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ETHANOL FERMENTATION WITH YEAST CELLS IMMOBILIZED ON GRAINS OF POROUS CERAMIC SINTER

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A study was undertaken to analyse the kinetics of the course of processes (batch, feed-batch and continuous) of ethanol fermentation with cells of *Saccharomyces cerevisiae* yeast immobilized on porous ceramic sinter and with free cells. The fermentation with free cells was treated as a reference process. Cells were immobilized with the use of porous ceramic sinter in the form of grains. Three grain fractions were applied: 1.2÷2.5; 2.5÷6.0; and 6.0÷10.0 mm. The study demonstrated that dynamics of batch processes with immobilized and free cells were similar in the logarithmic phase. In contrast, significant differences occurred in the initial phase. For the immobilized cells that period spanned considerably longer than for the free cells. In addition, it differed for each fraction of the support – being the shortest for the smallest grains and the longest for the biggest ones. Analyses of the feed-batch process demonstrated that the best kinetic parameters were obtained for the support with grain size of 2.5÷6.0 mm. The kinetics of the process was also found to depend on hydrodynamic conditions occurring in a reaction – flow rate of liquid through the layer of poured support. In turn, the continuous fermentation was carried out with the support's fractions characterized by the best kinetic parameters of feed-batch fermentation – 2.5÷6.0 mm in size. During the fermentation process, the concentration of yeast in the reaction medium was high, *ca.* 14 g/L – including *ca*. 13 g/L of immobilized cells. The high concentration of biomass enabled reaching reactor's productivity at a level of *ca.* 13 g/Lh, as compared to 2.7 g/Lh noted for the reactor with free cells (under the same conditions).

INTRODUCTION

Application of bioreactors with immobilized cells is one of the cost-saving means in ethanol production [Luong & Tseng, 1984; Harshbarger, 1995]. Those bioreactors are characterised by high productivity – as compared to batch and continuous fermenters operating with a biocatalyst in the free form [Demuyakor & Ohta, 1992]. High productivity is reached due to a high concentration of microorganism in the reaction medium. The process of immobilization consists in the physical binding of cells with an immobilized carrier or their accumulation in a constrained space but with maintaining their activity and viability. A number of methods for yeast immobilization have been known and described [Kourkoutas *et al.*, 2004; Mustranta, 1987]. Yet, not all of them are applicable. Depending on the method, immobilization evokes a variety of deviations in basic vital functions of cells [Bandyopadhyay & Ghose, 1982; Nawarro & Durand, 1977]. For these reasons, ceramic materials are often a focus of interest in the aspect of immobilization of biocatalysts [Cibis *et al.*, 2002; Horitsu & Morishita, 1992; Loukatos, 2000]. Special properties are also featured by aluminum ceramics [Kanellaki *et al.*, 1989; Zboromirska-Wnukiewicz, 2002]. Ceramic materials are characterised by high porosity and specific internal structure of pores. In heterogenous systems, including ethanol fermentation catalysed by yeast immobilized on a porous support, the rate of the whole process is determined, to a great extent, by diffusion [Zhang & Kennedy, 1996]. In the case of a porous support, two types of diffusion occur – internal and external one [Radovich, 1985; Tanaka, 1984]. The internal diffusion is linked with mass transfer inside the support, whereas the external one – with overcoming resistances in the boundary layer on the surface of the support.

The research was aimed at determining the effect of the size of ceramic sinter particles and hydrodynamic conditions on the productivity of a reactor and kinetics of ethanol fermentation catalysed by immobilized cells of yeast *Saccharomyces cerevisiae*.

MATERIAL AND METHODS

Ceramic support. Porous aluminum ceramics in the form of irregular grains was applied in the study. Use was made of three grain fractions with the following sizes: 1.2÷2.5; 2.5÷6.0; and 6.0÷10.0 mm. The fractions were obtained with a technique of sieve analysis. The major component of the ceramic material was aluminum oxide α–Al_2O_3 (97%). The ceramic material was characterized by the following parameters: total porosity – 0.75, pore size – 3÷1500 μm, volumetric density – 1.25 g/cm^3, and bulk density – 1.2 g/ mL

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[Bieniek *et al.,* 1982]. The support originated from the Institute of Building Engineering, Wrocław University of Technology, Poland.

