Continuous Production of 1,3-Propanediol Using Waste Glycerol with *Clostridium beijerinckii* NRRL B-593 Immobilized on Glass Beads and Glass Rushing Rings

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Among the economically viable and ecologically acceptable solutions for the safe disposal of waste glycerol coming from biodiesel production, the biotechnological conversion of glycerol into a very high-value-added bioplastic raw material, namely 1,3 propanediol (1,3-PDO) seems to be very promising. This process is easy and environmentally friendly since it produces no toxic wastes. The results indicate that glycerol by-product is as amenable for 1,3-PDO production as pure glycerol. The immobilization process has important advantages such as smaller reactor volumes, shorter fermentation times, and higher yields. This work involves the study of 1,3-PDO production with industrial raw glycerol via immobilized culture of *Clostridium beijerinckii* B-593 on glass beads and glass rushing rings with industrial glycerol. Thereby, maximum volumetric productivity of 7 g L^{-1} h^{-1} corresponding to a product concentration of 13 g L^{-1} was achieved at 2 hours of hydraulic retention time (HRT) on glass beads. It was demonstrated that continuous production of 1,3-PDO from waste glycerol is feasible and the immobilized bioreactor systems outcompeted the conventional suspended culture bioreactors in terms of both productivity and operational stability.

Key words:
1,3 propanediol, biopolymer, glycerol, biodiesel, immobilization, *Clostridium beijerinckii*

Introduction

Due to the significant worldwide interest in biofuels, there has been an outburst in biodiesel production, which has resulted in vast amounts of waste glycerol (10 % of total production by weight). Even though glycerol has many industrial uses such as pharmaceutics, cosmetics etc., the market is already saturated due to the tremendous amount of the by-product glycerol, and the invaluable part of this glycerol has become a waste material resulting in the most important environmental problem of biodiesel producers. The aim of this study was to produce a value added chemical that has significant environmental benefits. 1,3 propanediol (1,3-PDO) is a primary and basic organic monomer which is mainly produced in refineries.[1] 1,3-PDO is an important monomer for the synthesis of polymers, especially the new type of textile industry polymer, polytrimethylene terephthalate. It has long been known that a polymer prepared from terephthalic acid and 1,3-PDO has highly promising properties for large volume markets. This polymer called polytrimethylene terephthalate (PTT) is related to polyethylene terephthalate (PET) and polybutylene terephthalate (PBT) and is an important member of fiber industry. In addition, 1,3-PDO has been used mainly as a chemical intermediate in the production of polyester, polyether, and polyurethane.[2–4] Microbial 1,3-PDO production is of increasing interest as a result of the growing volume of glycerol generated by the oleo chemical industry. 1,3-PDO can be produced by fermentation of glycerol by *Klebsiella, Citrobacter, Clostridium, Enterobacter* and *Lactobacillus.*[5–9] In comparison to traditional methods for 1,3-PDO production, which generate toxic waste streams, the microbial pathway provides an attractive alternative.[10–11] For microbial 1,3-PDO production, the main metabolic pathway consists of two steps. These are, respectively, the oxidation and reduction steps. The 1,3-PDO metabolic pathway is induced only in the presence of glycerol. Total reaction takes two steps: first the glycerol converts 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratese, and second the NADH-dependent 1,3-PDO oxidoreductase converts 3-HPA to 1,3-PDO. Intracellular redox potential is one of the main factors that affect the synthesis of 1,3-PDO. Formation of 1,3-PDO provides balanced intracellular redox potential and glycerol deyhrogenase which is the first step of the oxidative branch of the glycerol metabolic pathway. This way, the microorganisms can use glycerol as a car-

