Madina Adilbievna Dzhakasheva et al /J. Pharm. Sci. & Res. Vol. 9(5), 2017, 751-756

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

# Immobilized Biocatalyst for Production of Red Table Wines

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**Abstract**

A method of obtaining high-yielding immobilized biocatalyst based on cells of mycelial fungus *Aspergillus awamori* (*a new strain of F-RKM 0719*) has been technologically developed and tested, which is able to secrete extracellular pectinases for long time during cultivation. The carrier of immobilized cells was cryogel of polyvinyl alcohol, which is a high strength macroporous viscoelastic gel material with good biocompatibility and stability in biological environments. Thanks to these properties, this carrier allows easy diffusion of components of the nutrient medium and metabolic products. An immobilized biocatalyst is obtained by gelation of polyvinyl alcohol with simultaneous immobilization of cells of *A. awamori F-RKM 0719*. To obtain the maximum enzymatic activity of immobilized biocatalyst, concentration of the carrier and concentration of the cells were found experimentally. The level of total accumulated pectolitic activity of the developed immobilized biocatalyst exceeds 3.5 times the level of strain *A. awamori F-RKM 0719* obtained in multi-stage breeding and used without immobilization. Thus, there is no need to isolate pectolitic enzymes from the cultural fluid, which significantly reduces the economic expenditures, and thus it is possible to reuse many times the immobilized biocatalyst that produces, outside the cell, the required balanced complex of pectolitic enzyme preparations in the process of technological operations in wine production.

**Keywords:** cell immobilization, polyvinyl alcohol cryogel, mycelial fungus *Aspergillus awamori*, pectolitic enzymes, red table wine, wine production.

## INTRODUCTION

Pectolitic enzymes play an important role in the physiological processes associated with destruction of plant cell walls or their modifications [1]. That is why they are widely used in food industry for production of juices and wines [2-4]. The use of pectolitic enzymes in the wine industry ensures high yield of juice and content of aroma-forming, coloring and other extractive substances [5, 6] in it; facilitates pressing of the pomace [7]; clarifies wine material [8]; improves resistance of the finished product to colloidal hazing [9].

At the present stage of development, searching for efficient producers of pectins [10] is important for the development of the scientific basis for the enzyme technology for processing industries of the agroindustrial complex. The most commonly used industrial pectinase-producing strains belong to genus *Aspergillus* [11, 12], and synthesize the rich complex of hydrolytic enzymes that ensure efficient degradation of polymers in the plant and microbial raw materials [1]. One of the most efficient approaches to obtaining and stabilizing enzymes and enhancing their economic attractiveness is immobilization with the use of insoluble carriers [13]. Cells immobilization, which means any restriction of their freedom of physical motion in space, has long been discussed as an efficient approach to solving the problems of intensifying and enhancing economical attractiveness of modern biotechnological processes [14], since biocatalytic systems based on immobilized cells feature high yield of the target products, resistance to negative factors (pH, temperature, cultivation medium components, etc.) [15], and long-term preservation of metabolic activity in case of their repeated use in biotechnological processes in the absence of visible lysis [16].

Efficiency of a biocatalyst is largely determined by the efficiency of supplying nutrients or substrates to immobilized population of cells, and the ease of removing metabolites [17]. These factors largely depend on the diffusion barriers created by the material of the carrier. One of the most promising methods of immobilizing enzymes is adding polyvinyl alcohol to cryogel; polyvinyl alcohol, due to its high strength, pronounced porosity, biocompatibility and stability in biological environments, is widely used in various fields of biotechnology [18]. Besides, cryogels are characterized by high hygroscopicity, which allows them to hold water inside the polymer substrate even in an anhydrous environment of polar organic solvents [19].

Our research is aimed at obtaining highly immobilized biocatalyst based on cells *A. awamori F-RKM 0719*, capable in the immobilized condition of secreting a complex of extra-cell pectinases for repeated use in wine production.

## MATERIALS AND METHODS OF STUDY

The culture of mycelial fungus *A. awamori* has been obtained as a result of multistage breeding and mutagenesis at the Department of Biotechnology of the South Kazakhstan State University (SKSU) n.a. M. Auezov [20]. This culture has been deposited in the Republican State Enterprise with the Right of Economic Management "Republican Collection of Microorganisms" of the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan, with registration number *A. awamori F-RKM 0719*.

