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Influence of Agitation Rate on Growth and Ribonuclease Production by Free and Immobilized *Aspergillus clavatus* Cells

R. J. MANOLOV

Institute of Microbiology, Department of Enzymes, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. B1.26, Sofia 1113, Bulgaria

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ABSTRACT

Aspergillus clavatus spores have been immobilized in poly(vinyl alcohol) criogel (PVAC) for ribonucleae (RNase) production. The enzyme productivity and growth of free (FC) and immobilized cells (IC) were studied in a bioreactor with agitation under different cultivation conditions. The influence of some medium compunds, aeration, and agitation speeds were investigated. Production of RNase was stimulated by the presence of reducing glucose, peptone, and soybean concentrations in the medium. From 42,000 to 45,000 U/L were produced by IC using optimized batch fermentation conditions. The RNase production by IC was 2.3 and 2.5 times greater than the same by FC in a bioreactor and control flasks, respectively.

Index Entries: Immobilized cells; *Aspergillus clavatus;* ribonuclease; poly(vinyl alcohol); cryostructurization; agitation; batch bioreactor.

INTRODUCTION

The use of living IC as multistep biocatalysts has attracted considerable interest in the past few years. The high potential of cofactor regeneration of IC leading to prolonged metabolic activity as well as the mechanical strength of the beads make immobilization processes attractive for many fields of application, especially enzyme production.

The RNase biosynthesis by different microorganisms has been studied by many researchers. Some genera, such as *Aspergillus* and *Penicillium* actively produce nucleases in complex media. *Aspergillus clavatus* 2286/8 strain was selected as a model organism since it has been reported to be a good producer of extracellular RNase among many different investigated strains (*1*). On the other hand, the RNase from *A. clavatus* has well studied primary (*2*), secondary, and three-dimensional structures (*3*) as well as an identified localization in the cells (*4*). Another reason to study carefully the process of extracellular enzyme biosynthesis is the great importance of these enzymes for molecular biology, biochemistry, and biotechnology.

Only a few studies have been published concerning the production of extracellular enzymes by immobilized fungal cells (*5–9*). Relatively limited information has been found in publications about the immobilization of fungal cells by freezing (cryostructurization) polymeric gels (*10*). Some investigations show that as a result of concentrated poly(vinyl alcohol) (PVA) or other polymeric gel aqueous solutions, after freezing and subsequent thawing have been received mechanically stable cryogels. Their mechanical properties, well developed microporous structure, and easy preparation make them interesting for application in biotechnological processes. In this paper, we have examined the influence of agitation speed, aeration, and medium compounds on RNase production and growth of FC and IC in PVAC beads.

MATERIALS AND METHODS

Chemicals

Poly(vinyl alcohol) was obtained from a polyvinylacetate factory (Yerevan, USSR); ribonucleic acid (RNA) from Biolar (Olaine, USSR); and glucose, peptone, and soybean (analytic grade) from Reachim (USSR).

Microorganisms

Aspergillus clavatus 2286/8, *A. pallidus,* and *P. brevicompactium* (32, 36, n54, and 3np9) strains were obtained from the microbial culture collection at the Bach Institute of Biochemistry (Moscow, USSR).

Culture Media

Stock cultures were maintained on Czapek Dox agar for 8 d at 25°C. Medium A (g/L) contained glucose, 50; peptone, 10; soybean, 5; KNO_3, 2; $MgSO_4 \cdot 7H_20$, 0.05; $CaCl_2 \cdot 2H_2o$, 0.1; pH 7 was used in the first- and second-stage inocula (*11*). In the batch experiments, media A, B (glucose, 30; peptone, 10; and soybean, 5 g/L), and C (glucose, 30; peptone, 5 g/L) with the same content of basal salts were used.

Shake Flask Studies

Inocula for the shake flask experiments were prepared by growing the microorganisms in a 750 mL flask containing 150 mL medium A for 48 h at 230 rpm and 25°C. To compare extracellular RNase activities produced by different strains, 750 mL flasks containing 150 mL medium were inoculated by addition of 10 mL 48-h-old cultures and incubated as described above. The cultures were harvested and assayed for RNase, β-galactosidase, β-glucosidase activities, growth, residual glucose, and pH. All of the experiments were carried out in duplicate.

