culture containing *Clostridium pasteurianum* [4]. 1,3-PD is an organic compound widely used in industry as a monomer for the production of polyesters, polyurethanes, polyethers, and as a raw material for solvents, adhesives, detergents, cosmetics and drugs [5]. Conversion of 1,3-PD from glycerol has been extensively studied in species of enterobacteria, clostridia and lactobacilli [6]. Numerous species of *Enterobacteriaceae* are able to convert glycerol into 1,3-PD. The most promising ones are *Klebsiella pneumonia* and *Citrobacter freundii* [7].

In biotechnological processes, in order to protect the cells against infection and accidental changes of the environment, immobilization is used [8,9]. Immobilization accelerates biotechnological processes and increases their yield, through better penetration of the substrate and high concentration of microbial cells. Immobilization of bacteria on a solid, porous matrices using adhesion is a natural process, often occurring in nature. This method is simple and widely used in many industrial processes.

It involves passive / natural immobilization usually occurring in bioreactors. Immobilization enables continuous fermentation processes. One of the most influential factors that impact on adhesion is type of carrier. Keramsite (leca) is a lightweight artificial porous aggregate manufactured by expansion of natural clay, classified as a ceramic material. Keramsite is nearly spherical granule, its internal structure is porous and closed by sintered surface [10]. Due to its high porosity and its sorptive characteristics can be used as a carrier for bacterial immobilization as a filter material and sorbent of organic compounds [11].

The aim of the study was the use of immobilization process for converting crude glycerol (Trzebinia Refinery, PKN Orlen Capital Group, Poland) to 1,3-PD using *C. freundii* immobilized on keramsite.

2. MATERIAL AND METHODS

2. 1. Material

Microorganism: Citrobacter freundii strain was isolated and obtained from the Faculty of Food Science and Nutrition, Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences (Poland).

Carrier for immobilization: Keramsite, fraction 2-4 mm (Liapor, Germany); before use mineral, porous carriers were sterilized in autoclave at 121 ºC; with 2,2 bar pressure for about 15 minutes;

Used mediums: TSA agar (Merck, Germany) for bacteria pre-grown; MacConkey agar (Merck, Germany) for plating method, all medium were prepared according to the Merck protocol. Broth "M" composition: 48g/L- K_2HPO_4 (Chempur, Poland), 12g/L- KH_2PO_4 (Chempur, Poland), 2g/L- $(NH_4)_2SO_4$ (Chempur, Poland), 0,4g/L- $MgSO_4 \cdot 7H_2O$ (Chempur, Poland), 0,1g/L- $CaCl_2 \cdot 2H_2O$ (Chempur, Poland), 0,004g/L- $CoCl_2 \cdot 6H_2O$ (Chempur, Poland), 2g/L- yeast extract (Merck, Germany), 2,5g/L- Aminobak (BTL, Poland), 1,5g/L

bacteriological meat extract (Merck, Germany) and 50g/L- crude glycerol (Trzebinia Refinery PKN Orlen Capital Group, Poland). Technical parameter of crude glycerol: content of glycerol- 80% [% (m/m)], water- 15% [% (m/m)], sulfated ash- 5% [% (m/m)], methanol- 0,3% [% (m/m)], MONG- 6% [% (m/m)] and density: 1,2 g/cm^3.

Column set for continuous production (Fig. 1): chromatographic column with thermo jacket XK26/20 (GE Healthcare Life Sciences, USA), thermostat (Julabo, Poland), peristaltic pump (MRC, Israel), magnetic stirrer with heating (DragonLab, China).

2. 2. Methods

Immobilization: Bacteria cells of *C. freundii* were pre-grown on TSA agar for 24h at 30°C. After incubation the biomass was suspended in sterile 0.85% NaCl solution. Then suspended biomass was added to sterile flask with broth "M" (in a ratio of 1:10) and stirred for 15 minutes (DragonLab, China). After stirring, medium with bacteria culture was added to two sterile flasks with sterile keramsite and incubated at 30 °C for 24h. Initial bacterial concentration for immobilization: $28 \cdot 10^6 \pm 28 \cdot 10^4$CFU/mL.