Activity of the pectolitic complex of enzymes was determined by the method of the current GOST R 55298-2012. For obtaining the immobilized biocatalyst, to the aqueous solution of polyvinyl alcohol (grade 16/1 NPO "Azot" (Ukraine)) at the concentration of 60-140 g/l, cell suspension (vegetative form) of strain *A. awamori F-RKM 0719* at the concentration of 0.4-1.0 g/l was added, which has been obtained after flushing spores from agar environment, exposed for 30 min at room temperature, stirred thoroughly until a homogeneous mass was obtained, from which spherical granules with the diameter of 1.4-1.6 mm were formed. Granulation was performed according to the method [21], where freezing was performed at -15°C, the granules were kept frozen for 24 h, after which the granules were thawed and dried at 8°C. After spores immobilization for forming immobilized mycelium, the granules were cultivated in specially selected conditions for preferential biosynthesis of a complex of pectolitic enzymes with granules concentration of 100-120 mg/ml in a 250 ml flask on a temperature-controlled nutator (220 rpm) at 30°C on nutrient medium I (pH 3.2) of the following composition, %: beet pomace: grape pomace: hydrolyzate of cotton sashes (1:1:1) – 3.0; lactose – 0.125; malt sprouts: extract from mycelium (1:1) – 1; $(NH_4)_2SO_4$ – 0.5%; $KH_2PO_4$ – 0.2; $MgSO_4$ – 0.1 (for determining the composition of the enzyme complex and productivity of the biocatalyst in the model conditions) and on nutrient medium II - grape pomace (for determining efficiency of the biocatalyst in the production environment). The specific cell growth rate was determined according to the recommendations of Perth [22].

The experiments were performed with grapes of variety Cabernet Sauvignon from the same plot, the same year, which had 20% of sugar and approximately the same content of coloring and tanning substances. The studied grapes were separated from the stems and crushed. The obtained pomace was sulfurized at the rate of 70 mg/l, heated up to 40-45°C, and subjected to fermentation for 6-24 h with the use of immobilized cells of *A. awamori F-RKM 0719* in the concentration of 5-12 g of granules per 1 liter of pomace, which were used repeatedly and loaded into a special perforated container mounted on the shaft of the mixing device in the thermal vinificator. At the end of the heat treatment and fermentation (6, 12, 24 h), samples were taken for analyzing the content for the mass concentration of sugars; the enzymatic reaction was stopped by diluting the sample in the 0.1 M acetate buffer solution (pH 5). The resulting must was fermented with the same race of yeast at 23-25 °C for 5 to 8 days. In the process of fermentation, the output of self-flowing and pressure fractions of must from the samples was monitored; must viscosity, and the content of suspended matters were measured. After separating self-flowing and press fractions of the must from the pomace, all factions of the must were sent for further fermentation until the content of sugars reached no more than 0.5 g/l. After complete cessation of fermentation, the wine material was removed from the yeast. Samples of ready wine materials were subjected to physicochemical analysis, and the results were used for making conclusions about efficiency and feasibility of using immobilized cells.

The mass fraction of solids in the must was determined gravimetrically, the relative viscosity - with the use of a viscometer through defining the flow time and mass concentration of total phenolic substances - using the Folin-Ciocalteau technique and mass concentration of anthocyanins - using the colorimetric method [23]. For obtaining the values of intensity ($I$) and hue ($T$) of the wine material, measurement was performed in quartz vessels with the 10 mm distance between the working faces. Distilled water was used for reference. Intensity ($I$) was determined as the sum of optical densities values at the wavelengths of 420 nm, 520 nm: ($I520 = A420 + A520$). Hue indicator ($T$) was calculated as the quotient of dividing A420 by A520: ($T = A420/A520$).

All experiments were repeated three times. Statistical reliability of the results was assessed with the use of applications "MathCAD" and "Statistica".

## RESULTS AND DISCUSSION

Earlier, at the laboratory of the Department "Biotechnology" of the SKSU n.a. M. Auezov, a new mutant strain of *A. awamori F-RKM 0719* was obtained as a result of multistage breeding, which synthesized a complex of pectinolitic enzymes with the activity of 2.1 units/ml [20].

Upon immobilization of cells of mycelial fungus *A. awamori F-0719 RKM* in cryogel of polyvinyl alcohol, the influence of concentration of polyvinyl alcohol and concentration of mycelial cells of fungus *A. awamori F-RKM 0719* on the process of biocatalyst formation were studied (Figures 1, 2).

