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Production of microbial enzymes by new method of cultivation of microorganisms

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Abstract:
We have developed efficient methods for long-term culturing and selection of highly active versions of the original cultures of micromycetes – producers of enzymes. 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 cultivation of filamentous microorganisms in subsurface conditions of growth, different shapes of mycelium are developed as a result. Forms of mycelium significantly affect productivity of crops (1) which vary in size from volumetric flaky mass to solid pellet of sizes 0.1 mm-10 mm, 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.

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

For the formation of enzymes we used 750 ml shaker bulbs with 100 ml of culture medium, which is seeded with a spore suspension of 2 ml of 5 – 7 day old culture grown on the stock with Capek's medium. Cultivation implemented on a shaker (280 rev / min

at 280C) for a prolonged period, with the replacement of the culture medium every 3 days and every 1-2 days afterwards.

Determination of enzyme activities

A-amylase activity was determined with two methods: a) by the ability of an enzyme to produce hydrolysis of starch until final dextrins, not polled by iodine (in units of AU) and b) by the saccharifying capacity (in units of the OS).

Determination of pectolytic activity (PCA) was performed on the number of unfissioned pectin before and after exposure to pectin diluting enzymes (PG and PL). Quantitative determination of the pectin is based on the obtainment of pectin acid copper salt.

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.

In our studies, producers of enzymes were selected micromycetes of the genus *Aspergillus* - *Asp.niger* and *Asp.oryzae* 3-9-15 - producers of pectinase and α-amylase. A comparative study of pectin diluting enzymes (PD) and α-amylase - free (FC) and immobilized cells (IC) showed that during the immobilization of filamentous-spongy culture, biosynthesis of enzymes is active and for a significant period of time (Figure 1). Stationary phase of enzymes formation increases 2.5 - 3 times.

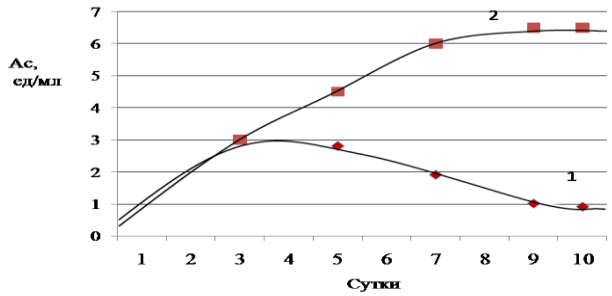

Figure 1 - Dynamics of α-amylase formation by different structures of mycelium. *A.awamory16*.
1 - by mass of cells growing through pellets, 2 – by filamentous-spongy mycelium immobilized on paralon.

Free cells have a limited development cycle; they form greatest amount of enzymes on 3-9 day (Fig. 2, 3).

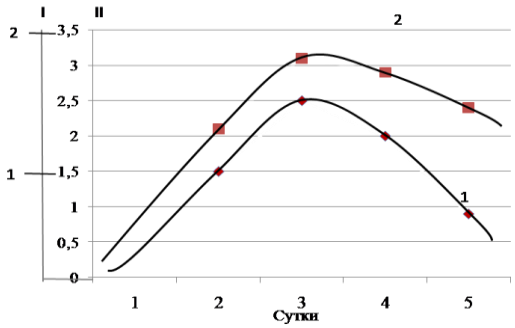

Figure 2 - Dynamics of α-amylase of batch culture *Asp.oryzae* 3-9-15,
I, 1 - Biomass, g; II, 2 – α -Amylase activity, units / ml.