Adhesion: After 24h of incubation, carriers from the first flask were rinsed with sterile NaCl solution, suspended in sterile broth "M", homogenised with sterile glass rod and vortex. From each suspension serial dilutions were made. Cell concentration was expressed as colony-forming units (CFU) per mL and determined by making serial decimal dilutions and plating on MacConkey agar. Results were presented as average values with standard deviation.

SEM: Before and after immobilization, microscopic analysis was performed using a microscope Vega 3 LMU (Tescan) scanning electron microscope (SEM). The tests were necessary to examine the porous structure of keramsite and to confirm the adhesion of *C. freundii* cells to the surface of the carriers. Analysis was performed at room temperature with tungsten filament, and an accelerating voltage of 20 kV was used to capture SEM images for both of the pure carriers samples and immobilized carriers. All specimens were viewed from the top.

Continuous process: After immobilization, carriers were rinsed with sterile 0.85% NaCl solution and transferred to sterile column set (not too closely packed) filled with sterile "M" broth. The temperature in the column was kept at 30 °C using thermo jacket connected to thermostat (Julabo, Poland). Continuous process of conversion glycerol to 1,3-PD was started with flow rate 0,3 mL/ min ($D = 0{,}36h^{-1}$) for 8 days.

Bioreactor: After immobilization, carriers were rinsed with sterile 0,85% NaCl solution and transferred to sterile bioreactor and filled broth "M". The temperature was kept at 30°C using thermo blanket. Static culture in bioreactor was started with stirring 150 rpm for 8 days.

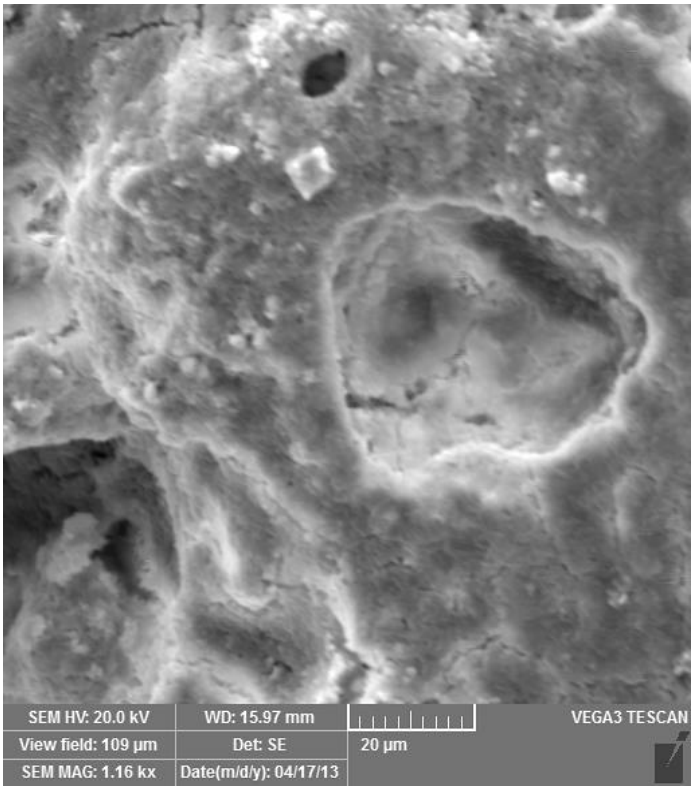
Chromatography analysis: total 1,3-PD and glycerol content was determined by HPLC (Knauer, Germany) using a Aminex HPX-87H organic acid analysis column and RI detector (Smartline S2300, Knauer, Germany). The injection volume of the sample was 10 µL. Quantitative determinations of 1,3-PD and glycerol were based on method of the external standard and calculated using the integrated computer program (Eurochrom, Knauer, Germany).The column, maintained at 25 °C, was eluted with 5 mM H_2SO_4 at a flow rate of 0,6 mL/min, samples ran for 30 minutes. Samples for chromatography analysis were taken every day after 24 h. Production of 1,3-PD and consumption of glycerol were obtained by

dividing the final concentration (g/L) by fermentation process time (h).