Yeast. *Saccharomyces cerevisiae* yeast originated from a collection of the Chair of Fermentation Technology, Wrocław University of Environmental and Life Science, Poland. The yeast were proliferated on YPG medium with the following composition: glucose – 20 g/L, peptone – 10 g/L, and yeast extract – 10 g/L, pH 5.0. Static culture (in test tubes) and dynamic culture (in flasks on a shaker) were run for 48 h at a temperature of 30°C.

Immobilization of yeast. In batch fermentation, a flask (0.3 L) containing 30 g of support and 0.1 L of yeast suspension with a concentration of 5 g/L (pH 4.0, containing $Al(NO_3)_3$ with a concentration of 2.5 mmol/L), was stirred for 24 h in a shaker at a temperature of 30°C). The amount of immobilized yeast was computed based on concentrations of biomass before and after the process. Before immobilization, the ceramic material was burnt at a temperature of 900°C (in a muffle furnace) for 24 h and rinsed with distilled water. In the case of feed-batch and continuous fermentation, the procedures of yeast preparation were the same as for batch fermentation. The support for immobilization was fixed inside the reactor on a mesh in the form of poured 20-cm high bed. Yeast suspension was added and circulated through support's bed. The process lasted for 24 h (at a temperature of 30°C).

Fermentation wort. The wort was prepared from a sterile aqueous solution of glucose (pH 5.0) containing 2 g/L of the yeast extract and mineral salts at the following concentrations (converted into 100 g of glucose): $MgSO_4 \times 7H_2O$ – 0.5 g, KH_2PO_4 – 5 g, and $(NH_4)_2SO_4$ – 2 g. Glucose concentration in worts varied depending on the fermentation method applied, *i.e.* 80 g/L for batch and continuous fermentation and 250 g/L for the feed-batch one.

Fermentations. *The batch fermentation* was run in 0.3-L flasks (fixed in a water bath of the shaker) containing 30 g of the support with immobilized cells (see: Immobilization) and 0.1 L of fermentation wort. The fermentation was begun in eight flasks. After three hours, the process was terminated in one flask which was then subjected to analyses, while the fermentation was continued in the other flasks. After another 3 hours, the fermentation was terminated in the next flask. The procedure was repeated until the last flask. Results of fermentation from one flask corresponded to one measuring point in figures depicting the course of the process (Figures 1-3).

The feed-batch fermentation was run in a column reactor with a working volumey of 0.26 L (diameter: 3.7 cm, height of liquid column: 24.2 cm). The support with immobilized cells in the form of a packed layer of bed (*ca*. 20 cm in height, set in a wire mesh) was fixed in a reactor. The content of support in the reactor depended on the size of grains (Table 1). Over the entire period of the process, the wort was recirculated through the bed layer (upwards). Circulation and fixed flow rate were provided by a membrane pump.

The continuous fermentation proceeded in a column reactor with a working capacity of 0.215 L. Column cross-section diameter reached 3.7 cm. Inside the column, on the whole working height of the reactor, there was mounted a an overflow pipe (external diameter of 1 cm, mounted vertically along column's axis). The layer of poured support formed a 20 cm high bed. Fresh culture medium was dosed in the bottom part of the reactor (under the bed), and collected by means of the overflow pipe. All fermentation processes were carried out at a temperature of 32°C.