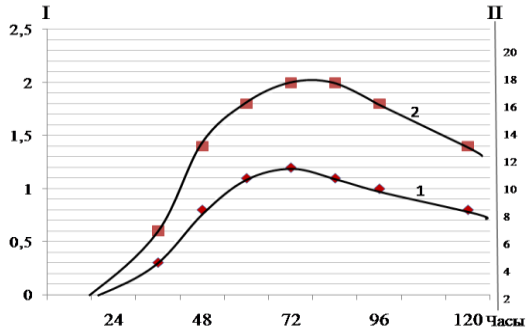

Figure 3 - Dynamics of the formation of PD enzymes of batch culture *A.awamory 16*.
I, 1 - mass of dry mycelium, g; II, 2 - RCA, U / ml

During subsequent culturing of FC, with the replacement of the environment within the same period of time, as that of the IC, there is autolysis of cells, which leads to a gradual fall of the enzymatic activity of the cultural fluid.

Thus, 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
Characteristics of the cultivation process of free and immobilized cells *A.awamory 16* on polyurethane substrate

Cultivation parameters	Free cells	Immobilized cells
Duration of cultivation, days	3	60 and more
Volume of used medium, ml.	100	6000
The intervals between the receipt of QOL and replace with a new batch of medium, days	3	1-2
Amount received QOL ml	85	4600
The total amount of enzymes after 60 days of cultivation in the immobilized culture in 4600 ml, PG activity by reducing substances, U / ml	-	16 468
Submarine U / ml	-	55 660
The total amount of enzyme in the cell-free in 3 days GHG units / ml Submarine U / ml	 180 200	 - -

With the accumulation of the substrate mycelium eventually biosynthesis enzymes begins to fade, due to lack of oxygen in a confined space for bulb cultivation. To restore the biosynthetic enzymes with the substrate removed all biomass. This concludes the first stage of the process, which takes up to 11-14 days. The enzymatic activity of PG and PL in QL immobilized cells, respectively, higher than in the quality of life of free cells in 2 and 6 times (Table 2). In addition, in a batch culture free cells maximum formation of enzymes observed on day 3, while the immobilized cells stably high fixed for 11-14 hours.

Table 2

Activity of pectin-resolving enzymes of free and immobilized cells in A.awamori 16 (on the third day after the removal of culture mycelium from the substrate)

Enzyme	Free cells	Immobilized cells
PG activity for reducing substances	1.54	5.5
PG activity viscosity, U / ml	1.8	3.58
PME, U / ml	.22x103	.13•103
PL, U / ml	2.0	12.1
Pectin resolving activity (PcA) U/ml	18.0	120.0

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, namely, 60-80 U / ml QoL in crop productivity 20 - 30 U / g of mycelium. 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 (Figure 4).

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.

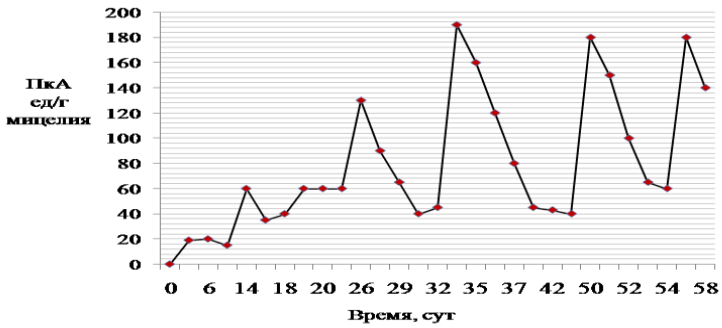

Figure 4 - The dynamics of productivity of the immobilized culture A.awamory 16

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.

Table 3
Specific productivity of free and immobilized cells *A.awamori 16* (on the third day of cultivation)

Table 4
Volumetric efficiency of immobilized culture *A.awamori 16* (after 60 days of cultivation in 4600 ml of KH) and free cells (three days in 100 ml of QoL)

Table 5
Cultivation parameters of immobilized and free cells *A.awamori 16* (for 60 days)

The observed increase in enzyme activity of QoL on all stages of cultivation of immobilized biomass, elongation of the phase of active enzyme formation from 3 to 11-14 days, a new type of curve dynamics of

culture productivity, can be explained by the fact that immobilization creates favorable conditions for the formation of selective culture, activity of which is greater than the initial 2 to 10 times or more.

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 30 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. 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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