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Elaboration of Method of Long-Term Culturing and Selection of Enzyme Producers

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Abstract
On the basis of the conducted researches on pectin lyase and proteolytic enzyme biosynthesis by *Penicillium* and *Asperaillus* micromycetes we have developed efficient methods for their cultivation and selection. We theoretically substantiated and experimentally confirmed an advantage of growing micromycetes in a new filament-spongy immobilized growth structure on the substrate relative to the traditional method of deep cultivation of free cells in the form of pellets. When comparing a traditional with our innovative method of cultivation, many advantages of the latter are revealed, above all being the possibility of the formation of new highly selective cultures in the long process of their growth with modified culturally - morphological properties.
***Key words*:** immobilization, enzymes, cultivation, productivity, selection, pectinas, amylase

I. Introduction

During the subsurface cultivation different types of mycelium microorganisms relating to *Penicillium* and *Asperaillus* are developed in shape of solid pellet or volumetric flaky mass. Forms of mycelium significantly affect productivity of microorganisms. The size of pellets can vary from 0.1 mm to 10 mm; they are inaccessible to nutrients oxygen.

The formation of any form of mycelium depends on the physical and chemical conditions of the culture environment (2,3). On solid surfaces cystophore of micromycetes grow linearly without creating spathella, by twisting into pellets. In subsurface conditions of growth such spathella forms a loose filamentous structure with favorable access to nutrients and oxygen. We have immobilized such structure on the carrier using adsorption materials (4,5). In conditions of the immobilization on the substrate and growth in liquid environment, micromycetes form a loose filamentous-cancellous structure of mycelium with good availability to nutrients and oxygen.

For the immobilization of microorganisms, we used the least costly and simplest method of adsorption immobilization, eliminating the use of toxic chemicals. However, this method has not found wide application due to its several disadvantages: low strength of cells retention on carriers, the limited amount of biomass adsorbed by the carrier unit and others. Nevertheless, we have offset some of these shortcomings by using solid substrates with high adsorption surface, on which satisfactory immobilization of investigated cultures was achieved. In all these works there is no comparative characteristic of the cultivation process of free and immobilized cells and the appraisal of cultivation methods was not given on the following criteria: the stability of the process, the concentration of the resulting product, the volumetric efficiency, as well as the maximum productivity of the culture. This work is dedicated to the development of new methods of long-term culturing and selection of enzyme producers and their comparative evaluation.

Study the formation of pectin lyase and proteolytic enzyme biosynthesis by different structures of growth mycelium (pellets and cellular tissue) of *Penicillium* and *Asperaillus* micromycetes, as well as the increase of their productivity due to formation of highly active selective alternatives represents theoretical and practical interest.

II. Materials and Methods

Microorganisms

Producers of pectinase used were *Aspergillus awamory 16* and *Penicillium cyclopium*. All cultures were maintained on agar slants with Czapek's medium. Inoculum was a suspension of spores of 5 -7 day culture diluted in sterile distilled water at a concentration of spores 1,3x107 cells / ml.

Environment

Capek's medium contained: $NaNO_3$ – 0.15 g.; Sucrose - 2.0 g; KH_2PO_4 – 0.1 g; $MgSO_4$ – 0.05 g; KCl – 0.05 g; $FeSO_4$ - 0,001 g per liter.

Environment for *Aspergillus awamory 16* contained: Glucose - 2 g; $(NH_4)_2SO4$ - 0,15 g; MgSO4 – 0.05 g; KCl – 0.05 g; KH_2PO_4 – 0.1 g; $FeSO_4$ – 0.001 g per 100 ml .

Environment for *Asp.oryzae* 3-9-15 contains: Sucrose - 2 g; $NaNO_3$ – 0.15 g; KH_2PO_4 – 0.1 g; $MgSO_4$ – 0.05 g; KCl – 0.05 g; $FeSO_4$ - 0,001 g 100 ml.

The formation of enzymes

Determination of pectin lyase enzymes was performed by spectrometric method using apple pectin for determination of PMGL and pectic acid – fot PGL on the Albersheim's method. Determination of pectolytic activity (PA) was was assayed by the Anson method with some modifications. The given experimental data is an arithmetic mean of 2-3 experiences executed in triple frequency.