Analytical methods. The concentration of free yeast was determined with the spectrophotometric method using Beckman apparatus type 560 (at λ = 560 nm). The concentration of immobilized yeast was assayed with the gravimetric method. After the fermentation process, the sinter was rinsed in distilled water and dried at a temperature of 105°C (until constant weight). The amount of yeast immobilized on the sinter was computed as a difference between the masses of dried sinter before and after the fermentation. Free cells are those not bound with the support and constituting a suspension in the fermentation solution. Their concentration was determined as dry matter content of yeast in a unitary volume of a fermenting solution and expressed in g/L. In turn, the concentration of immobilized cells meant dry matter content of yeast permanently bound with the support (sinter) and was expressed as that of free cells (*i.e.* in g of dry matter of yeast per L of fermenting wort). They were calculated based on dry matter content of yeast bound by a specified mass of support in the fermenter (determined with the gravimetric method – as above) in wort's volume in the reactor. Glucose and ethanol were determined by means of high performance liquid chromatography HPLC Varian ProStar with RI detector. Conditions of measurement were as follows: HPX-C column, temp. 85°C, eluent H_2O, pressure on the column 53.754.7 bar, and injection – 10 μL.

RESULTS AND DISCUSSION

Evaluation of the kinetics of the ethanol fermentation process was based on kinetic parameters characterizing the course of the following fermentation processes: batch, feed-batch and continuous. Analyses were conducted under the same conditions for fermentation processes with immobilized and free cells. In the case of trapping the cells in pores of a porous support of key significance are conditions linked with mass exchange between the cells and solution. If the cell is trapped in external pores of the support, then diffusion of both nutrients to the cells and metabolites to the solution is impaired. To what extent it will affect the course of the process is determined, among others, by the size of support grains and hydrodynamic conditions occurring in the reactor. The size of support has a significant effect on diffusion of substrates and products outside the porous material [Tanaka *et al.,* 1984]. In turn, the hydrodynamic conditions affect diffusion through the boundary layer between support surface and the solution [Bourassa & LeDuy, 1987]. Both those aspects were taken into account in the reported study. Three fractions of the support were used in the form of irregular particles with the sizes of: 1.2÷2.5; 2.5÷6.0; and 6.0÷10.0 mm. The hydro-

dynamic conditions applied were variable through a change in the flow rate of fermenting wort through the support's bed. Since the character of flow is determined by the linear velocity, the rate circulating wort was expressed as the velocity counted per empty cross-section of the reactor (Table 1). The course of the batch fermentation (Figures 1-3) indicates that the size of grain affected the dynamics of the process. Out of the fermentation processes with cells immobilized cells, the best dynamics was observed for the process with the support grains of 1.2÷2.5 mm, whereas the worst one – for that with grains 6.0÷10.0 mm in size. Those differences are due to the length of the initial stage of the process. Definitely the shortest period was observed in the case of free cells. For the immobilized cells that stage was found to elongate along with the increasing size of grains. In turn, in the logarithmic phase

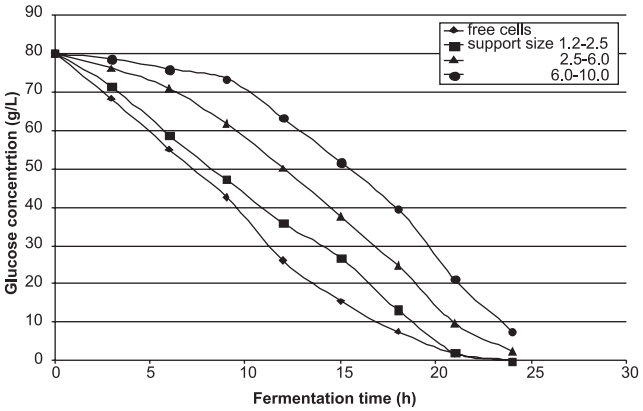

FIGURE 1. Changes in glucose concentration during batch fermentation.

TABLE 1. Results of feed-batch fermentation.