As a result of the experiments it was found that the concentrations of polyvinyl alcohol less than 60 g/l deteriorates not only the physico-mechanical properties of the polymer; due to the increase in the medium size of pores (over 5.0 μm), germination of hyphae outside of the carrier occurs, which leads to their partial separation when particles of immobilized biocatalyst are mixed, and to contamination of the cultural medium. When the concentration of polyvinyl alcohol exceeds 120 g/l, development of biomass is inhibited due to hampered diffusion of substrates and products caused by small cross-section of the pores (less than 0.5 μm), which results in lower production and yield of pectins.

When the initial concentration of the suspension of cells of mycelial fungus *A. awamori F-RKM 0719* is less than 0.6 g/l, biocatalyst features much lower pectolitic activity, and at the concentration of 0.8 g/l, incomplete inclusion of all cells into cryogel-polyvinyl alcohol is observed during biocatalyst formation, which leads to their partial washing out when the obtained granules are placed into the cultural liquid. The highest synthesis of pectinase (7.4 u/ml) was observed when the concentration of cells of the mycelial fungus was 0.8 g/l.

Figure 3 shows the influence of granules concentration on the maximum biosynthesis of extracellular pectinases by immobilized cells of strain *A. awamori F-RKM 0719* when they are cultivated in liquid nutrient environment I.

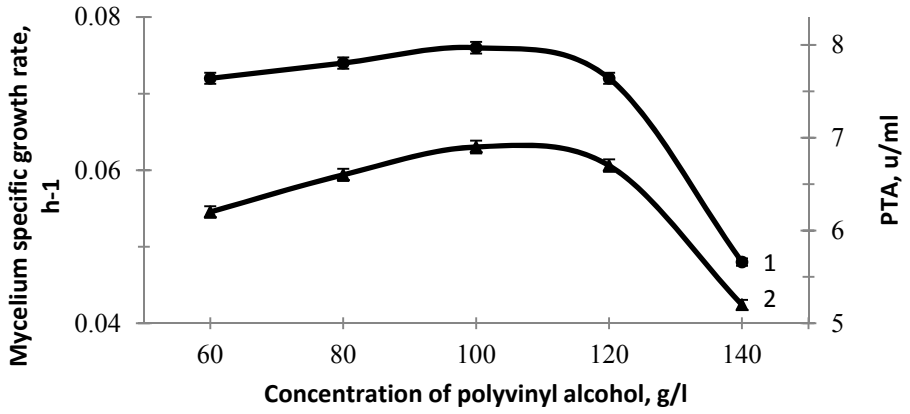


**Figure 1 – Characteristics of forming immobilized biocatalyst depending on the concentration of polyvinyl alcohol (Curves denomination: 1 - mycelium specific growth rate, h-1; 2 – total pectolitic activity, u/ml. Conditions: initial concentration of cells in the suspension is 0.5 ml/l)**

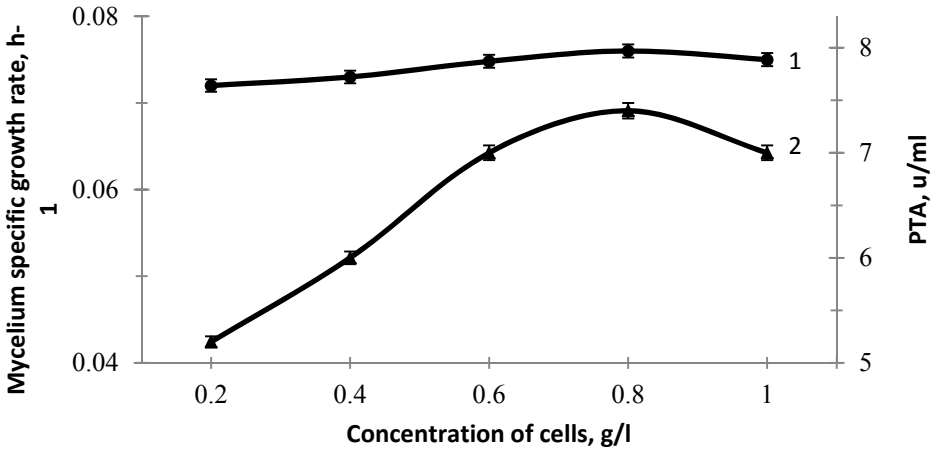


**Figure 2 – Characteristics of forming immobilized biocatalyst in dependence on the concentration of cell suspension. (Curves denomination: 1 - mycelium specific growth rate, h-1; 2 – total pectolitic activity, u/ml. Conditions: initial concentration of polyvinyl alcohol is 100 g/l)**