Immobilization of *A. clavatus*

The generated spores (stock culture) were washed out with sterile water and agitated; spore concentration was reduced to $2–4.10^6$ spores/mL by adding sterile water. The spore suspension was mixed with 11.1% water solution of PVA (1:10 w/w) and the mixture was poured into a cylindrical dural column, then frozen in MK70 ultracryostate (MLW, Iena, (Germany) (*10*). In a fixed time, the samples were thawed for 1 h at 22°C and washed several times with sterile water and medium. The PVAC beads (3 mm in diameter) with entrapped fungal cells were incubated for 60 h on a 230 rpm rotary shaker at 25°C and then used as an inoculum (10% v/v) for the bioreactor.

Fermentation Conditions

The 10 mL of 48-h-old 160 mL seed cultures in medium A were used to inoculate secondary seed cultures in 750 mL flasks containing 150 mL medium A, B, or C. After 60 h of growth (for conditions, *see above*), the entire content of each secondary culture was used to inoculate a bioreactor that contained 1.9 L medium A, B, or C. Fermentations were carried out in a baffled 5 L stirred tank bioreactor (Biotec, Sweden). After inoculation, the temperature was controlled at 25°C, and air was sparged at 1 or 0.5 vvm. The dissolved oxygen partial pressure (pO_2) was measured with polarographic oxygen probes.

Analytical Methods

The RNase activity was determined by the modified Anfinsen method (*12*) using as a substrate RNA recrystallized by a formerly described method (*13*). The reaction mixture (0.4 mL 0.2*M* potassium phosphate buffer, pH 8; 0.4 mL enzyme sample; and 0.5 mL RNA, 0.8% solution), was incubated for 24 min at 25°C. The reaction was stopped by adding 0.5 mL uranylacetate (0.75% solution in 25% $HClO_4$). The vol was increased to 3 mL by adding distilled water and incubated for 30 min at 4°C. The precipitate was filtered and 0.1 mL of it suspended in 3 mL distilled water. The optical density was measured at 260 nm.

Table 1
Production of RNase, β-Galactosidase, and β-Glucosidase by Six Strains in medium A in Shake Flasks (t=4 d; T=25°C)

Strain	Growth, g/L	RNase, U/L	β-Gal, U nNF/L	β-G, U nNF/L
A. clavatus	10.0	68,000	18.4[a]	25.7[a]
A. pallidus	9.8	32,000[a]	7.9	67.7
P. brevicompactum 32	11.0	35,000	6.8[a]	77.1
P brevicompactum 36	10.5	30,000	5.1[a]	5.1[a]
P. brevicompactum n54	9.0	9,000[a]	6.8[a]	6.6[a]
P. brevicompactum 3np9	8.3	6,000[a]	7.1	8.2[b]

[a]Enzyme activity (t=5 d).
[b]enzyme activity (t=6 d).

The β-galactosidase and β-glucosidase activities were determined by the method of Kuby and Lardy (*14*). Reducing sugars were determined by the method of Somogyu (*15*). Cell dry biomass was determined by drying at 60°C for 24 h (up to constant wt). The dry wt of IC was determined after boiling at 70°C for 10 min, as described above.

RESULTS

Shake Flask Experiments

The RNase production rate has been determined for all fungal strains in media A, B, and C. In shake flask experiments, *A. clavatus* produced approx twice more quantity of RNase than *A. pallidus* and *P. brevicompactum*. The strains *P. brevicompactum* n54 and *P. brevicompactum* 3np9 exhibited nearly background RNase, β-galactosidase, and β-glucosidase activities under all cultivated conditions tested (data not shown). Only *A. clavatus* showed significant enzyme activities. The results are summarized in Table 1. Similar but lower values were observed for the other experiments in media B and C. Thus, the extracellular RNase production exhibited by *A. clavatus* led to its being chosen for further experiments with FC and IC in a batch bioreactor.