Parameter	Symbol	Unit	Fermentation with immobilized cells									Fermentation with free cells		
			Sizes of support fractions (mm)											
			1.2 ÷ 2.5			2.5 ÷ 6.0			6.0 ÷10					
			Linear rate of wort flow through support bed (m/s)											
			0.03	0.1	0.3	0.03	0.1	0.3	0.03	0.1	0.3	0.03	0.1	0.3
Initial concentration of glucose	$[S]_p$	g/L	141.0	121.6	122.9	128.5	120.5	132.6	120.3	122.5	125.6	133.0	121.6	115.7
Final concentration of glucose	$[S]_k$	g/L	101.0	65.9	74.6	86.0	68.9	86.9	80.7	77.0	82.2	115.9	95.1	90.3
Mean concentration of glucose	$[S]_{śr}$	g/L	121.0	87.7	98.7	107.2	94.7	109.7	100.5	101.6	103.9	124.4	108.4	103.0
Initial concentration of ethanol	$[P]_p$	g/L	31.0	40.4	39.1	37.9	40.8	42.1	44.5	47.7	44.3	30.5	45.1	43.6
Final concentration of ethanol	$[P]_k$	g/L	50.4	66.9	62.0	58.5	65.2	65.5	63.7	69.3	64.7	37.7	55.9	54.0
Mean concentration of ethanol	$[P]_{śr}$	g/L	40.7	53.6	50.5	48.2	53.0	54.3	54.1	58.5	54.5	34.1	50.5	48.8
Initial concentration of free yeast	$[X]^w_p$	g/L	10.0	11.3	11.4	13.4	14.1	14.3	10.3	11.4	11.4	2.1	5.4	5.1
Final concentration of free yeast	$[X]^w_k$	g/L	11.5	13.6	13.7	14.6	15.7	15.9	11.3	12.9	12.8	2.6	6.6	6.3
Mean concentration of free yeast	$[X]^w_{śr}$	g/L	10.7	12.4	12.5	14.0	14.9	15.1	10.8	12.1	12.1	2.3	6.0	5.7
Concentration of immobilized yeast	$[X]^u$	g/L	4.3	4.3	4.3	11.3	11.3	11.3	8.0	8.0	8.0	-	-	-
Summary yeast concentration	$[X]^c_k$	g/L	15.8	17.9	18.0	25.9	27.0	27.2	18.3	20.9	20.8	2.6	6.6	6.3
Mean total yeast concentration	$[X]^c_{śr}$	g/Lh	15.0	16.7	16.8	25.3	26.2	26.4	18.8	20.1	20.1	2.3	6.0	5.7
Rate of glucose utilization	r_s	g/Lh	3.33	4.64	4.02	3.54	4.30	3.81	3.30	3.79	3.62	1.42	2.21	2.12
Rate of ethanol production	r_p	g/Lh	1.62	2.21	1.91	1.72	2.03	1.86	1.60	1.80	1.70	0.60	0.90	0.87
Rate of yeast proliferation	r_x	g/Lh	0.12	0.19	0.20	0.10	0.13	0.14	0.08	0.12	0.12	0.04	0.11	0.10
Yeast productivity	υ_p	1/h	0.108	0.132	0.114	0.068	0.077	0.070	0.085	0.090	0.085	0.260	0.150	0.153
Specific growth rate	μ	1/h	0.008	0.011	0.012	0.004	0.005	0.005	0.004	0.006	0.006	0.017	0.018	0.017
Ethanol yield	$Y_{p/s}$	g_{et}/g_{gluc}	0.485	0.476	0.475	0.486	0.472	0.487	0.485	0.475	0.470	0.422	0.407	0.410
Yeast yield	$Y_{x/s}$	g_{yeast}/g_{gluc}	0.036	0.041	0.050	0.028	0.030	0.037	0.024	0.032	0.033	0.028	0.050	0.047
Reactor productivity	Q_p	g/Lh	1.46	1.94	1.80	1.76	1.96	1.96	1.66	1.80	1.68	0.60	0.89	0.86
Support mass in the reactor	m	G	150			120			100			-		
Working volume of reactor	V_r	L	0.260			0.260			0.260			0.026		
Wort volume in reactor	V_b	L	0.145			0.140			0.160			0.026		
Glucose concentration in culture medium	$[S_o]$	glL	250			250			250			250		
Volume of dosed medium	ΔV_o	L	0.050			0.050			0.050			0.050		
Frequency of medium dosage	Δt	h	12			12			12			12		
Rate of medium dosage	V_o	L/h	4.2×10^{-3}			4.2×10^{-3}			4.2×10^{-3}			4.2×10^{-3}		
Dilution rate	D	1/h	0.029			0.030			0.026			0.016		