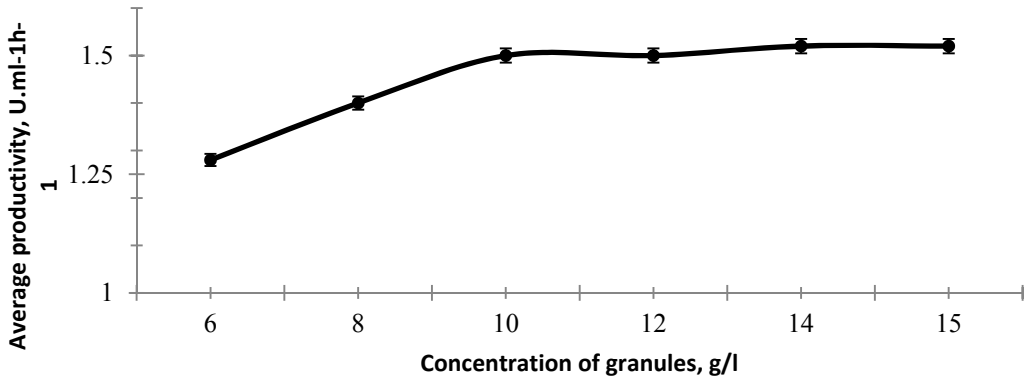


**Figure 3 – The influence of granules concentration on the maximum biosynthesis of extracellular pectinases by immobilized strain *A. awamori F-RKM 0719***

Figure 3 shows that the maximum average productivity of the process in terms of pectolitic activity is 1.5 units $ml^{-1}$ $h^{-1}$ at the granules concentration of 10-12 g/l, and, since further increasing the content of granules in the volume of the cultural fluid does not result in an increased productivity, we chose the concentration of 10 g/l as the optimum concentration for production.

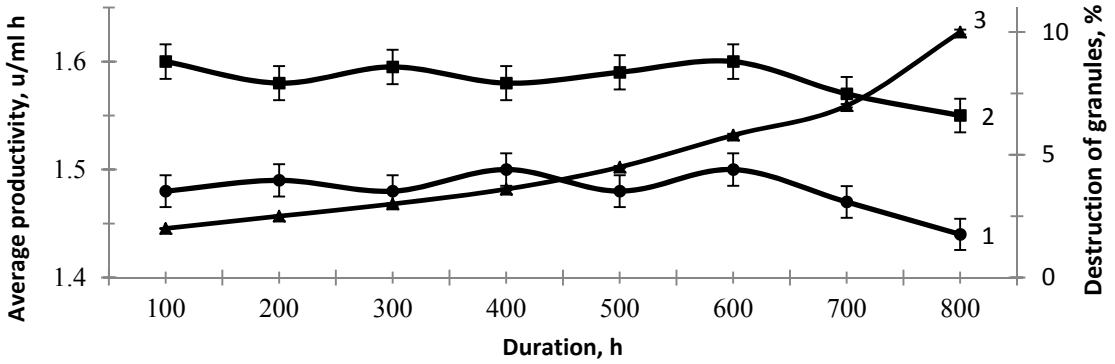


**Figure 4 – The influence of duration of using cells of strain *A. awamori F-0719 RKM* in immobilized state (Curves denominations: 1 - cultivating in nutrient medium I; 2 - cultivation in nutrient medium II; temperature - 28°C; without mixing; 3 – destruction of the granules in nutrient medium II)**