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bon source.[12–14] Unlike the *Klebsiella* species, *Clostridium* species synthesizes glycerol dehydratese independent from the coenzyme B12.[6, 15] The use of immobilized cell culture systems has many advantages over suspended cells, such as high cell inventory in the reactor, prevention of cell washout, robustness in bio-production. Larger surfaces resulting in higher microbial inventory by immobilization techniques also provide shorter reaction times in comparison to suspended culture bioreactors. Therefore, higher yields and production rates in less reactor volumes with shorter fermentation times are possible.[16] There is a limited number of immobilization studies in the literature. Jun et al. (2011) reported 1,3-PDO productivity of 1.61 g L^{-1} h^{-1} with immobilized culture of *K. pneumonia.*[17] Zhao *et.al* (2006) used encapsulated *K. pneumonia* and reported a productivity of 4 g L^{-1} h^{-1}.[18] Papanikolaou *et al*. (2007) studied suspended cell culture system in which 1,3-PDO productivity of 1.33 g L^{-1} was achieved by *Clostridium sp*. Papanikolaou *et al.* (2005) also studied a two-stage suspended continuous fermentation with *Clostridium butyricum* strain and obtained 5.5 g L^{-1} h^{-1} volumetric productivity.[19–20] Immobilized culture of *Citrobacter freundii* DSM 30040 on modified polyurethane carrier particles was used for continuous glycerol fermentation, and a productivity of 8.2 g L^{-1} h^{-1} for 1,3-PDO was reported.[21] There are also some literature reports in which a productivity of 3.0 g L^{-1} h^{-1} was reported with genetically modified organisms (*Clostridium sp*.).[1,21] 1,3-PDO could also be produced via genetically modified organisms such as *E. coli* strain from glucose to directly 1,3-PDO as it is done by DuPont and Genencor International, Inc. So far, this is the only example of successful commercialization of the biotechnological 1,3-PDO production. The base strain, *E. coli* K12, produces 1,3-PDO at titers of over 130 g L^{-1}.[22] In this paper, the immobilization capacity of *C. beijerinckii* B-593 on glass beads and glass rushing rings with industrial glycerol was comparatively studied. The volumetric productivities were found to be approximately 4 times higher than those of suspended cultures. This is the first time that immobilized cell culture of *C. beijerinckii* B-593 is reported for continuous 1,3-PDO production with glass bead and glass rings.

Materials and methods

Strains and sources of microorganisms

Clostridium beijerinckii NRRL B-593 was kindly provided by ARS Culture Collection (NRRL), USA. The microorganism was activated from lyophilized cultures in Differential Reinforced *Clostridial* Broth (DRCM) and then incubated anaerobically at 37 °C for 2 days.

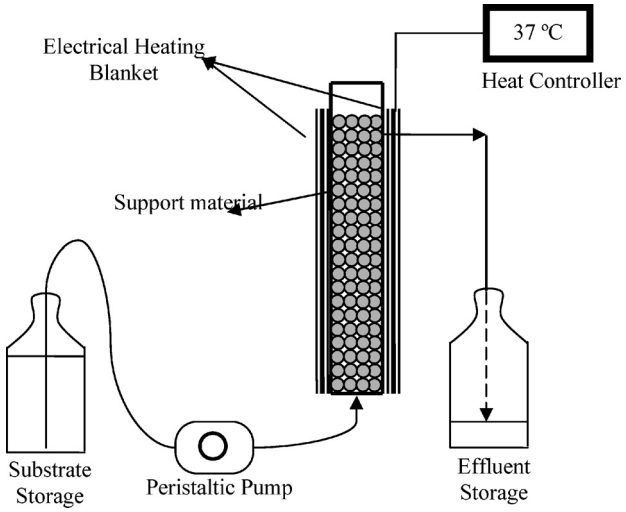
Media and growth conditions

The fermentation medium used in each study consisted of (g L^{-1}) 45 waste (industrial) glycerol as substrate, 1 K_2HPO_4, 0.5 KH_2PO_4, 2 $(NH_4)_2SO_4$, 0.2 $MgSO_4 \cdot 7H_2O$, 0.015 $CaCl_2 \cdot 2H_2O$, 1 yeast extract, 0.005 $FeSO_4 \cdot 7H_2O$, 2 $CaCO_3$, and 2 ml L^{-1} trace element solution. The trace element solution consisted of (mg L^{-1}) 70 $ZnCl_2$, 100 $MnCl_2 \cdot 4H_2O$, 60 H_3BO_3, 200 $CoCl_2 \cdot 2H_2O$, 20 $CuCl_2 \cdot 2H_2O$, 25 $NiCl_2 \cdot 6H_2O$, 35 $Na_2MoO_4 \cdot 2H_2O$, and 0.9 ml L^{-1} HCl (37 %). [23]