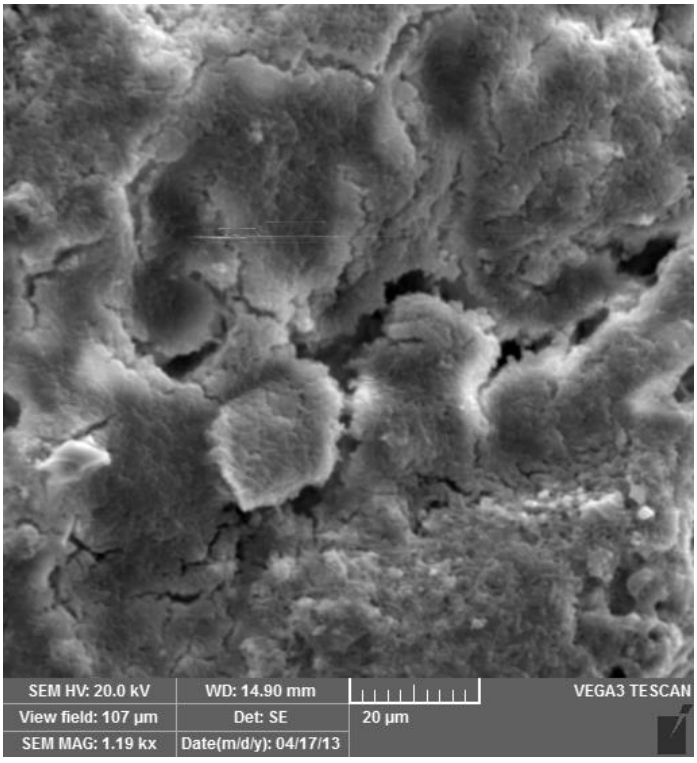
3. RESULTS AND DISCUSSION

Processes that are aimed at immobilizing of bacterial cells, are more and more widely used in the bioreactor process [12], and the immobilization using microbial adhesion, successfully found application in the manufacture of vinegar already in the eighties [13]. The adhesion may be influenced by many factors, including environment (pH, temperature) and biological (age cultures, the ability to flocculate). However, apart from the above-mentioned factors, an important parameter is the selection of a suitable media for the immobilization [14]. Selected mineral carrier is material obtained during the process of baking clay. Because of its physical properties (porosity, mechanical strength, the ability to adsorb) and relatively low price (177zł/1m^3), it has been successfully applied for the immobilization of microorganisms.

The first purpose of the study was immobilization of *C. freundii* cells on keramsite surface, the process was performed in triplicate to determine the "initial adhesion", which amounted $32 \cdot 10^7 \pm 28 \cdot 10^5$ CFU/ mL. Parallel to the chromatographic analysis, which was used to determine the concentrations of substrate and product in medium, viability of bacteria cells was determined. The results of the study using scanning electron micrographs revealed the porous structure of keramsite (Fig. 1). A network of bacteria rapidly colonized the matrix. After 24 h of immobilization the biofilm on the keramsite was macroscopically visible.



(a)



(b)

Figure 1(a,b). Scanning electron micrograph of *C. freundii* immobilized on Keramsite

Continuous production and static culture- concentration of C. freundii during processes:
The obtained results (Fig. 2) show that the highest growth of microorganisms during continuous production was observed in the first three days of the ongoing process. The highest concentration of bacteria was observed after 72 hours of bioconversion: $92 \cdot 10^7$ $\pm 12 \cdot 10^5$ CFU/mL, whereas after 5-day: $25 \cdot 10^7$ $\pm 87 \cdot 10^5$ CFU/mL. By the following days, the concentration of microorganisms in fermentation broth, remained on the same level. It was caused by the fact that during the continuous process in column, the nutrients were constantly supplied. Slight differences in the amount of microorganisms in the broth provide the correct functioning of the process and of the same good condition bacterial culture.