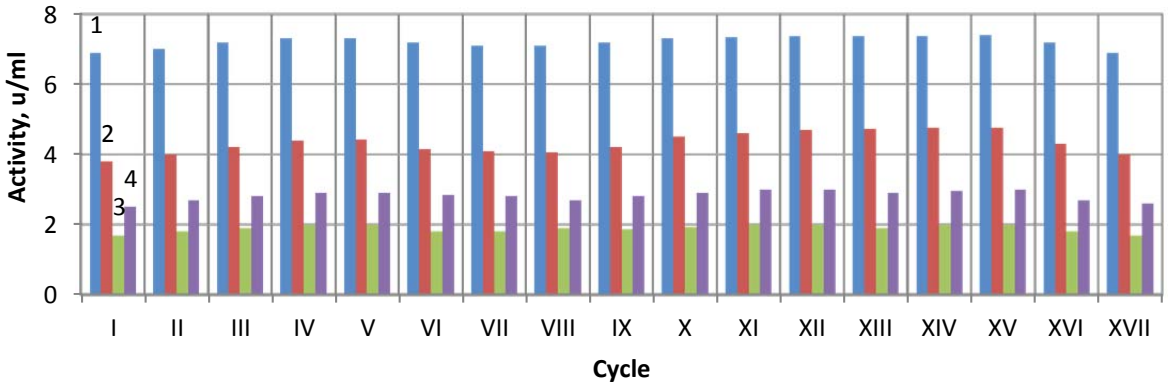


**Figure 5 – Activity of the enzyme complex in case of repeated usage of immobilized strain *A. awamori F-0719 RKM* in the process of maceration. The duration of one working cycle of immobilized biocatalyst is 12 hours (the optimum duration of maceration). (Designations curves, enzymes activity, u/ml: 1 - pectolitic; 2 – polygalacturonic; 3 – pectinesterase; 4 - pectin lyase)**

It has been experimentally established (Figure 4) that the average pectolitic activity in the cultural fluid during cultivation in nutrient medium I is approximately 1.5 u/ml×h 1.6 u/ml×h when cultured in medium II for 600 hours, then it starts decreasing.

The thus prepared highly active immobilized biocatalyst based on cryogel of polyvinyl alcohol at the concentration of 100 g/l containing cells of strain *A. awamori F-0719 RKM* in the initial concentration of 0.8 g/l is characterized by long and stable biosynthesis of a complex of pectolitic enzymes in the process of maceration (Figure 4, 5). The maximum pectolitic activity of the developed immobilized biocatalyst is 7.4 u/ml. In the production conditions, activity of immobilized biocatalyst started decreasing after the 17-th cycle, i.e. its use with the maximum efficiency is limited to 200-220 hours.

For the purpose of assessing the efficiency of obtaining pectinases, Table 1 shows the comparative characteristics of the conditions and the products of cultivating free and immobilized cells of mycelium.

**Table 1 – Comparative characteristics of the conditions and the products of cultivating free and immobilized cells of *A. awamori F-0719 RKM* in nutrient medium I**

| Parameters of cultivation | Free cells | Immobilized cells |
|---|---|---|
| Duration of cultivation, h | 84 | 600 (220)* |
| The maximum duration of pectinase synthesis, h | 70-80 | Continuously for 600 (220)* |
| Pectolitic activity, u/ml | 2.10 | 7.40 |
| Polygalacturonic activity, u/ml | 1.25 | 4.77 |
| Pectinesterase activity, u/ml | 0.90 | 2.00 |
| Pectin lyase activity, u/ml | 1.30 | 2.95 |
| Specific activity, u/mg of protein, u/mg of immobilized biomass | 87.3 | 465 |

Note: *600 hours – laboratory conditions, 220 – production conditions in a thermal vinificator with stirring at the temperature of 45 °C

The obtained experimental data indicate the advantages of using cells of *A. awamori F-0719 RKM* in immobilized state, a 3.5 times increase in the biosynthesis of a complex of pectolitic enzymes, and a 5.3 times increase in the specific activity allow efficient and repeated use of immobilized cells of the producer as the source of pectolitic enzymes in wine production. In addition, the obtained immobilized biocatalyst is characterized by markedly elevated polygalacturonic and pectin lyase activities, which are aimed at releasing the coloring and phenolic substances from the peel of grapes, and at destruction of pectin and facilitating of grape pomace pressing.

Efficiency of immobilized cells of *A. awamori F-0719 RKM* for high-quality table red wines has been studied. Before the first use, the biocatalyst was subjected to aging in nutrient medium I for 10 hours. The used method of thermal vinification provides a higher flexibility. First, the processes of extraction and fermentation are separated, since colored must is fermented without the pomace. Secondly, the temperature profiles may be adjusted; if necessary, the grapes partially affected by mold may be successfully processed, which is impossible with the use of classic pomace fermentation. Third, multi-variance and route of processes are easily resolved. The use of thermal vinification ensures high economical efficiency, the route of the process with complete mechanization and automation of operations, inactivation of harmful microorganisms, reduces the dosages of sulfitization and high quality of red table wines. The choice of the duration of maceration is explained by the fact that in case of a prolonged contact of pomace with the developed immobilized biocatalyst (over 12 hours) wine materials become oversaturated with polyphenols, which tinctures excessive astringency and roughness to the taste of wine. If maceration continues for less than 10 hours, less significant changes in transparency and viscosity are observed, which indicates insufficient hydrolytic processes. During the research, the main indicators that characterize efficiency of enzyme preparations have been determined (Table 2).

