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CULTIVATION CONDITIONS PREFERABLE FOR YEAST CELLS TO BE IMMOBILIZED INTO POLY(VINYL ALCOHOL) AND USED IN BOTTLED SPARKLING WINE PRODUCTION

An effective approach to the improvement of the biochemical status of yeast cells before their immobilization was applied to develop a biocatalyst with a high level of cell metabolic activity and viability that could be used for sparkling wine production. According to the approach, the wine-containing medium (pH 3.0) routinely used for the accumulation of champagne yeast biomass under aerobic conditions was replaced by a semi-synthetic one (pH 5.6), usually applied for aerobic yeast growth. The variation of temperature and pH conditions of cell growth showed a modification of the fatty acid pool of yeasts and its importance for the further immobilization of cells into a poly(vinyl alcohol) cryogel. Measurement of the specific intracellular ATP concentration by the bioluminescent method revealed the growth phase favorable for yeast immobilization. The main characteristics of the sparkling wine obtained after four-week fermentation with application of both the free and immobilized cells were similar but, according to the detected energetic status, the viability level of the immobilized cells was considerably higher compared to the free yeast. The CO₂ pressure accumulated in the bottles with immobilized cells (up to 500 kPa) after fermentation also appeared notably higher than in the bottles with free yeast.

Keywords: Immobilized yeast; Poly(vinyl alcohol) cryogel; Intracellular ATP concentration; Sparkling wine production.

Nowadays, an active search for new methods and biotechnological approaches to the enhancement of sparkling wine (SW) production is of high interest [1–3]. The remuage and disgorgement should be noted to compose 43–50% of the total expenses of bottled champagne production [4]. The use of immobilized yeast allows significant simplification of these long technological stages of SW production and, thereby, provides the total process with new economic benefits, offering products of high quality at accessible prices.

A search for effective methods that guarantee the production of a metabolically active immobilized yeast biocatalyst for wine champagnization was recently undertaken [5–7]. It is obvious that the efficiency of a biocatalyst developed on the basis of immobilized cells often depends on the physiological state of the cells taken for immobilization, since all manipulations with microorganisms within the main procedure are stressful for them [8].

The content of the growth medium can have a substantial effect on the biochemical and metabolic features of the yeast. For instance, both the initial sugar and ethanol concentrations can significantly decelerate the yeast growth and biomass yield [9]. Consequently determination of the cell growth conditions, which guarantee the accumulation of yeast biomass

appropriate for the further development of a highly effective biocatalyst for SW production, was the main objective of the investigation.

Poly(vinyl alcohol) cryogel (PVA CG), being one of the most attractive polymers appropriate for the immobilization of a wide variety of microbial cells [10–12], was used in this study to entrap champagne yeast cells. This macroporous viscoelastic hydrogel resulted from the freezing and subsequent thawing of the polymer in which the relevant cells were suspended [10]. It should be noted that the perspectives of PVA CG application for the immobilization of various types of yeast cells have already been described in the literature [12–14]. The high chemical, mechanical and microbiological stability of this carrier along with its macroporous structure, which enables good mass-transfer features, was shown to be the main advantage of PVA CG [10, 12, 15].

The results of applying both free and immobilized champagne yeast cells, grown in different nutrient media, were analyzed after four-week fermentation.

ATP being the main source of cell energy was chosen as the major criterion of cell viability in this study, since routine cell colony counting, commonly used to estimate the cell viability, was not appropriate in the case of cells entrapped into PVA CG. The problem is that the recovery of immobilized yeast from PVA CG is known to be impossible inasmuch as the polymer partially penetrates and forms a gel structure in the yeast cell walls [11, 12]. Generally, the bioluminescent analysis of intracellular ATP concentrations is one of the contemporary highly sensitive methods used to quantify the viable free and immobilized cells [16–18].

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MATERIALS AND METHODS

Microorganism and cultivation conditions

Saccharomyces cerevisiae strain Champagne-39 was used for SW production. The yeast cells were maintained at 4–8°C on a fermentable wort containing 2% agar.