Reactor configuration and Immobilization

A packed-bed column reactor, 30 cm high with an i.d. 4.5 cm (total volume of 280 ml), was filled with glass beads (3.0 mm radius), and a second column reactor having the same dimensions was filled with glass rushing rings (10 mm radius and 10 mm height) as the immobilization support (Fig. 1). The working volume of the bioreactors filled with glass beads and rushing rings were 110 ml and 230 ml, respectively. In addition to these bioreactors, another column reactor with no immobilization support was also operated as a control reactor in order to compare the 1,3-PDO productivity. The total

Fig. 1 – *Glass Beads and Glass Rushing Rings support materials and plug flow fermentor view before immobilization process for continuous 1,3-PDO production*



F i g . 2 – *Schematic diagram of the immobilized systems used in this study*

weight of the immobilization materials was 407.7 g for glass beads and 141.6 g for rushing rings, respectively (corresponding to equal total surface area in each bioreactor). Fig. 2 shows a schematic diagram of the system. After washing twice with distilled water, all immobilization materials were left to dry overnight at 37 °C. Sterilization was done by autoclaving (121°C, 1 atm, 30 min). The reactors were allowed to cool down, and were then fed with sterile culture medium (DRCM-Differential Reinforced *Clostridial* Medium). Inoculation of the fermenters was done by injecting 1 % (v/v) of an anaerobic (10 h) culture of *C. beijerinckii* B-593. In order to establish immobilized cells on the material, the reactors were continuously recycled with a dilution rate (D) of 0.05 1 h^{-1} for 1 week. The system was incubated at 37 °C with heating blankets. After attachment process was completed, all the reactors were continuously fed by fermentation medium. The continuous production of 1,3-PDO was initialized at the 260th hour of the whole process. During the fermentation period, hydraulic retention times (HRT) varying between 2 – 16 h were examined for 40 days.

Substrate preparation and use

Waste glycerol was obtained from a biodiesel production plant in Torbali, Izmir, Turkey. According to information from the manufacturer, biodiesel production process is based on base catalyst. The substrate was kept at 4 °C until used. Before each use, crude glycerol was heated up to room temperature (25 °C) in order to defrost. High Pressure Liquid Chromatography (HPLC) was used to determine the glycerol content, which was found to be 50 % (w/w) as verified by the manufacturer. No pretreatment except for dilution to the desired feeding concentration was done. Results showed that crude glycerol was suitable for use in microbial production and that there was no need for pretreatment. This stock solution was diluted down to the required initial substrate concentration (~45 g L^{-1} glycerol) before feeding for fermentation of 1,3-PDO.

Analytical methods

The samples collected from the bioreactors were centrifuged at 10000 rpm for 15 minutes before analysis. The supernatant after centrifugation was filtered through 0.22 μm filters. The residual glycerol and the products such as 1,3-PDO, 2,3-BD, lactic acid, acetic acid, succinic acid, and ethanol were measured by high performance liquid chromatography (HPLC) (Agilent 1100) with an Phenomenex Rezex RHM Monosaccharide (H+) 300 x 7.8 mm ion exchange column, using an Agilent 1100 Series G1362A Refractive Index Detector. The column temperature was 65 °C, detector temperature was 45 °C, and the injection volume was 15 μL. A solution of 5 mM H_2SO_4 was used as mobile phase at 0.8 ml min^{-1} flow rate. BET surface area method was used for surface area measurement of glass beads. Total suspended solids (TSS) measurements were carried out in accordance with Standard Methods.[24] In order to determine the biomass inventory throughout the reactor (suspended section, immobilized section and reactor walls), all the reactors were first emptied, and then all the support materials and walls of reactors were rinsed with Phosphate buffered saline (PBS) three times to remove all the biomass from surfaces. Furthermore, sonication was also applied for any remaining biomass on the surfaces. Cell attachment was documented with scanning electron microscope (SEM) (Philips XL-30S FEG/FEI Quanta250 FEG). All the samples were coated with gold palladium using a Magnetron Sputter Coating Instrument before analysis in Izmir Institute of High Technology.