Based on the obtained data for the production of red table wines, immobilized biocatalyst in the concentration of 11 g of granules/l of pomace may be recommended.

Thus, a conclusion may be made about the efficiency of using immobilized cells of *A. awamori F-0719 RKM* in wine production. The use of the obtained immobilized biocatalyst ensures an increased yield of must from grape pomace, facilitates pressing of grape pomace and brightening of wine due to decreasing viscosity and the content of solids in the must, and obtaining wine materials enriched in colorants and phenolic substances.

Figure 6 shows a diagram of a comparative tasting assessment of the studied red table wines after enzymatic treatment with immobilized cells with the reference samples.

The wines obtained as a result of enzymatic treatment are characterized by rich, complex aroma and intense, harmonious, styptic taste, which is the result of deep hydrolysis of polyphenols.

**Table 2 - The main indicators that characterize efficiency of using immobilized cells of *A. awamori F-RKM 0719**

| Indicators | Reference | Dosage of fermentation preparations, granules / l of pomace | | | | | |
|---|---|---|---|---|---|---|---|
| | | 5 | 6 | 7 | 9 | 11 | 12 |
| Overall yield of must and the yield of self-flowing must, ml | 560 (410) | 665 (417) | 671 (422) | 678 (431) | 682 (430) | 687 (437) | 687 (438) |
| Suspended solids, g/100 ml | 3.2 | 2.8 | 2.7 | 26 | 2.4 | 2.3 | 2.3 |
| Relative viscosity | 1.61 | 1.56 | 1.51 | 1.46 | 1.43 | 1.40 | 1.41 |
| Phenolic compounds, mg/l | 1.830 | 1.925 | 1.970 | 2.032 | 2.095 | 2.180 | 2.180 |
| Anthocyanins, mg/l | 520 | 528 | 579 | 593 | 615 | 675 | 672 |
| Color intensity | 1.19 | 1.20 | 1.22 | 1.24 | 1.25 | 1.26 | 1.25 |
| Tint | 1.09 | 1.06 | 1.02 | 0.99 | 0.96 | 0.94 | 0.95 |

Note: *tenfold reuse of the biocatalyst, time of maceration is 12 hours

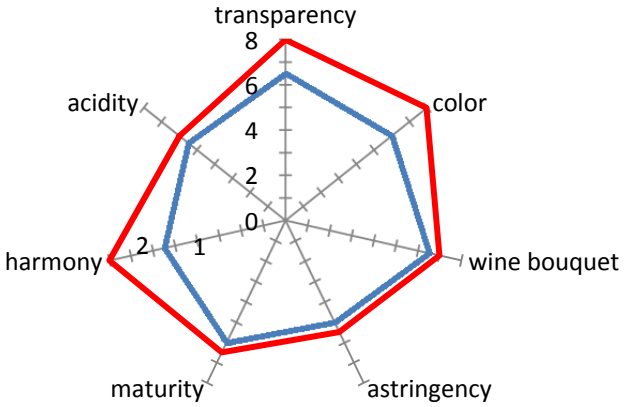


**Figure 6 - Organoleptic diagram (Designation: 1 - reference; 2 – immobilized cells of *A. awamori F-RKM 0719)**

## CONCLUSION

The advantages of the method of immobilization based on the polyvinyl alcohol cryogel are in the fact that the polyvinyl alcohol has no negative impact on pectolitic enzymes biosynthesis by immobilized cells; on the contrary, viability and enzymatic activity of the latter increase due to the porous structure of the matrix of the carrier (pores cross-section is 0.5-1.2 μm), which ensures easy diffusion of components of the nutrient medium and the metabolic products. In addition, the polyvinyl alcohol cryogel is a visco-elastic non-friable material which is not subject to abrasive wear, has good characteristics during long-term cultivation (up to 600 h), and can accept any form of granules, which is suitable for various reactors with various operating profiles. The developed biocatalyst has significantly increased its productivity in terms of pectolitic activity (7.4 u/ml), compared to free cells. The use of immobilized cells of *A. awamori F-RKM 0719* in obtaining red table wines is economically and technologically substantiated, since due to the possibility of using them repeatedly (up to 17 times), the actual consumption is 10 times less than that of the conventional enzyme preparations, with the cost of its cleaning being comparable with the cost of producer immobilization. The enzyme-heat treatment during the process of maceration significantly increases the yield of highly colored must, improves the process of lightening and filtration of must, and ensures the maximum extraction of anthocyanins and phenolic compounds, which allows obtaining harmonious red wines with a strong fruit notes in the high quality aroma.

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