These results have proved that keramsite could be used as carrier for continuous bioconversion processes. The other results of vitality of bacteria cells were obtained during process of bioconversion in standard bioreactor. The highest growth was noticed after first 24 hours ($52 \cdot 10^7$ $\pm 12 \cdot 10^6$ CFU/mL). Significant differences were observed after second day of the process. The highest concentration was observed after 72 hours ($65 \cdot 10^7$ $\pm 15 \cdot 10^6$ CFU/mL). During next day, amount of bacteria cells in bioreactor was decreasing. The decrease of bacterial cells could have been caused by consumption of nutrients in fermentation broth (e.g. proteins). The decrease of bacterial concentration could have been also caused by increase of byproducts (e.g. lactic acid, acetic acid). After 120 hours of bioconversion the increase of viability of *C. freundii* has stopped, and the almost constant number of bacterial cells was

observed. These results suggest that immobilization protects bacteria against acidic environmental conditions and that the carriers with bacterial cells could be used in a next bioconversion process after total exchange of the medium.

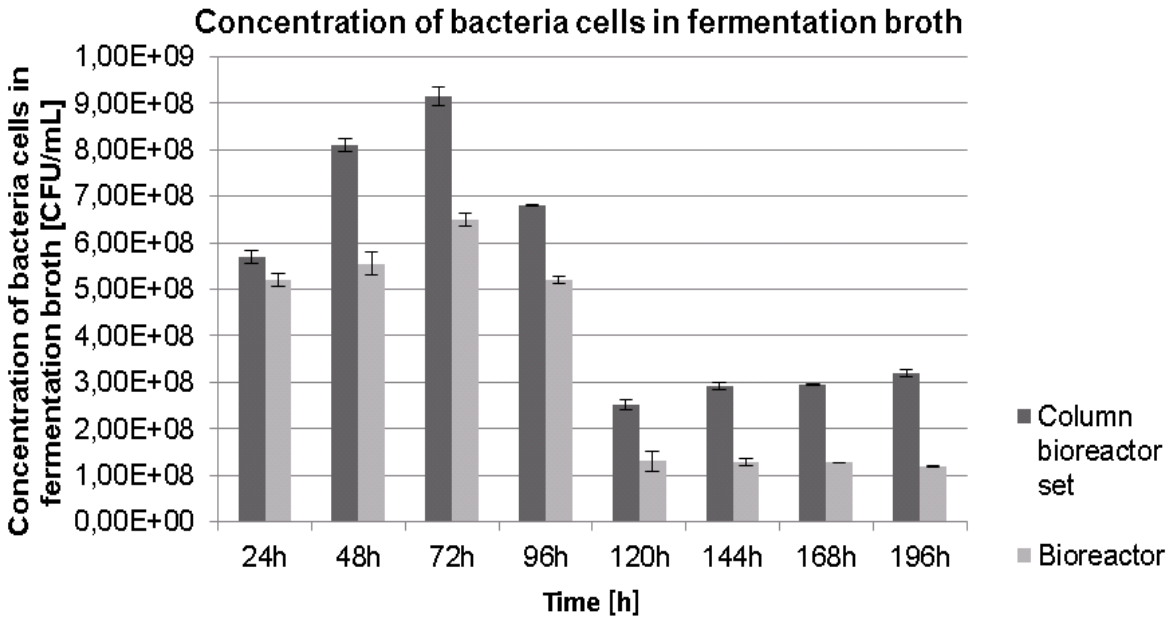


Figure 2. The viabilyty of *C. freundii* cells

Consumption of glycerol:

Chromatographic analysis performed during the bioconversion process, confirmed the high consumption of crude glycerol by the selected strain of *C. freundii.* After 24 hours of process the concentration of glycerol in fermentation broth decreased to 16,5g/L (1,4g/Lh^{-1}) in column bioreactor (Fig. 3), and to 21,1 g/L (1,2g/Lh^{-1}) in bioreactor (Fig. 3).