To accumulate a certain amount of cell biomass necessary for biocatalyst production, the yeast cells were grown in two media in parallel: i) a semi-synthetic medium (pH 5.6) with the following content (g/l): glucose – 2.5; yeast extract – 2.0; NaCl – 1.0; (NH₄)₂SO₄– 2.0; MgSO₄·7H₂O – 1.0; KH₂PO₄– 13.5, and ii) a wine-containing medium (pH 3.0) with the following main components: 2.4 g/l sugar; 10.5% ethanol, organic acids (totally 7.0 g/l). Both media were sterilized by filtration through antimicrobial Corning filters (0.20 Mm, Corning Inc, Germany).

The yeast cells were cultivated on a shaker (Lab-therm, Adolf Kühner, Switzerland) under aerobic conditions with constant agitation (220 rpm) at 15°C, 20°C and 30°C. The yeast biomass was separated from the cultivation media by centrifugation (10,000 g, 20 min, Beckman J2-21 centrifuge, USA). The obtained cell precipitate was used for immobilized biocatalyst production.

Immobilization of yeast cells

PVA was purchased from NPO Azot (trade mark 16/1, Severodonetsk, Ukraine). The champagne yeast cells were immobilized by the following procedure: the cell precipitate was mixed with 11% PVA aqueous solution. The yeast concentration in the final homogeneous mass was 2.0% (w/w). The suspension of cells in the polymer solution was dripped onto a special device with round holes (2 mm diameter) and frozen for 2 days in the refrigerator (–20°C). The formed granules were thawed and washed with 0.9% NaCl.

Fermentation

Both the free and immobilized yeast cells were placed into 350 ml champagne bottles filled with a mixture (300 ml, pH 3.0) of champagne wine material and liqueur that finally contained 10.5% (v/v) ethanol and 24 g l⁻¹ sugar. Initially equal amounts of granules (20 g l⁻¹) were placed in all champagne bottles. Concurrently, free yeast cells were used in the investigation of fermentation as a comparative control. Initially, the amount of free and immobilized cells in each bottle was actually the same and equal to (1.0±1.5) × 10⁶ cell ml⁻¹. The fermentation was carried out under anaerobic conditions at 20°C for four weeks.

Analytical methods

Determination of the intracellular ATP concentration

The intracellular ATP concentration in the free and immobilized yeast cells was determined by the

bioluminescent method using a luciferine–luciferase reagent (Moscow, Russia) [19]. To extract ATP from the free yeast cells, an aliquot (0.1 ml) of cell suspension in the fermentation medium was treated by 0.9 ml of dimethylsulfoxide (DMSO). The determination of the ATP concentration in the immobilized cells included the treatment of previously weighed granules (100–300 mg) with 1 ml of DMSO. Cell extracts (50 μl) from the free and immobilized cells were transferred to a cuvette with an aliquot of luciferase reagent (50 μl), and the intensity of the bioluminescence was immediately measured on a microluminometer 3560 (New Horizons Diagnostic, USA). The ATP concentration in the samples was calculated using calibration curves plotted with ATP standards.

Other methods

The fatty acid pool (FAP) was analyzed according to a known procedure [20]. The carbon dioxide concentration accumulated during yeast fermentation was detected according to international methods of analysis of wine and musts [21]. The total acidity of the sparkling wines (expressed as g tartaric acid per liter) was determined by titration with 0.1 M NaOH. The volatile acidity (expressed as g acetic acid per liter) was determined by titration of the distillates obtained after steam distillation of the sparkling wine samples with 0.1 M NaOH [22].

The sugar and ethanol concentrations were determined using a Shimadzu LC-9A HPLC system. A Shim-park column (SCR 101 N), refractive index detector, distilled water as the mobile phase and 1-butanol as the internal standard were used [23].

To determine the dry matter content in the free cells, an aliquot of cultural broth was centrifuged (10,000 g, 20 min, Beckman J2-21 centrifuge, USA), the wet biomass precipitate was weighed and dried to constant weight at 80°C. The granules with immobilized cells were also dried to constant weight at 80°C. The amount of dry matter of polymer carrier was determined from the dry mass of granules with immobilized cells to calculate the dry weight of entrapped biomass.