Batch Cultivation

The influence of agitation directly on growth rate and yield is complicated by its other effects on mixing and oxygen transfer. Although fungi may suffer greater shear damage when the stirring speed is raised, the greater oxygen transfer may result in a higher titer. In such fermentations, there will be an optimum stirring speed. The influence of agitation speed on FC growth and RNase production was studied in the range of 200–600 rpm in medium A at an aeration rate of 1 vvm. Figure 1 shows

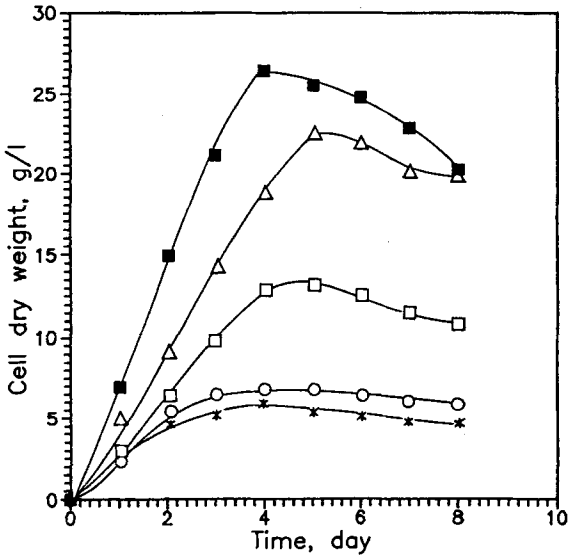

Fig. 1. Time-course of cell dry wt during batch fermentation of *A. clavatus* at several stirring speeds. ○ 200 rpm; □ 300 rpm; △400 rpm; ■ 600 rpm; * control.

that cell growth was influenced at speeds higher than 300 rpm. At higher agitation rates, FC biomass increased exponentially with a specific growth rate of 0.18 h^{-1} for 400 rpm and 0.27 h^{-1} for 600 rpm. Local oxygen limitation is thought to be responsible for the lower growth at 200 rpm. At higher speeds, lysis of the cells was obtained after 130–140 h of cultivation, but at 200 rpm, the culture actively produced RNase up to the end of the process.

The rate of product formation also can be affected by mixing and oxygen transfer. Figure 2 illustrates the time-curve of RNase production. There was substantial differences in enzyme production during the batch fermentations. At the low agitation speeds, the RNase production curves started to increase after 24–36 h, just when the dissolved oxygen became to 20–40%. Maximum RNase (33,000 U/L) was obtained at 130 h at 200 rpm. The enzyme production at the high agitation rates varied from 4,000 to 10,000 U/L because of mycelial destruction and cell lysis.

Minimum aeration and agitation conditions were chosen to avoid mixing difficulties and mass transfer limitation by maintaining the aeration at 0.5 vvm. In order to investigate the effect of agitation on RNase production, additional experiments were done at an aeration rate of 0.5 vvm at speeds of 150 and 200 rpm. The apparent dependence of RNase production rate on aeration rate, based on the results of fermentations on medium A, was examined further on medium B. Figure 3 shows that enzyme synthesis was influenced not only by agitation rate but by aeration also. In the experiments with 0.5 vvm air in a bioreactor, the time-course of RNase shows a maximum of 45,000 U/L at 144 h, which is approx

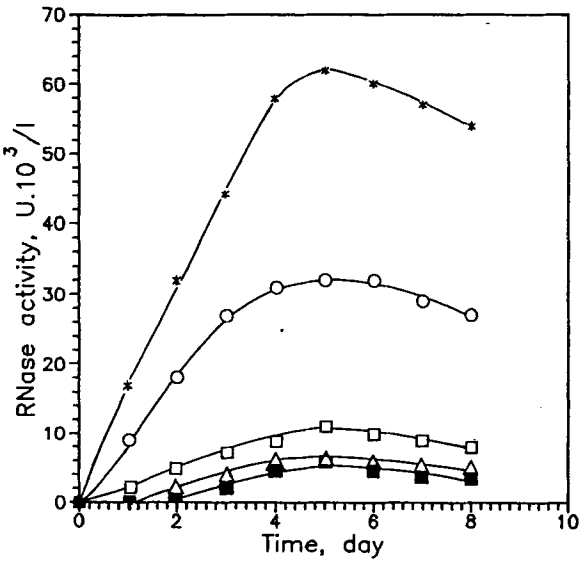

Fig. 2. Time-course of RNase concentration during batch fermentation of *A. clavatus* at several stirring speeds. ○ 200 rpm; □ 300 rpm; △400 rpm; ■ 600 rpm; * control.