Results

Glass beads and glass rushing rings have been used as immobilization materials for many biotechnological applications except for 1,3-PDO fermentation.[25–27] Glass beads and glass rushing rings possess suitable surface characteristics and also provide enhanced total surface area in the bioreactor. They are especially suitable for effective microbial colonization and immobilization in order to achieve higher productivities of 1,3-PDO in smaller reactors; it is a novel way of immobilizing active biomass of *C. beijerinckii* B-593.[28,29] ORP measurement was performed online on the effluent line of

fermenter (without contact with air). ORP (oxidation reduction potential) measurements indicated that the fermentation medium was sufficiently anaerobic (–300±50 mV; Thermo-scientific αlpha-pH800)

The pH in the bioreactor was kept around 6.0–6.5 during fermentation (data not shown). The inoculation of each bioreactor was carried out with biomass coming from suspended culture system, continuously operated in our lab. Fig. 3 shows the growth curve of *C. beijerinckii* B-593, grown in the DRCM at 37 °C. *Clostridium sp.* are known to be resistant to impurities like methanol free fatty acids and salts coming from biodiesel. [30] As seen in Fig. 3, the organism showed smooth growth following a 2h lag period. The biomass needed for inoculation of each packed-bed and suspended culture bioreactors were harvested at the end part of the logarithmic growth phase (8–10 h). After inoculation, cell counts were also carried out in suspended culture (effluent of the immobilized reactors) in order to follow the immobilization (Fig. 4). A decrease in cell count from the suspended part of reactor indicated that a significant portion of the inoculation had immobilized, which was also monitored by the decrease in the suspended solid matter in the effluent of the reactors. All these measurements indicated that the immobilization step of fermentation was successful. Fig. 5 illustrates the SEM images of glass surfaces before and after immobilization. SEM images showed that immobilization was successful and support the results given above (Fig. 4). It was seen that greasy structure of glycerol resulted in blurry images. *C. beijerinckii* is rod-shaped bacteria and under greasy structure this shape is distinctive. Immobilization was calculated by considering the cell inventory on the support material, reactor surface and suspended part of bioreactor at the end of the study. Table 1 shows immobilization ratio for both of the support materials used in this study. The immobilization ratio of 99.5 % shows that the immobilization process was very successful.

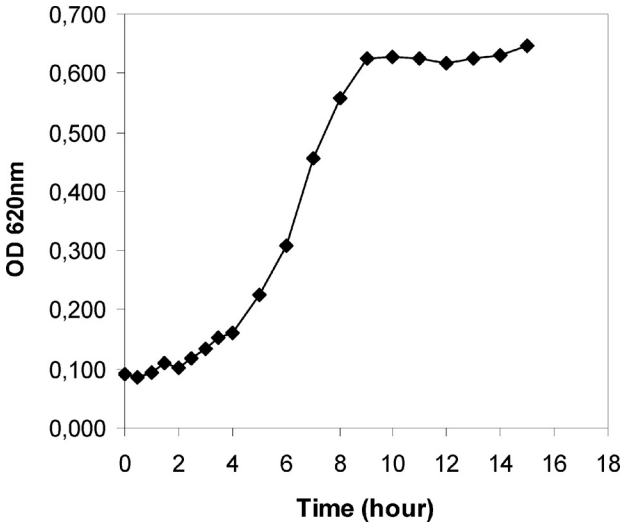


Fig. 3 – *Growth curve of C. beijerinckii NRRL B-593*

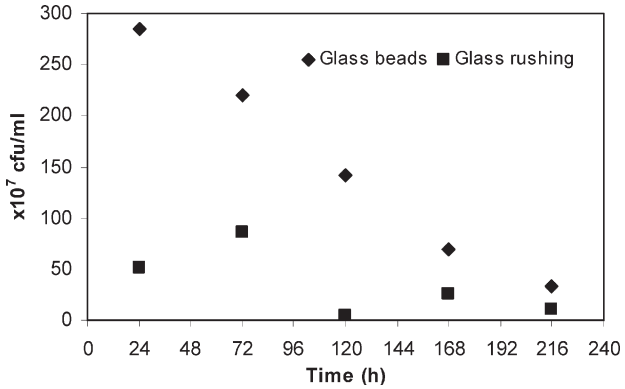


Fig. 4 – *Biomass concentration changes in suspended culture during immobilization*