The kinetic parameters of cell growth, namely, the specific rate of growth (μ , h⁻¹) and the time of cell doubling (t_d , h) of the free yeast cells, grown under different pH and temperature conditions, were estimated according to Pirt's recommendation [24].

RESULTS AND DISCUSSION

Cultivation conditions

To investigate the influence of the initial state of the yeast taken for immobilization on the features of the biocatalyst designed for SW production, cells were grown concurrently in a wine-containing medium at varied pH (3.0, 5.0 and 7.0) and in a semi-synthetic medium with constant initial pH 5.6 (Fig. 1). The yeast cells were comparatively cultivated at three

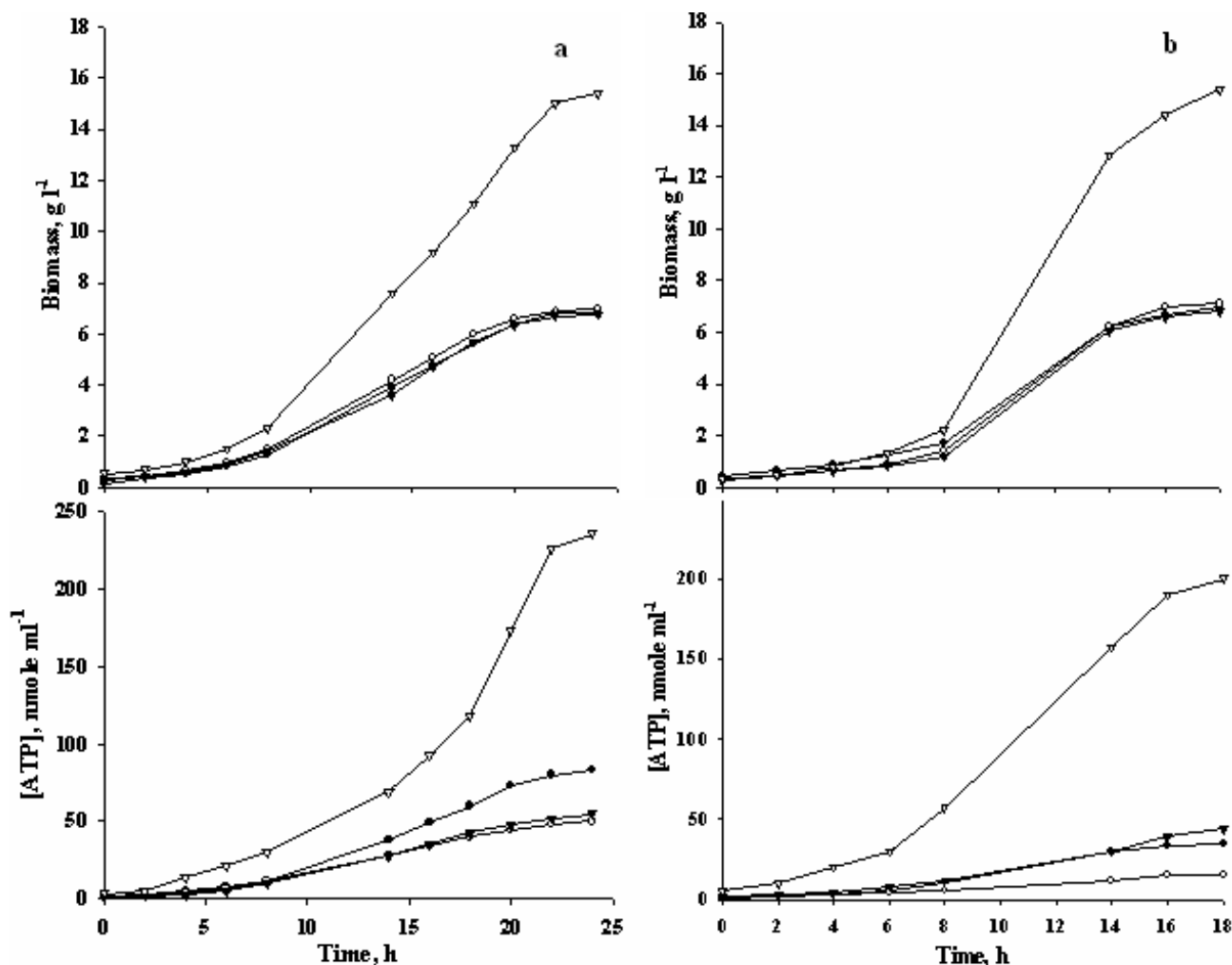


Figure 1 (a,b). The growth of yeast cells and the accumulation of the total intracellular ATP concentration in cells growing in various nutrient media at 20°C (a) and 30°C (b). Symbols ●, ○ and ▼ designate cells in the wine-containing media at pH 5.0, 7.0 and 3.0, respectively; symbol ▽ designates cells in the semi-synthetic medium (pH 5.6)

temperatures, 15°C, 20°C and 30°C, since the first one is widely used in practice for wine production, the second one is concurrently employed for yeast growth and the fermentation process, and the third temperature is optimal for maximal yeast biomass accumulation [25–27]. An approach to biocatalyst elaboration based upon the accumulation of viable yeast biomass under aerobic conditions for their further immobilization was applied in the study. It was taken into account that the cells presumably spend consumed substrates for their proliferation and biomass accumulation under aerobic conditions.

The physiological state of the free yeast was analyzed throughout the cell cultivation by the bioluminescent method of intracellular ATP detection, and ATP was used as a characteristic of cell viability (Fig. 1). It was determined that the cell growth correlated with the total ATP concentration estimated in the cells suspended in the culture broth (Fig. 1). The maximal ATP concentration was determined at the end of the