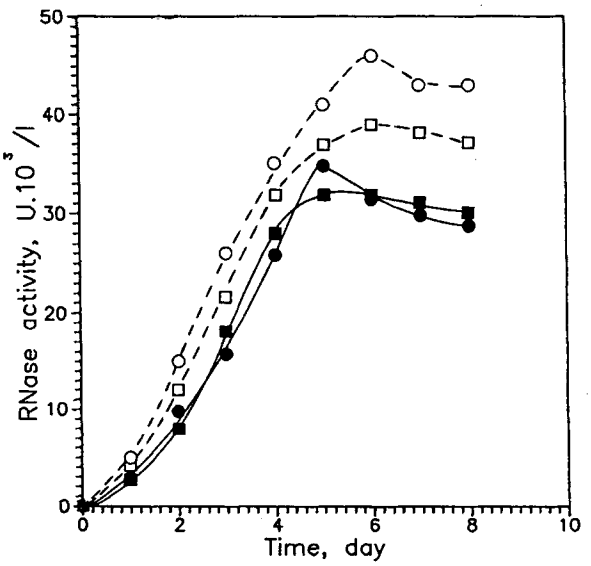

Fig. 3. Biosynthesis of RNase at 1 vvm (——) and 0.5 vvm (---) air flow at batch fermentation on medium B. ○ 150 rpm; □ 200 rpm; ● 150 rpm; ■ 200 rpm.

10,000 U/L higher than in the case with 1 vvm aeration rate. On the other hand, lower stirring speeds (150 rpm) did not affect productivity, although growth of mycelial cells was lower (4.5–5.1 g dry wt/L, data not shown). In that way, 150 rpm agitation speed and 0.5 vvm aeration rate were chosen for further experiments.

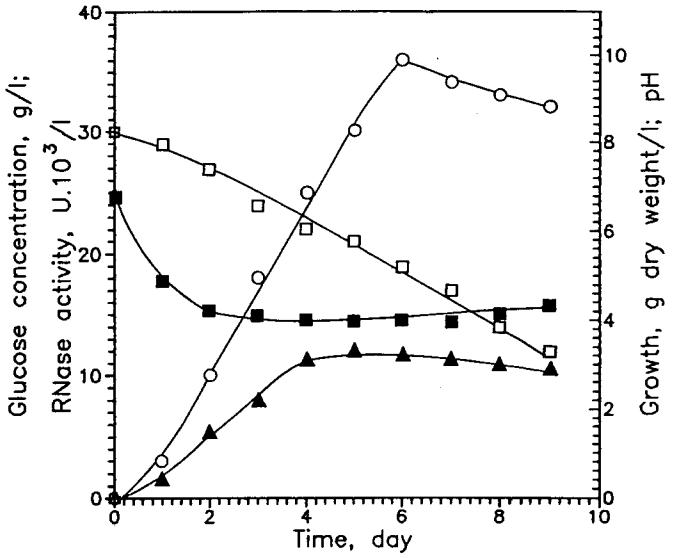

Fig. 4. Batch cultivation of free *A. clavatus* cells at 150 rpm agitation speed and 0.5 vvm aeration in medium C. RNase activity (○); growth (▲); glucose concentration (□); and pH (■).

Effect of Medium Compounds on Growth and Enzyme Activity

To test the effect of initial concentrations of the main compounds of medium A on growth and enzyme production, both FC and IC were grown in medium C at 150 rpm and 0.5 vvm aeration. *A. clavatus* FCs were grown under these conditions, the final biomass ranged from 2.9 to 3.2 g dry wt/L, and RNase activity typically reached 36,000 U/L (Fig. 4). The enzyme production rate was similar to that for fermentation in medium B where the same aeration rate of 0.5 vvm was used. The values for specific growth rate (0.026 h^{-1}) and RNase activity in the bioreactor were significantly higher than those in control flasks. Although no direct measurements of dissolved oxygen have been made in control flasks, it is generally accepted that aeration in the bioreactor at the chosen conditions (150 rpm and 0.5 vvm) is better than in controls. Requirements for sufficient oxygen supply for RNase biosynthesis by fungi have not been found in the literature.

As is known, every immobilization method influences in a different way the functions of IC and their ability for enzyme production. The use of IC of *A. calvatus* in PVAC of immobilized *A. calvatus* in PVAC cells as a model system depends on their ability to be exploited for a long period of time. For that reason, the agitator of the bioreactor was coated by a thin plastic film for IC protection during cultivation. The results in Fig. 5 are for batch fermentation at the conditions as above. The maximum RNase activity of IC in bioreactor (42,000 U/L) was considerably higher (162%) than that in control flasks.