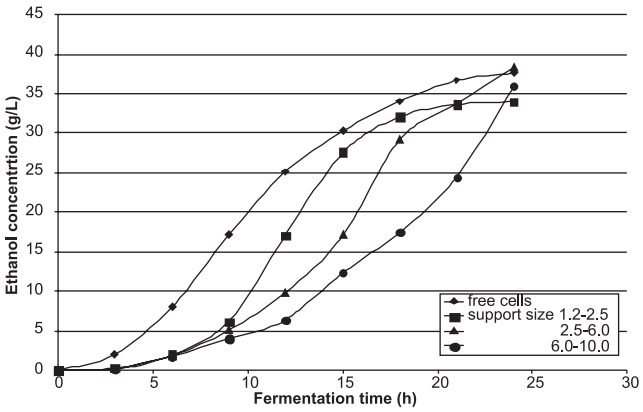

FIGURE 2. Changes in ethanol concentration during batch fermentation.

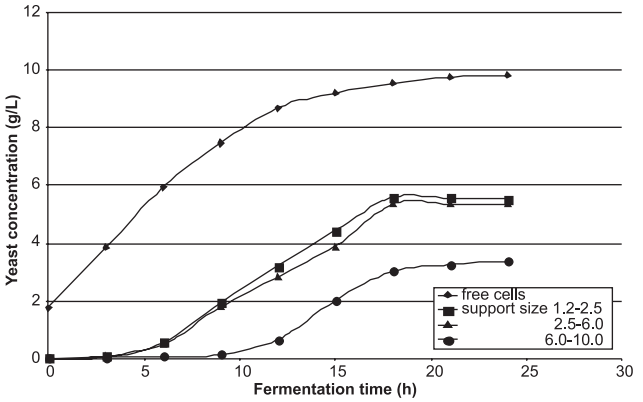

FIGURE 3. Changes of yeast (free) concentration during batch fermentation.