III. Results and Discussion

Study of the growth of filamentous microorganisms and their formation of hydrolytic enzymes are closely related to the method of their cultivation. Apical nature of micromycetes' growth during normal subsurface cultivation of free cells causes the formation of masses of mycelium growing in the form of pellets. Form of mycelium formed during submerged culturing directly affects crop productivity.

Since metabolic reactions depend on the concentration of nutrients, cells on the surface of filamentous pellets have the maximum access to nutrients. Therefore, cells of mycelium growing in the form of a spathella have greatest access to nutrients for the longest period. Spathella in submerged culturing transforms into filamentous-spongy structure, in which hyphae of mycelium grow linearly, are accessible to nutrients and oxygen. For the formation of the spathella we have created conditions of adsorption of inoculum on the surface of a monolithic substrate with a large adsorption surface on which culture retained and grew linearly along the surface for a long time. In conditions of aeration and mechanical agitation on a shaker, filamentous sponge-like structure is formed which produces enzymes for a prolonged period.

By the end of culturing of the immobilized cells of P. Cyclopium enzymatic activity of PMGL and PGL of cultural liquid increased in 1,8-2 times in comparison with free culture (tab. 1) . Frequency of receiving a target product has gradually increased. If at periodic culturing the maximum of a target product is formed for 4 days and only once, at an immobilization of culture of PMGL from 15,8 pieces/ml to 26,0 pieces/ml and PGL from 16,0 pieces/ml to 28,5 pieces/ml is formed (tab. 1), that is the enzyme biosynthesis increases twice with receiving enzymes repeatedly.

The traditional method of microorganism's growth cultivation for 3-4 days is follower by the pause of the process because of fall of enzymes biosynthetic associated with lysine culture. Our method of culturing microorganisms extends continuously on the substrate without interruption for a period of 12-15 days to 2-4 months with multiple target enzymes every 1-2 days (Table 1). If during the periodic cultivation, the desired product can be obtained only once in three days, then we get the desired product during immobilization repeatedly and continuously for a long time every 1-2 days to produce more of the cultural fluid.

Table 1
Comparative data on formation of pectin lyase enzymes *P.Cyclopium* at cultivation of free and immobilized culture.

Method of cultivation	PL Enzymes, piece /ml	Duration of cultivation, days							
		4	7	10	13	16	19	21	24
Periodic, free culture	PMGL	12,9	0	-	-	-	-	-	-
	PGL	15,9	0	-	-	-	-	-	-
Semicontinuos, immobilized culture	PMGL	15,8	22,0	24,0	23,5	20,05	21,5	23,0	26,0
	PGL	16,0	23,1	25,0	24,5	22,2	22,5	26,0	28,5

Besides, at PL enzymes biosynthesis by the immobilized culture of *P. Cyclopium* labor costs were reduced by 12 times, in as much time the sowing material was reduced. The filtration of the cultural liquid is excluded, which emission was increased by 10 times.

Table 2
Advantages of *P. Cyclopium* cultivation in immobilized state before periodic culturing of free cells.

№	Cultivation parameters	Free cells	Immobilized cells
1	Duration of cultivation, days	4	40 and more
2	Volume of received culture liquid, ml.	85-90	900-1000
3	The number of necessary passages	13	1
4	Terms of active enzymes formation, (days)	4	7-13
5	Duration of a stationary phase of enzyme formation (days)	1	6-7
6	Frequency of receiving target products (1 day later)	4	3-4
7	Activity of cultural liquid PMGL PGL	12,9 15,9	26,0 28,5

8	Operation on cultural liquid filtration	Required	Not required
9	Save time and money on equipment sterilization, preparation of a sowing material (repetition factor)	-	12

At an immobilization and long cultivation of *P. Cyclopium* on a substrate, the culture was subject to changes on morphological and zymoplastic properties. In the course of studying of population variability of culture the active alternative was selected - the strain of *P. Cyclopium* 2-11 forming pectin lyase enzymes 6,2-6,6 times more actively than initial culture.