Significant differences in glycerol reduction were observed after 48 hours. The average consumption for glycerol during 8 days of the process was about 1,52g/Lh^{-1} in column bioreactor. It was also observed that the consumption of glycerol after 120 hours of process was almost on the same level what corresponds to the results of the viability of bacterial cells. It proved that immobilization of bacteria on porous carrier before continuous process run was appropriate step. After 96h in standard bioreactor (static culture), microorganisms used the substrate because the lack of glycerol in fermentation broth was observed. The similar results concerning glycerol consumption by bacterial cells during continuous process were obtained by Gungormusler [15]. Gungermusler immobilised *Klebsiella pneumoniae* on ceramic balls and ceramic rings and used it in continuous conversion of glycerol to 1,3-PD, average consumption of glycerol obtained by his team was for ceramic balls 31,8g/L and 33,2g/L for ceramic rings. Gonzalez-Pajuelo and his team [16] have showed that consumption of glycerol also depends on type of glycerol. Their studies showed that, consumption of raw glycerol (65%) during conversion was higher about 13%, than consumption of raw glycerol (92%) and about 24% higher than consumption of commercial glycerol (87%). It is possible to increase

consumption of glycerol, by some modifications (temperature, nutrients in broth). Luthi-Peng and his team [17] had stimulated the consumption of glycerol by *Lactobacillus reuteri*, by addition of glucose to MRS broth.

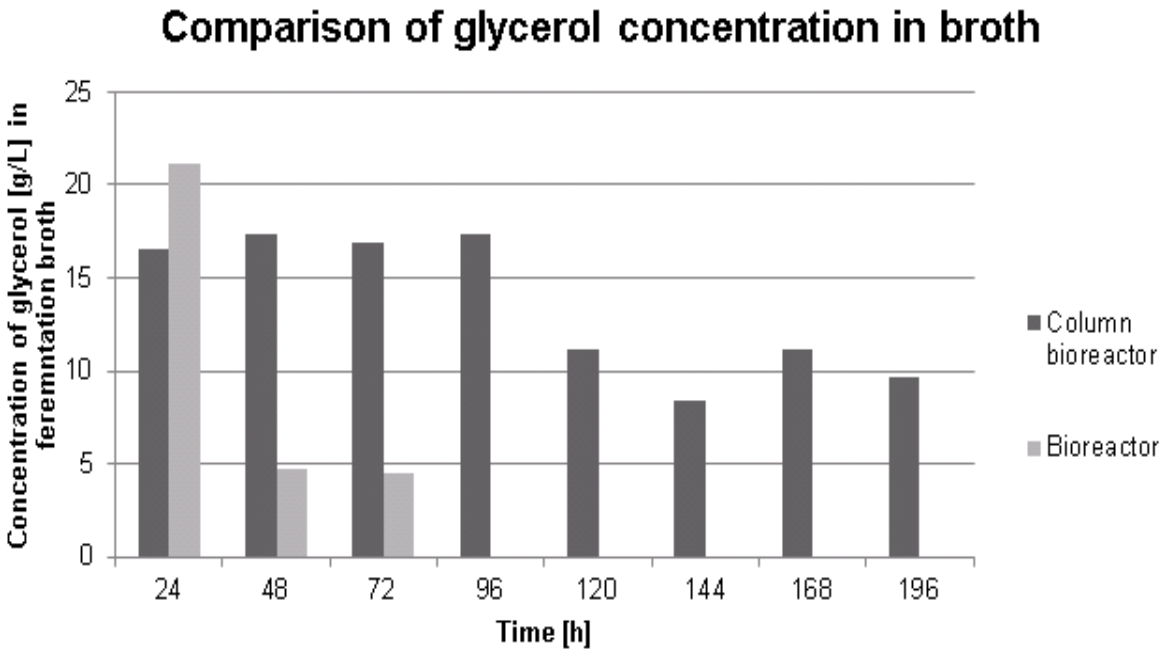


Figure 3. The concentration of glycerol

1,3-Propanediol production:

Figure 4. shows the production of 1,3-PD by *C. freundii* immobilized on keramsite during bioconversion in column bioreactor and standard bioreactor. The results showed that it is possible to obtain 11,3 g/L of 1,3-PD after first 24 hours in the case of column bioreactor and 8,5 g/L of this product in the case of standard bioreactor with static culture.

It could be noted that productivity of 1,3-PD yield (g 1,3-PD/g glycerol) was 12% higher in first system. After 48 hours the productivity of 1,3-PD was only 0,07 for standard bioreactor and 0,47 for column what proved better yield of continuous bioconversion. Also statistical evaluation confirmed significant differences between production of 1,3-PD in bioreactor and in column (tab.2). The average productivity (during 8 days) of 1,3-PD for column was 0,39 that was 26% higher than average productivity in standard bioreactor. The average production of 1,3-PD for *C. freundii* in column bioreactor was 14,1g/L, the mean daily production of 1,3-PD was 0,59g/Lh^{-1} which was 56% higher than average daily production in standard bioreactor. The results of the study have also showed that in the case of static bioreactor, 1,3-PD was produced by *C. freundii* even after 96 hours of process when the lack of glycerol in medium was observed. These experiments demonstrated that bacterial cells could use the own metabolites as a carbon source during 1,3-PD production. The results obtained after bioconversions in both conditions are tempting to suggest that adhesion of bacterial cells on surface of keramsite is proper process that could be used to immobilize of

microorganisms before bioconversion in column or in bioreactors. As will be argued below, the immobilization was key process for the others scientists [15,18]. The authors used bed-packed bioreactor- system for the conversion of glycerol to 1,3-propanediol with immobilized *Clostridium beijerinckii* strain [18] and *Klebsiella pneumoniae* [15]. The results obtained by them demonstrated that immobilization increases the yield of the process compared to cell-free culture (yield doubled). Although bioconversion processes had different parameters and different bacterial strains had been used, the production of 1,3-PD was similar to obtained results. Average production of 1,3-PD for *Klebsiella pneumoniae* [15] was approximately 13,4g/L for bacteria immobilized on ceramic rings and about 13,7g/L for ceramic balls. The differences were observed in productivity. The average productivity of 1,3-PD for *Klebsiella pneumonia* was 22% higher than *Citrobacter freundii* what can be caused by different HRT value (HRT for *C. freundii* was 2,8h while for *K. pneumoniae* was 4h).