the slope of glucose and ethanol curves indicates that rates of all processes were similar, and in the case of the support with the smallest grains – higher than for the processes run with free cells. The elongated time of the initial phase of the processes with immobilized cells, as compared to fermentation with free yeast, may be explained by the occurrence of resistances linked with diffusion of culture medium components to the external pores of the support containing the cells. The low concentration of free cells (Figure 3) and alike rates of the processes indicated that the increase of biomass occurred mainly outside the support. In addition, it shows that the cells were effectively immobilized and their activity was preserved. Based on the feed-batch fermentation, analyses were carried out for the effect of the size of porous support grains and hydrodynamic conditions on the kinetics of the process and productivity of the reactor. Usability of the material as a support for cell immobilization is evaluated in terms of possibilities of increasing biocatalyst concentration in the reaction medium. Results of those analyses should consider, most of all, the impact of yeast concentration. In order to eliminate the effect of the concentration of a restrictive substrate (sugar) on the rate of the process it was assumed that during assays of the feed-batch fermentation the concentration of glucose in the medium would account for *ca.* 100 g/L. At a high concentration of sugar, the rate of the process is determined, chiefly, by the concentration of a biocatalyst. Results of those analyses were presented in Table 1. The mean (total) concentration of yeast in the fermentation wort was high (15.0÷26.4 g/L) – several times higher as compared to the process with free cells (2.3÷6.0). The concentration of immobilized cells $[X]^u$ (being a part of total concentration) depended on the size of support grain. The highest amount of yeast was observed on sinter 2.5÷6.0 mm (11.3 g/L), and the lowest one – on the fraction 1.2÷2.5 mm (4.3 g/L). The concentration of glucose had a decisive effect on kinetic parameters of the process and reactor's productivity. For the process run with immobilized cells, fermentation rate and reactor productivity were almost threefold higher as compared to the fermentation with free yeast. The rate of the process and reactor productivity were also found to depend on the linear velocity of wort flow through the bed. Increasing the flow rate from 0.03 to 0.1 m/s evoked a remarkable increase in the productivity (Q_p) for all fractions of the support. Fermentation with immobilized cells was characterized by a high coefficient of ethanol yield ($Y_{p/s}$), *i.e.* 0.470÷0.487, that did not depend on support size nor on the rate of circulated wort. Amongst all immobilized cells, the highest activity (v_p, μ) was demonstrated for yeast immobilized on the sinter with the smallest grains and was twice as high as that reported for the other two fractions. The sinter fraction of 2.5÷6.0, characterised by the best kinetic parameters, was used in the further stage of the study, namely in continuous fermentation. The main goal of cells immobilization is increasing the productivity of a reactor. The productivity Q_p (defined as D[P]) is determined by ethanol concentration in attenuated wort and by dilution rate. At an assumed degree of sugar attenuation, the concentration of a product [P] will be constant. Therefore, in order to improve productivity, the rate of dilution should be increase. At a constant working capacity of the reactor, it means increasing the rate of culture medium dosage. It is possible only through increasing the rate of fermentation. At an assumed (constant) degree of sugar attenuation, the only means of accelerating the rate of reaction is maintaining a high concentration of yeast in the fermenting medium. Yet, such a procedure is likely to be successful only in continuous reactors. For this reason, investigations on the evaluation of a new method for cells immobilization should be validated by experiments based on applying that solution in a continuous reactor. In that context, analyses were also conducted for the continuous process of fermentation. Results of those analyses were summarised in Table 2. Apart from the fermentation with immobilized cells, assays were also carried out for the continuous process with free cells – which proceeded under the same conditions and in the same column reactor. Productivities of the reactor with immobilized cells were five times higher than those of the reactor with unbound cells. At similar dilution rates (0.253 1/h – immobilized cells and 0.224 1/h – free cells), the productivity of the reactor with immobilized yeast (8.7 1/h) was threefold higher as compared to the reactor with free cells (2.7 1/h). That effect was yielded through providing a high concentration of yeast – 13.47 g/L – as compared to yeast concentration of 1.64 g/L in the reactor with free cells. Worthy of notice are high values of ethanol yield coefficients $Y_{p/s}$ (0.461 and 0.471). The fermentation activity of immobilized yeast (v_p, μ) was lower than that of free cells. The diminished activity of immobilized cells is due to

TABLE 2. Results of continuous fermentation.

Parameter	Symbol	Unit	Immobilized cells		Free cells	
			Rate of medium dosage (L/h)			
			0.024	0.048	0.024	0.048
Glucose concentration	[S]	g/L	5.1	25.9	41.5	50.0
Ethanol concentration	[P]	g/L	34.5	25.5	15.4	12.3
Concentration of free yeast	$[X]_w$	g/L	0.73	1.4	1.51	1.64
Mass of yeast immobilized on support (after fermentation)	X_n	g/100 g support	1.0	1.0	-	-
Concentration of immobilized yeast	$[X]_u$	g/L	12.74	12.74	-	-
Total yeast concentration	$[X]_c$	g/L	13.47	14.14	1.51	1.64
Rate of glucose utilization	r_s	g/Lh	18.95	27.37	4.31	6.72
Rate of ethanol production	r_p	g/Lh	8.73	12.90	1.72	2.75
Rate of yeast proliferation	r_x	g/Lh	0.185	0.708	0.169	0.367
Specific productivity of yeast	υ_p	$g_{et}/g_{yeast}h$	0.648	0.912	1.139	1.677
Specific rate of yeast growth	μ	$^1/h$	0.014	0.050	0.112	0.224
Ethanol yield	$Y_{p/s}$	g_{et}/g_{gluc}	0.461	0.471	0.399	0.409
Yeast yield	$Y_{x/s}$	g_{yeast}/g_{gluc}	0.010	0.026	0.039	0.055
Reactor productivity	Q_p	g/Lh	8.7	12.9	1.7	2.7
Working capacity of reactor	V_r	L	0.215		0.215	
Wort volume in reactor	V_b	L	0.095		0.215	
Glucose concentration in culture medium	$[S_o]$	g/L	80		80	
Support mass in reactor	m_n	G	120		-	
Dilution rate	D	1/h	0.253	0.506	0.112	0.224