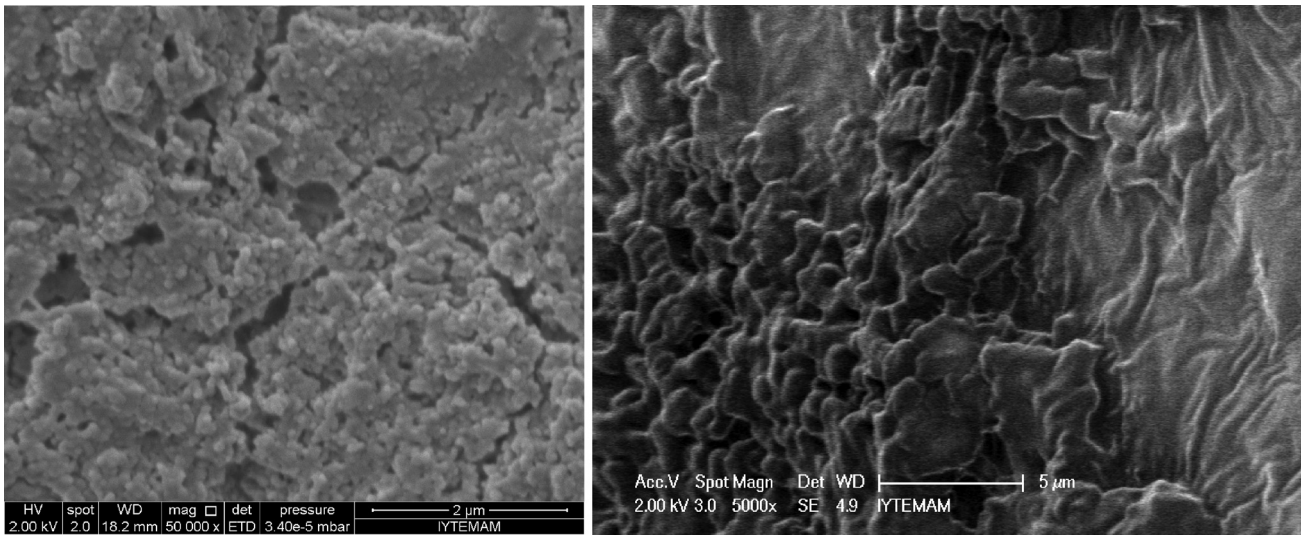


Fig. 5 – *SEM images of glass surfaces (Left: before use, right: after immobilization)*

Table 1 – *Immobilized and suspended portion of each immobilized bioreactor as biomass mg L^{-1}*

	Suspended Part	Immobilized part	Immobilization ratio (%)
Glass rushing rings	877	146204	99.4
Glass beads	1033	207302	99.5

Fig. 6 shows the average 1,3-PDO concentrations obtained at different HRT conditions for each reactor, namely; glass bead, glass rushing ring and suspended culture reactor (up-flow reactor). As seen in Fig. 6, in terms of 1,3-PDO concentrations, the highest 1,3-PDO was produced at HRT of 8 h for all reactors. On the other hand, the suspended cell culture system resulted in the lowest 1,3-PDO concentration (almost twice less than immobilized bioreactors). The highest 1,3-PDO concentrations at 8 h HRT were measured as 28, 30 and 24 g L^{-1} for glass beads, glass rushing rings and suspended bioreactors, respectively. As the hydraulic retention time increases from 8 h up to 12 h, product concentrations started to decrease and reached the lowest values at HRT of 16 h for all immobilized bioreactors, and the suspended bioreactor, which seems to prefer longer retention times. It was seen that 1,3-PDO production was adversely affected by long HRT's. Also, Fig. 6 shows that immobilized fermenters are more robust than the suspended fermenter as expected. Interestingly, all immobilized bioreactors produced almost the same level of 1,3-PDO at HRT of 2 h, while the suspended culture bioreactor was vulnerable to cell washout due to the short retention times in the bioreactor. Fig. 7 shows the volumetric 1,3-PDO productivities. It is quite clear that as the HRT decreased, the volumetric productivities for all bioreactors increased. Fig. 7 clearly shows that 2 hours HRT provides the highest productivity values for all bioreactors. The highest volumetric productivity was obtained with