logarithmic phase of yeast growth independently of the medium type used for cell cultivation. The end of the exponential growth phase concurred with the 30th hour of cultivation at 15°C (data not shown), whereas the same stage of cell growth was observed two times earlier (at the 15th hour of cultivation) when the cultivation temperature was 30°C (Fig. 1). The application of 20°C as the temperature of yeast cultivation enabled the production of cells at in the same phase of growth after 23 h of cultivation.

It appeared that the lower the temperature of cultivation used, a lower specific rate of champagne yeast growth was observed (Table 1).

The pH variation of the wine-containing medium had no notable influence on both the specific rate of cell growth and the time of cell doubling at all the temperatures tested for cell cultivation (Table 1). Independently of the temperature regimes used for cell growth, the final yields of cell biomass in the same medium were actually similar. The use of a

Table 1. Parameters characterizing the growth of yeast cells under various cultivation conditions^a

Temperature	Medium ^b	μ , h ⁻¹	t _d , h	Maximal accumulated biomass, g l ⁻¹
15°C	A	0.11±0.01	6.3±0.6	6.5±0.3
	B	0.12±0.02	5.8±0.8	6.6±0.4
	C	0.10±0.01	6.9±0.6	6.4±0.3
	D	0.14±0.02	4.9±0.8	15.0±0.5
20°C	A	0.15±0.02	4.60±0.3	6.8±0.2
	B	0.16±0.03	4.30±0.1	6.9±0.3
	C	0.15±0.02	4.60±0.3	7.0±0.2
	D	0.18±0.01	3.85±0.2	15.4±0.4
30°C	A	0.2 ±0.02	3.15±0.2	6.8±0.1
	B	0.19±0.01	3.65±0.3	7.0±0.2
	C	0.2 ±0.02	3.15±0.2	7.1±0.2
	D	0.2 ±0.018	2.50±0.1	15.4±0.3

^aEach value is an average of three replicates.

^bWine-containing media with pH 3.0 (A), 5.0 (B) and 7.0 (C); semi-synthetic medium with pH 5.6 (D).

semi-synthetic medium enabled the accumulation of two times more biomass during yeast cultivation compared to the wine-containing medium (Table 1).

Taking into account the obtained results, 30°C seemed to be the preferable temperature for sufficiently rapid and productive accumulation of yeast biomass under aerobic conditions in the semi-synthetic medium.

Fatty acid content of yeast biomass

Cultivation conditions are known to significantly influence the biochemical content of cells thereby affecting the final features of the immobilized biocatalyst. Special attention was paid to the FAP of the yeast biomass since this parameter in many aspects predetermines the plasticity of the cell membrane and, hence, the viability of the cells in the "freezing-thawing" processes [28]. Therefore, the FAP could appear to be crucial for the viability of yeast cells immobilized into PVA CG.