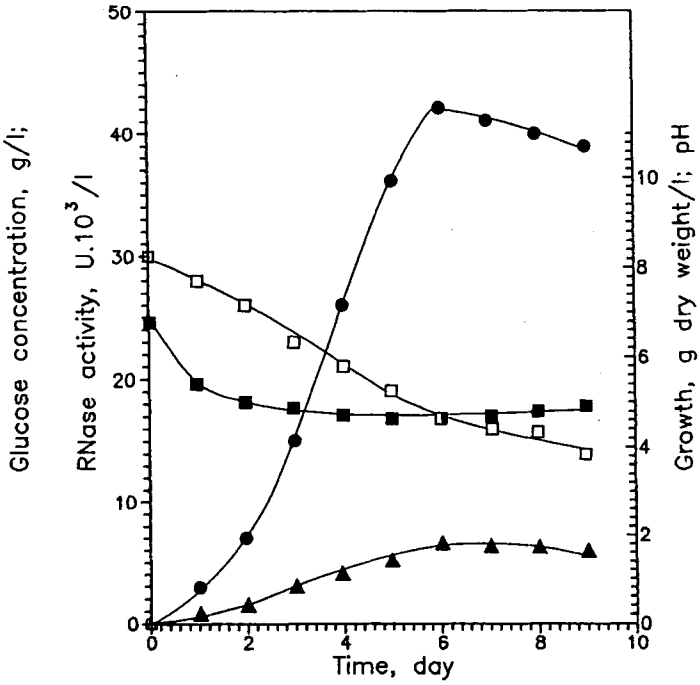

Fig. 5. Batch cultivation of immobilized *A. clavatus* cells at 150 rpm agitation speed and 0,5 vvm aeration in medium C. RNase activity (●); growth (▲); glucose concentration (□); and pH (■).

The morphology data for experiments with IC at different aeration and agitation rates in the bioreactor were compared with those obtained from shake flask controls. The data from batch cultivations show how the morphology changed within each fermentation. The PVAC beads overgrew at 48–60 h with a specific growth rate of 0.011 h^{-1} and increased their vol 1.5–2.5 times in 120–140 h. In control flasks and batch experiments in media A and B, free mycelial fragments have been observed over 96-h cultivation, which indicates the susceptibility of the mycelia to shear forces at a particular time. When medium C was used for IC cultivation at finally chosen conditions, no free mycelial fragments were observed in the culture broth.

The PVAC beads were gradually enveloped in a mycelial veil, increased their volume 2.3–4.2 times, and did not stick together at the end of the process, as did the previous fermentations in media A and B. The scanning electron micrograph of the state of hyphae twined around the surface of the PVAC carrier is shown in Fig. 6. If the mycelial hyphae grew on the PVAC surface without interaction between beads, IC cultivated in media A or B were twined around the carrier with high density, resulting in limited surface area. In this way, cells could not grow enough to affect enzyme production and separated in the medium as free mycelial

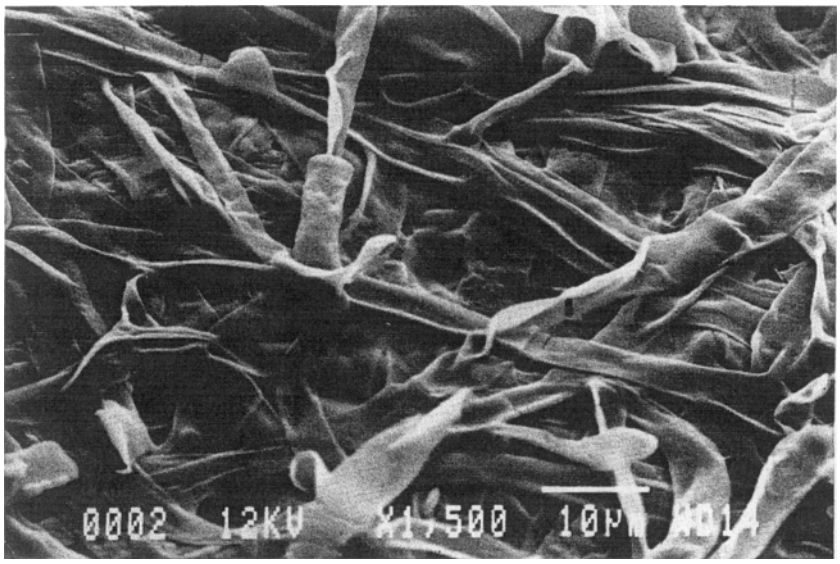

Fig. 6. Scanning electron micrograph of surface of immobilized *A. clavatus* cells. Culture time 8 d, medium C, agitation speed rate 150 rpm and aeration 0.5 vvm. Bar = 10 μm.