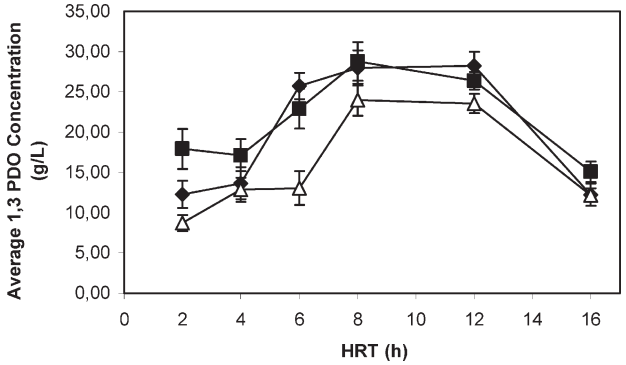


Fig. 6 – *Average 1,3-PDO concentrations at different HRTs for all immobilized and non-support material suspended fermenter. (square: rushing rings, diamond: glass beads, empty triangle: suspended)*

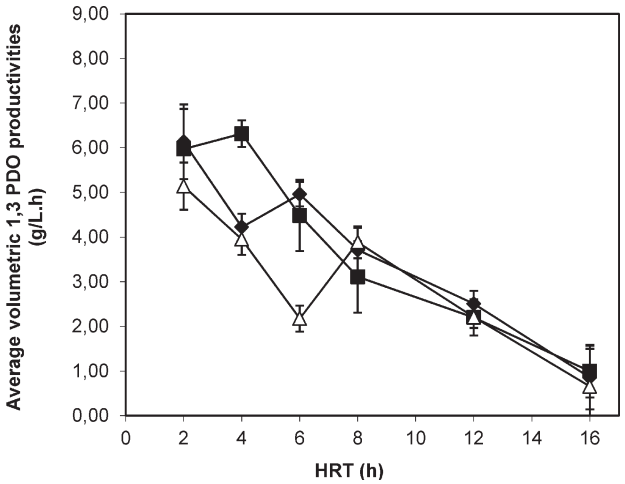
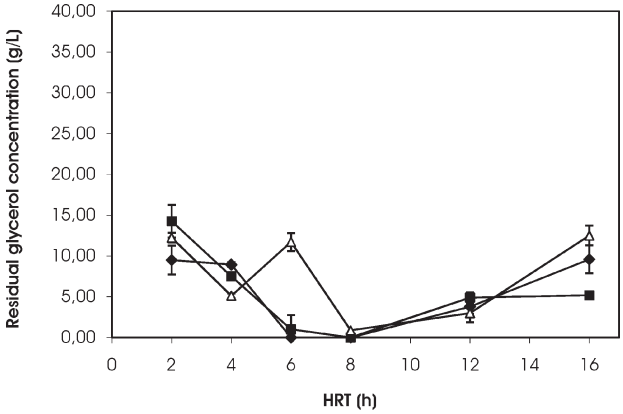


Fig. 7 – *Average volumetric 1,3-PDO productivities of the immobilize and non-support material suspended fermenters. (square: rushing rings, diamond: glass beads, empty triangle: suspended)*

glass rushing rings, which reached a maximum value of 6.4 g L^{-1} h^{-1} at 2 h HRT. Glass beads and suspended bioreactor showed almost identical volumetric productivity values; 6.1 and 5.0 g L^{-1} h^{-1}, respectively. On the other hand, suspended culture bioreactor is susceptible to cell washout, and therefore needs longer thus larger bioreactor volumes. Ceramic rings and pumice stone support material were studied for 1,3-PDO production with *C. beijerinckii* by Gungormusler *et.al* (2011)[31] and similarly the maximum 1,3-PDO concentration was reached at 30 g L^{-1}at 8 h HRT. In parallel to this study, high volumetric productivity value was observed at short HRT with other support material by Gungormusler *et.al* (2011) [31]. The highest volumetric value was 6.4 g L^{-1} h^{-1}, while the study made by pumice stone material present 12 g L^{-1} h^{-1}. Pflugmacher and Gottschalk (1994) reported similar observations for 1,3-PDO production with immobilized bioreactors.[16] M. Gonzalez-Pajuelo et al. (2006) also obtained parallel results in which shorter hydraulic retention times resulted in higher 1,3-PDO productivities with continuous culture of *Clostridium sp*. [6] Both the literature reports and the results obtained in this study clearly indicate that HRT plays an important role as an operational parameter. Papanikolaoua et al. (2007) reported 1,3-PDO productivity value of 1.3 g L^{-1} h^{-1} for continuous culture of *Clostridium sp*. and the recombinant *E. coli* produces 1,3-PDO at the rate of 3.5 g L^{-1} h^{-1}, titer of 135 g L^{-1}, and a weight yield of 51 % in D-glucose fed-batch 10 L fermentations. [1] Typical results obtained by wild-type strains in anaerobic fermentation of glycerol 3.0 g L^{-1} h^{-1}, 78 g L^{-1}, and 55 %, respectively. Undoubtedly, this is the most successful application of metabolic engineering in 1,3-PDO production, which are signifi-