Comparative analysis of the FAP in cells grown at 20°C and 30°C (Table 2) showed that the unsaturated fatty acids (UFA) in the yeast biomass obtained at the lower temperature in the semi-synthetic medium constituted almost 90% of the total amount of fatty acids as opposed to the cells grown at 30°C where the amount of UFA was less than 40%. It is known that a high general level of unsaturation of the membrane lipids allows the cells to maintain the permeability and fluidity of their membranes and thereby guarantee good adaptation of the microorganisms to low temperature [29].

It was determined that the yeast cells grown at 20°C were characterized by biomass containing a higher concentration of unsaturated lipids that showed lower thawing temperatures and provided higher membrane flexibility than the saturated analogues.

It should be noted, that the application of 15°C for yeast growth resulted in an average 5% increase in the

Table 2. Fatty acid content of the yeast biomass harvested after cell cultivation under various conditions

Fatty acids (Carbon chain length : Double bond)	Relative content, %							
	20°C				30°C			
	A ^a	B	C	D	A	B	C	D
Σ C 10:0+14:0	3.99	0.63	3.99	0.88	1.22	1.17	14.69	12.87
C 14:1	0.3	0.18	0.3	0.42	0.08	*	0.42	*
C 15:0	0.64	0.34	0.65	0.23	0.30	0.44	*	4.92
C 16:0	9.32	7.68	9.47	6.39	26.66	28.48	27.16	24.80
C 16:1	41.91	36.74	42.56	55.00	26.11	22.97	8.72	10.89
C 17:0	0.2	0.07	*	0.18	0.11	*	*	1.16
C 18:0	3.2	4.22	3.46	2.53	25.12	25.34	19.02	6.51
C 18:1	38.74	48.8	37.98	33.51	19.51	21.20	16.30	19.19
C 18:2	0.44	0.31	0.37	0.10	0.26	0.30	2.05	6.16
C 18:3	*	0.05	*	0.01	*	*	7.30	*
C 20:0	*	0.04	*	0.03	0.04	*	0.38	0.51
C 20:1	0.14	0.34	*	0.16	0.45	*	1.22	*
C 20:3	*	0.02	*	*	0.02	*	1.41	*
C 20:4	*	0.23	*	0.05	*	*	0.54	0.73
C 22:0	*	0.18	*	0.03	0.04	*	0.79	0.53
C 22:6	1.12	*	1.22	0.46	0.04	0.05	*	5.37
C 23:0	*	0.17	*	0.02	0.04	0.05	*	6.36
All fatty acids	100	100	100	100	100	100	100	100
Σ Unsaturated fatty acids	82.65	86.67	82.43	89.71	46.47	44.52	37.96	42.32

^aSee notation of the nutrient media used for yeast cultivation in Table 1. * Undetermined.

UFA content in the cells compared to the results obtained at 20°C in the semi-synthetic medium (data not shown). So, the lower temperature (15°C) of cell cultivation guaranteed the higher concentration of UFA in the cells, whereas the higher temperature (30°C) provided the rapid accumulation of yeast cells. Thus, 20°C appeared to be a compromise temperature for the sufficiently rapid growth of biomass with a concentration of UFA in the cells sufficient for their further immobilization into PVA CG.

Fermentation activity of the yeast cells

Cells grown at 20°C under aerobic conditions till the end of the logarithmic phase of growth were immobilized and used for bottled SW production. The fermentation ability of the free and immobilized yeast cells grown under the same conditions was concurrently analyzed. Two types of yeast were taken for the investigation: i) cells grown in the semi-synthetic medium (pH 5.6), and ii) yeast grown in a wine-containing medium (pH 3.0). The latter one was used as a control. The fermentation activity of the yeast was analyzed by measuring the CO₂ pressure accumulated in the bottles after four-week fermentation.

It was determined (Table 3) that the cells previously grown in the semi-synthetic medium possessed higher fermentation activity compared to the control. This behaviour was observed for both free and immobilized forms of the yeast (Table 3). The intracellular ATP concentrations determined in both the free and immobilized cells, after four-week fermentation, showed a twofold higher final level of the parameter in the yeast grown initially in the semi-synthetic medium (Table 3).