After removing mycelium from the substrate the second stage of the process of biosynthesis begins with a fall in activity of QOL. After 2-4 days after removal of mycelium, even trace amounts of old cells remained in the pores of the carrier, are capable of producing as much enzyme as 2-3 g. of young mycelium on the first stage of cultivation. Furthermore, the patterns observed in the first phase are also repeated on the second phase. On the third stage, activity increased 10-12 times compared to that of batch culture. In the process of multi-stage cultivation was observed to steadily increasing producing capacity of culture, which is maintained for the duration of culture - within 60 days.

Therefore, during the immobilization of *A.awamori 16* increased activity of QOL enzymes 2 to 6 times in the first stage and 10 to 12 times in the third, as well as the elongation of the phase of active enzymes formation takes place, as compared to batch culture fom 3 to 11-14 days. In addition to the activation of culture, stabilization of culture-producing ability of the fungus was observed, which is maintained throughout the period of cultivation (60 days). Frequency of obtainment of the desired product is gradually increasing. If the periodic cultivation of target product was obtained on the third day, during immobilization it was obtained in 1-2 days, depending on the number of aggregated mycelium.

The most informative criteria of any industrial production of biologically active substances, including enzymes, are the specific productivity and volumetric efficiency cells. Figures for both parameters in immobilized cells are higher than those of the free 2 to 6 times (Table 3, 4). Specific productivity of immobilized cells, as well as PR enzymes activity, increases compared to that of culture of free cells 1.5-6 times (see Table. 3). Table 5 presents data on the benefits of cultivating micromycetes in the immobilized state.

Based on the method developed long-term culturing of micromycetes a new method of microorganisms selection has been worked out without the use of mutagens of physical and chemical nature. The essence of the developed method of selection lies in creating a variety of different options on the substrate during prolonged cultivation of microorganisms immobilized on a substrate, among which a highly active one is formed. Prerequisite for the formation of the active option is stress, which culture is experiencing in the long process of growing. This mechanical damage of hyphae in removing accumulating mycelium from the substrate, conditions change nutrient to poor environment to complete the process of long-term cultivation. In order to identify the active option, isolates are selected in different periods of cultivation for capacity analysis of a particular enzyme formation. Revealed selective culture exceeded the original cultures of enzyme activity tenfold. Thus, highly active producer of pectin culture - P.cyclopium was obtained, exceeding original 10-15 times.

Application of adsorptive immobilization by the developed method provides significant advantages in cultivation of the producer in the immobilized state as compared to the traditional process of microbial synthesis on the base of free cells in the periodic culture conditions.

Table 3 **Advantages of *A. awamori 21/96* cultivation in immobilized state before periodic culturing of free cells.**

№	Cultivation parameters	Free cells	Immobilized cells
1	Duration of cultivation, days	3	30-36 and more
2	Volume of received culture liquid, ml.	85-90	900-1000
3	The number of necessary passages	12	1
4	Duration of an active proteoletic enzymes formation (days)	3	9-11
6	Frequency of receiving target products (1 day later)	4	3-4
7	Activity of cultural liquid (PA, pieces/ml)	6,5	7,7
8	Operation on cultural liquid filtration	Required	Not required
9	Save time and money on equipment sterilization, preparation of a sowing material (repetition factor)	-	12 and more

In this case, culture productivity increases 4 times on average, a term of producer cultivation also increases 20 times, the stage of active enzyme formation extends, and multiple use of initially immobilized culture is provided.

Also, raw materials for preparation of media and inocula are saved, as well as labor costs – operations on recharging fermentation vessels involved in the removal of biomass from the system are excluded. The process of enzyme activity is simplified due to the fact that quality of life does not require filtration due to immobilization of biomass substrate. For the same reason, the system has only vegetative mycelium. Absence of spores in the cultivation of vegetative immobilized mycelium maintains air quality. All these benefits of micromycetes immobilization can significantly impact the overall economy and culture of enzymes biosynthesis, and dramatically reduce the cost of the product.

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