cantly lower than those obtained in this study. It is obvious that immobilized systems are more robust in terms of resistance to microbial wash out.[22] Gungormusler *et.al* (2011) reported that *Clostiridium beijerinckii* produced 10 g L^{-1} 1,3-PDO from 40 g L^{-1} initial crude glycerol in suspended batch reactor. Yield and volumetric productivity were 0.60 mol mol^{-1} and 0.5 g L^{-1} h^{-1}, respectively.[32] These data showed that 1,3-PDO production via immobilized cell culture presented higher performance in terms of volumetric productivity and robustness.

Table 2 shows glycerol consumption ratio, product transformation ratio as mol 1,3-PDO mol^{-1} glycerol, by-products and pH values. As seen in Table 2, shorter HRT values resulted in lower glycerol consumption in parallel to the study by Biebl (2001).[33] On the other hand, HRT between 6 and 12 h resulted in almost complete utilization of glycerol (Fig. 8) in immobilized bioreactors, while the suspended bioreactor could not consume as good as the immobilized ones. Table 2 also shows the production of volatile fatty acids and alcohols at varying HRT values. As seen in Table 2, main by-products are acetic acid, lactic acid, succinic acid, 2,3 BD and ethanol, which need to be separated during the purification of 1,3-PDO from the fermentation broth. In parallel to the previous study by Gungormusler *et.al* (2011), intermediate production was also monitored at trace amount at short HRT (2,4 and 6 h).[31] As seen in Table 2, ethanol formation occurred. Chatzifragkou *et.al* (2010) reported that the ethanol addition did not affect the microbial bioconversion of glycerol into



F i g . 8 – *Residual glycerol concentration for different HRTs at the immobilized and non-support material suspended fermenter. (square: rushing rings, diamond: glass beads, empty triangle: suspended)*

T a b l e 2 – *1,3-PDO yield and byproducts at different HRTs*

Immobilized Material	HRT (h)	1,3-PDO Yield mol 1,3PDO/ mol Glycerol	Glycerol Consumption ratio	HSuc (g L^{-1})	HLac (g L^{-1})	HAc (g L^{-1})	2,3BD (g L^{-1})	EtOh (g L^{-1})	pH
Glass Beads	16	0.42	79	0.72	0.00	0.00	0.00	2.00	6.3
	12	0.69	92	1.08	0.48	0.61	0.35	4.66	5.5
	8	0.65	100	0.27	0.00	0.00	0.00	5.81	5.9
	6	0.35	100	0.00	0.00	0.00	0.00	3.51	6.3
	4	0.43	80	0.00	0.00	0.00	0.00	0.00	5.8
	2	0.30	78	0.00	0.00	0.00	0.00	0.00	6.4
Glass Rushing Rings	16	0.46	89	0.37	0.00	0.06	0.00	8.13	6.4
	12	0.73	98	0.53	0.67	0.74	0.00	0.00	5.8
	8	0.77	100	0.14	0.00	0.00	0.00	0.00	5.8
	6	0.66	93	0.00	0.00	0.00	0.00	0.00	6.9
	4	0.55	83	0.00	0.00	0.00	0.00	0.00	6.5
	2	0.66	73	0.00	0.00	0.00	0.00	0.00	6.5
Suspended	16	0.46	72	0.53	0.00	0.00	0.56	8.03	6.1
	12	0.77	93	0.97	1.47	0.76	1.48	2.53	5.8
	8	0.77	98	0.32	0.00	0.00	1.22	0.00	5.8
	6	0.55	74	0.00	0.00	0.00	0.00	0.00	8.1
	4	0.34	89	0.00	0.00	0.00	0.00	0.00	6.2
	2	0.38	73	0.00	0.00	0.00	0.00	0.00	6.9