Independently of the type of medium initially used for cell cultivation, the characteristics of the immobilized cells, namely, the fermentation activity and intracellular ATP concentration, were than those of the free yeast (Table 3). This fact confirmed the advantages of using yeast in the immobilized form for SW production compared to the free state.

The main chemical characteristics of SW samples obtained after four-week fermentation conducted using

Table 3. The parameters determined after four-week fermentation realized with the application of free and immobilized yeast cells^a

Sample	Medium ^b	CO ₂ pressure, kPa	ATP, nmole mg ⁻¹ cell dry matter
Free yeast cells	A	380±10	2.7±0.3
	D	430±20	6.9±0.2
Immobilized yeast cells	A	450±20	27±5
	D	500±10	50±4

^aEach value is an average of three replicates.

^bSee notation in Table 1.

both free and immobilized yeast cells were analyzed. The comparison was done for cells initially grown in a semi-synthetic medium (pH 5.6) at 20°C under aerobic conditions. The results testified to the evident similarity of the wine characteristics obtained using the free and immobilized yeast: residual sugar – 1.6±0.1 g l⁻¹, ethanol – 11.2±0.1 % (v/v), total acidity – 7.45±0.05, volatile acidity – 0.8 g l⁻¹, SO₂ – 102.45±0.05 g l⁻¹. The slightly lower concentration of residual sugar and a slightly higher ethanol concentration observed in the wine with immobilized yeast were due to the metabolically better state of the cells.

CONCLUSION

The approach applied in this study to the production of an active biocatalyst with immobilized yeast cells for wine champagnization included the aerobic cultivation of cells in a semi-synthetic medium at 20°C up to the end of the logarithmic phase of growth and the further entrapment of biomass into PVA CG. The cultivation conditions used for biomass accumulation before its immobilization could affect the features of the biocatalyst and, thereby, the results of SW production. These data should be taken into account by biotechnologists developing yeast-immobilized preparations to be used in practice.

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IZVOD

USLOVI KULTIVACIJE POGODNI ZA IMOBILIZACIJU ČELIJA KVASCA U POLIVINIL ALKOHOLU I NJIHOVO KORIŠĆENJE U PROIZVODNJI PENUŠAVIH VINA FERMENTACIJOM U BOCI

(Naučni rad)

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U radu je na efikasan način izvršeno poboljšanje biohemijskog stanja ćelija kvasca pre imobilizacije sa ciljem dobijanja biokatalizatora sa visokim nivoom metaboličke aktivnosti i viabilnosti, koji bi se dalje koristio za proizvodnju penušavog vina. Ovaj pristup je podrazumevao da se hranljivi medijum koji je sadržao vino (pH 3.0) i koji se uobičajeno koristi za akumulaciju biomase šampanjskog kvasca zameni sa polusintetičkim (pH 5.6) koji se uobičajeno koristi za aerobni rast kvasca. Variranje temperature i pH tokom rasta dovelo je do promena u sadržaju pula masnih kiselina ćelija kvasca, a ovo je imalo bitan efekat na imobilizaciju ćelija u polivinil alkohol kriogelu. Merenjem koncentracije specifičnog intracelularnog ATP-a bioluminiscentnom metodom otkriven je stadijum rasta kvasca pogodan za imobilizaciju ćelija. Glavne karakteristike penušavih vina dobijenih nakon četvoronedeljne fermentacije pomoću, s jedne strane imobilisanih, a sa druge suspendovanih ćelija, bile su slične, ali je na osnovu detektovanog energetskog statusa ćelija viabilnost imobilisanih bila znatno viša od viabilnosti suspendovanih ćelija kvasca. Pritisak akumuliranog ugljendioksida u bocama sa imobilisanim ćelijama nakon fermentacije (do 500 kPa) takođe je bio viši od onog u bocama sa suspendovanim ćelijama.

Ključne reči: Imobilisani kvasac, Polivinil alkohol kriogel, Koncentracija intracelularnog ATP, Proizvodnja penušavog vina.