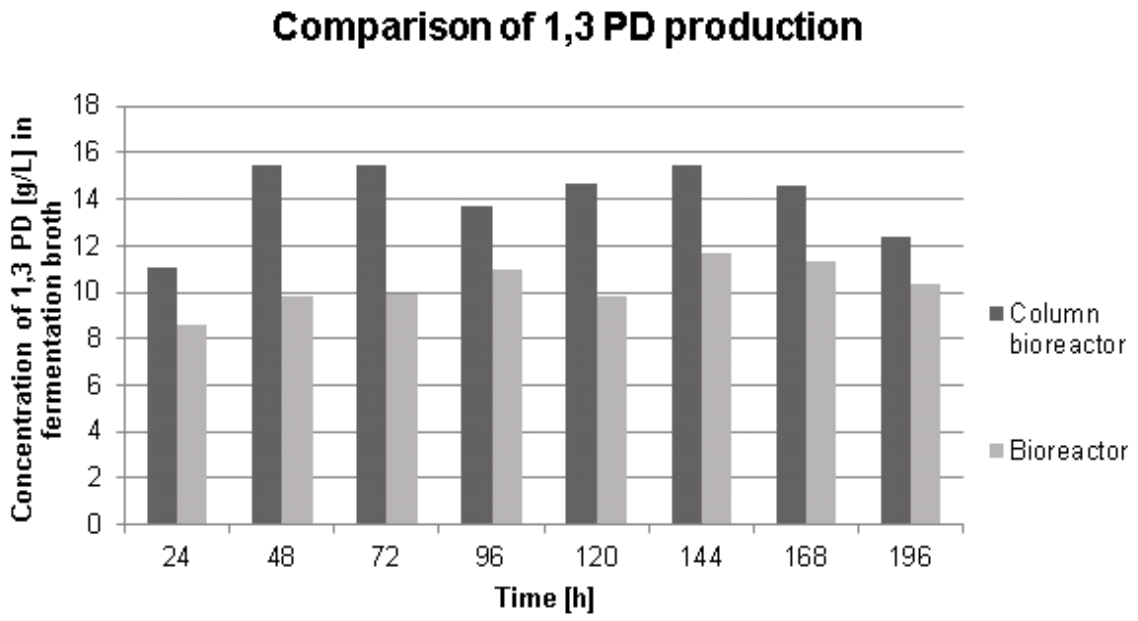


Figure 4. The concentration of 1,3-PD

Similar results on the yield of bioconversion 1,3- propanediol by immobilized *C. freundii* were obtained by using immobilized *Clostridium beijerinckii*. Although in case of *C. beijerinckii* the used HRT was 16h, productivity was the same as obtained by *C. freundii* 0,39 with HRT 2,8. It shows that productivity of bioconversion process is influenced by many factors. Papanikolaou [6] demonstrated that 1,3-PD production was closely related to the cell growth and is a growth-associated by-product. Gonzalez-Pajuelo [17] has shown that initial concentration of glycerol has impact on production of 1,3PD. He doubled production of 1,3-PD from 1,59g/Lh^{-1} to 3,1g/Lh^{-1} by increased addition of glycerol about 100%. The impact initial concentration of glycerol on production of 1,3-PD has been also confirmed by Zhao [19].

Zhao and his team caused increasing of productivity of 1,3-PD from 2,96g/Lh^{-1} to 5,74g/Lh^{-1} by 3 fold increase concentration of initial glycerol. Papanikolaou [20] confirmed that high inlet substrate concentration positively affected the biosynthesis of organic acid. Due to the fact that production of 1,3-Propanediol is closely related to cell growth and as Gungormusler [15] demonstrated, 1,3-PD production rates were higher in immobilized cells than suspended cells, the immobilization has become increasingly applied. Despite the fact that Pflugmacher and Gottschalk [21], Zhao [19] and Mizielińska [22,23] reported significantly higher 1,3-PD production rates using adhesion on PUF and gel entrapment, especially on PUFs with additives as wood chips and peanut shells, the use of mineral carriers seems to be good alternative. Lower cost of such mineral carrier and possibility of multiple using, can decrease cost of the processes.

As was emphasized in this study the productivity of 1,3-PD yield obtained in column bioreactor was more efficient than feed-batch process in which standard bioreactor was used. Summarizing the feed-batch process it can be concluded that the use of keramsite for the immobilization of *C. freundii* cells was important because it could have make production in long-term operations possible. As was reported by authors [22,23] reiterated fed-batch bioconversion by using immobilized strains could be not so time-consuming and not as expensive as conversion in bioreactor containing free cells.

4. CONCLUSIONS

- The results have shown that bioimmobilization of *C. freundii* strain on surface of keramsite is proper process for bioconversion of raw glycerol to 1,3-propanediol using bed-packed (column) bioreactor and the static culture in standard bioreactor.
- The viability of bacterial cells affects on yield of bioconversion process significantly.

Acknowledgments

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