their high concentration (12.74 g/L). Despite so high concentration, their activity was only twofold lower as compared to that of the free cells. Low values of a yeast yield coefficient $Y_{x/s}$ (0.010 and 0.026) demonstrate that metabolism of the immobilized cells changed towards increased production of ethanol – which is indicated by $Y_{p/s}$ values.

CONCLUSIONS

Experimental results obtained confirmed the usability of porous aluminum ceramics as a support for immobilized yeast cells in the process of ethanol fermentation. The support did not evoke a reduction in the activity of cells, which indicates its inertness towards yeast. Kinetics of the fermentation process depended on the size of support grains and on the flow rate of the reaction liquid through the support's bed. This points to the need for the recognition of those correlations and, on their basis, for the selection of optimal values. Application of porous ceramic support enabled maintaining a high concentration of yeast in the fermenting medium over the entire process, which resulted in a high productivity of the reactor. The effect of support grain size and flow rate of the liquid through its bed is linked with diffusion; in the first case – with diffusion inside the grains, in the second – with resistances in the boundary phase support-solution. Both these phenomena seem to determine the rate of the whole process. Thus, further actions should be aimed at a detailed description of those relationships and at reducing their effects.

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FERMENTACJA ETANOLOWA Z KOMÓRKAMI DROŻDŻY UNIERUCHOMIONYMI W ZIARNACH POROWATEGO SPIEKU CERAMICZNEGO

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Badano przebiegi procesów (okresowego, okresowo-dolewowego i ciągłego) fermentacji etanolowej z komórkami drożdży *Saccharomyces cerevisiae* unieruchomionymi na porowatym spieku ceramicznym i wolnymi – w aspekcie kinetycznym. Fermentację z komórkami wolnymi traktowano jako procesy porównawcze. Do unieruchamiania komórek użyto porowatego spieku ceramicznego w formie ziaren. Stosowano trzy frakcje o rozmiarach: 1,2÷2,5; 2,5÷6,0; 6,0÷10,0 mm. Stwierdzono, że dynamiki procesów okresowych z komórkami unieruchomionymi i wolnymi są podobne w fazie logarytmicznej. Natomiast istotne różnice występowały w fazie początkowej. Okres ten dla komórek unieruchomionych trał znacznie dłużej niż wolnych. Różny był również dla każdej z frakcji nośnika – najkrótszy dla najmniejszych ziaren i najdłuższy dla największych. Badania procesu okresowo-dolewowego wykazały, że najlepsze parametry kinetyczne uzyskano w przypadku nośnika o rozmiarach ziaren 2,5÷6,0 mm. Stwierdzono również, że kinetyka procesu zależy także od warunków hydrodynamicznych panujących w reaktorze – szybkości przepływu cieczy przez warstwę usypanego nośnika. Do fermentacji ciągłej wybrano frakcję nośnika o najlepszych parametrach kinetycznych fermentacji okresowo-dolewowej – o rozmiarach 2,5÷6,0 mm. Podczas fermentacji stężenie drożdży w podłożu reakcyjnym było wysokie ok. 14 g/L – w tym ok. 13 g/L to komórki unieruchomione. Wysokie stężenie biomasy pozwoliło osiągnąć produktywność reaktora na poziomie ok. 13 g/Lh – przy produktywności 2,7 g/Lh dla reaktora z komórkami wolnymi (w tych samych warunkach).