fragments. Thus, any parameter that is a function of agitation speed, aeration rate, or supporting medium will show a positive correlation with the rate of RNase production.

DISCUSSION

The results presented here for *A. clavatus* as the best RNase producer show a trend for enzyme synthesis, with a greater reduction occurring at higher agitation speeds because at speeds of about 600 rpm, mycelia are shorter and less branched, although total biomass is not affected. By all fermentations carried out in medium A, the rate of RNase production fell down when higher agitation speeds were used (Table 2).

In some experiments, the agitation speed was changed during the fermentation process in order to investigate its effect on enzyme synthesis. The aim of these experiments was by cultivation at high agitation speeds, e.g., 600 rpm, to accumulate enough biomass to produce at low agitation speed (e.g., 200 rpm) more RNase. In fact, this did not occur. The results of the experiment show that regardless of accumulated biomass, the mycelia cultivated at 600 rpm agitation speed do not have the same enzyme productivity as the hyphae grown at low agitation speeds of about 200 rpm.

Table 2
RNase Production by Free and Immobilized Cells at Different Agitation and Aeration Conditions

Medium	Agitation, rpm	Aeration, vvm	Time period, h	RNase production, U/L		Growth, g dry wt	
				FC	IC	FC	IC
A	600	1.0	132–144	4000	—	26.8	—
	400	1.0	120–136	5100	—	22.5	—
	300	1.0	120–144	10,000	17,800	13.5	8.3
	200	1.0	120–132	33,000	20,000	6.8	7.2
	200	0.5	120–130	32,000	23,400	7.1	8.1
	200–600	1.0	148	4,000	—	18.2	—
B	150	1.0	120–130	35,000	28,900	5.2	5.6
	200	1.0	120–138	32,000	—	5.0	—
	150	0.5	140–146	45,000	33,000	4.5	5.0
	200	0.5	144–148	38,400	—	4.7	—
C	150	1.0	144–150	31,200	34,700	4.1	2.6
	200	1.0	144–146	29,000	—	3.4	—
	150	0.5	132–144	36,100	42,000	3.4	1.8
	200	0.5	134–146	34,000	—	3.8	—

The data presented in Table 2 show how RNase production in medium B changed with time in each fermentation when FC or IC were used. In medium B, FC produce more RNase than IC do. On the other hand, reduction of the aeration rate to 0.5 vvm increases the RNase productivity of FC and IC. Maximum RNase activity of FC (45,000 U/L) was obtained at 150 rpm and 0.5 vvm, which is 12,000 U/L more than the activity of IC. Quite the contrary, by cultivation in medium C, IC synthetize more enzyme than FC do. As shown in Figs. 4 and 5, medium C contributes to lower growth rate, which influences the enzyme synthesis capability of mycelial cells. The specific productivity of IC in medium C (23,340 U/g dry wt) was 2.3 and 2.5 times higher than that of FC in the bioreactor and of IC in the controls, respectively. The observed differences in RNase production can be attributed to the shear forces in the bioreactor and to the resultant morphological changes during fermentation.

SUMMARY

All of the experiments show that fungal spores could be successfully immobilized in poly(vinyl alcohol) by the method of cryostructurization that does not influence the viability of the cells. The PVAC has good mechanical properties and microstructure, which permits the mycelial hyphae to easily pass from innerspace to the surface of the carrier. It was

established that mycelial length and RNase production are reduced at high agitation rates. It is clear that medium C is suitable for cultivation of free and immobilized *A. clavatus* cells, and its use permits IC to save their activity as well as avoid any sticking between them. It also can be concluded that at lower agitation and aeration rates, fungal cells immobilized in PVAC could be successfully used as well for batch as for continuous cultivation in other type bioreactors—column or airlift reactors.

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