HSuc: Succinic acid, HAC: Acetic acid, HLac:Lactic acid, 2,3 BD: 2,3 Butanediol, EtOH:Ethanol

1,3-PDO, even when imposed at relatively high concentrations in batch-bioreactor operations.[30] Lactic acid and 2,3 BD, which are the main by-products of 1,3-PDO production are also the main regenerator of NAD+. Alkaline fermentation medium inhibit 2,3 BD formation and lactic acid becomes primary by-product as lactate.[34] In the oxidative pathway, which is responsible for the energy and supply of biomass precursors, in order to decarboxylate pyruvate and to form acetyl-CoA, *Clostridium* species utilizes pyruvate:ferredoxin 2-oxidoreductase (CoA-acetylating), instead of pyruvate dehydrogenase with NAD as a cofactor. Thereby, this process generates NADH to be used in the 1,3-PDO formation pathway. 1,3-PDO formation in the cell utilizes NADH from the oxidative glycerol pathway so intracellular redox potential is balanced.[13,35] Table 3 shows the comparison of literature and experimental value in terms of productivity and yield. The yields obtained in this study are similar to literature of both immobilized and suspended process. Maervoet[36] reported that the maximum theoretical yield calculated for the anaerobic fermentation of PDO from glycerol as sole carbon source is 0.875 mol mol^{-1} glycerol. The yields obtained in this study are below this maximum value.

As a result, it was demonstrated in this study that immobilized cells could further enhance the productivity of the entire process in parallel to some literature reports.[16,17,37] Furthermore, the immobilization materials reported in this study are very suitable for the purpose of 1,3-PDO production

Conclusion

Vast production of 1,3-PDO at industrial-scale is needed and research on the biotechnological 1,3-PDO production have been intensively carried out as it is for this study. In this regard, valorization of crude glycerol into a value-added chemical, namely 1,3-PDO is important. The majority of the literature work aimed to enhance the 1,3-PDO production in a way to increase the volumetric productivity and to obtain high product concentration. [19, 22, 30] In this study, 1,3-PDO production from waste glycerol using immobilized *C. beijerinckii* B-593 was comparatively studied. For this purpose, both immobilized and suspended bioreactor configurations were comparatively tested under continuous operating conditions. It was concluded that immobilized bioreactor configuration outcompeted its suspended counterpart in terms of both volumetric productivity and operating stability (cell washout). It was also demonstrated that it is possible to produce 1,3-PDO effectively in 4 times smaller bioreactors using immobilized bioreactor configuration in comparison to suspended, which resulted in twice less 1,3-PDO volumetric productivity. In addition, it was also concluded that both glass materials are quite suitable for the purpose of immobilization of *C. beijerinckii*. Furthermore, HRT is a significant operational parameter directly affecting the bioreactor performance. Immobilized cell system not only provides stable productivity but also continuous production of 1,3-PDO in a smaller reactor, which eliminates higher capital costs.

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Table 3 – *Comparison between immobilized and suspended production of the present study and reported in the literature*

Immobilized / suspended	Max. Volumetric productivity g 1,3PDO/h	Yield mol 1,3PDO/mol glycerol	Microorganism	Condition	Reference
Glass Bead	6.10	0.69	*C. beijerinckii*	Anaeorobic	this study
Glass Ruching	6.40	0.77	*C. beijerinckii*	Anaeorobic	this study
Ceramic ring	7.90	0.78	*C. beijerinckii*	Anaeorobic	31
Pumice Stone	12.00	0.79	*C. beijerinckii*	Anaeorobic	31
Suspended	5.10	0.71	*C. beijerinckii*	Anaeorobic	31
Suspended	5.0	0.77	*C. beijerinckii*	Anaeorobic	this study
Suspended	1.66	0.69	*C. butyricum*	N2 infusion anaerobic	38
Suspended	1.66	0.70	*C. butyricum*	Self-generated anaerobic	38
Suspended	3.47	0.75	*C. butyricum*	Anaerobic	39

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