TECHNOLOGY OF ALCOHOL OXIDASE PRODUCTION FROM YEAST *CANDIDA BOIDINII* FOR USE IN FUNCTIONAL FOODS INTENDED FOR WITHDRAWAL SYNDROME ALLEVIATION

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(Received March 31, 2014; Accepted in revised form April 30, 2014)

Abstract: The present article considers the increasing popularity of beverages containing ethyl alcohol and fermentation products with the population of Russia. Statistical data show that alcoholic beverages with ethanol concentration exceeding 40 vol. % account for the largest share of the market. The development of a procedure for the production of alcohol oxidase from the yeast Candida boidinii is reported; the enzyme is intended for use in the manufacturing of functional foods for withdrawal syndrome alleviation. A procedure for the disruption of cell walls of the yeast Candida boidinii in a planetary ball mill PM 400 and methods for the removal of ballast substances reducing the catalytic efficiency and the specific activity of the enzyme preparation are presented.

Keywords: withdrawal syndrome, foods, beverages, alcohol oxidase, catalase, technology, purification

UDC 641:613.2:577.152.1
DOI 10.12737/5460

INTRODUCTION

The popularity of drinks containing ethanol and fermentation products with the population of Russia has been increasing recently. According to statistical data, alcoholic beverages with ethanol concentration above 40 vol. % account for the largest share of the market, while beer accounts for 25% only, and the share of sparkling wine and wine-based beverages is even lower. However, a trend towards a decrease in the popularity of vodka, whiskey, and cognac is evident, and the demand for low-alcohol beverages, such as beer, cocktails, and effervescent beverages, is growing rapidly [1, 2].

Increased alcohol consumption has an obvious negative impact going beyond the deterioration of physical and psychological health. Heavy alcohol use affects the welfare and health of people in the immediate environment of the drinker and has a negative impact on the society as a whole.

The state policy concerning alcohol production and consumption has always been a major factor affecting the consumption of alcohol by the population of Russia. All the documented increases in alcohol consumption and changes of consumption patterns towards increased use of strong beverages were related to actions of the state. Attempts to reduce regulation and relegate responsibility for alcohol production and consumption or increase profits from these processes have always led to increases in alcohol consumption and the share of strong beverages [3].

Alcoholic beverages are known to mankind since ancient times: wine was consumed in ancient Egypt (7-3 centuries BC), and both wine and beer were common in medieval Russia. Rye (vodka produced from grain) appeared in Russia in the mid-15th century, and illegal distillation of alcohol was widespread by the 1800s already. Statistical data show that the level of alcohol consumption in Russia is among the highest in the world. The annual consumption of ethyl alcohol per capita was 9.3 liters in 1992, while in the early 21st century it reached 15-18 l, a value significantly higher than those for the European countries.

Ethanol is used during medical procedures to sanitize the skin and sterilize surgical and medical instruments, because it induces denaturation of cytoplasmic proteins of various pathogens. The chemical structure and pharmacological properties of ethyl alcohol allow for classifying it as a central nervous system depressant. A general depression of physiological and emotional processes (withdrawal syndrome or hangover) is a consequence of alcohol consumption. The main symptoms of withdrawal syndrome are high blood pressure, chills, headache, hand tremors, loss of appetite, dry mouth, and depression. Withdrawal syndrome usually develops within a few hours after consumption of alcoholic beverages and lasts up to 2–3 days. An adverse consequence of withdrawal syndrome is repeated consumption of alcohol, which provides for a certain relief, but ultimately leads to poisoning of the organism and development of alcohol dependence [4].

Ingested ethanol is rapidly absorbed by diffusion in the stomach; the maximum concentration of alcohol in the blood is attained after 60–90 min. Notably, the rate of absorption varies and is affected by a multitude of different factors. The liver is the main site of ethanol metabolism, but the stomach epithelium can contribute to this process as well. Ethanol is dehydrogenated by alcohol dehydrogenase to form ethanal (acetaldehyde), which is subsequently converted to acetate by aldehyde dehydrogenase [5]. Oxygenase, peroxidase, catalase, and alcohol oxidase also play an important role in the

metabolism of ethanol in a living organism. According to Rumyantsev et al. (1981), consumption of ethanol results in increased concentration of acetaldehyde, which disturbs the functioning of a number of metabolic systems [6].

The quantity and variety of foods and pharmaceuticals intended to reduce the toxic effect of ethanol and its metabolites (acetaldehyde) on a living organism are currently insufficient in Russia, and therefore the development of competitive domestic harmonizing ingredients intended to bind toxic products of ethyl alcohol metabolism and eliminate them from the body is of high relevance and practical importance.

The interaction of chemicals and elements is the material basis of life and technological processes. The rate of biochemical reactions occurring in the body is normally very high due to the catalytic effect of enzymes. Therefore, enzymes and enzyme-based products receive considerable attention nowadays. Enzyme preparations are widely used in chemical, medical, pharmaceutical, and food industries. The world production of enzyme preparations for medical and food purposes increased significantly during the recent years, with more than half of the preparations obtained from microbial sources [7].

The market of enzymes and enzyme preparations for food industry has the highest capacity. Addition of enzyme preparations to the products of food industry offers new opportunities for the use of these products. Firstly, the use of enzymes allows for an increase in production volumes, and since there is a steady demand for foods produced using enzymes, these foods enable successful competition in the market. Secondly, the use of enzyme preparations allows for a considerable improvement of the quality of functional foods. Thirdly, improvement of working conditions in the production facilities becomes possible. Fourthly, the use of enzymes in food processing can contribute to increased food digestibility and nutrient availability, and to improvement of organoleptic properties of the food. Fifthly, the creation of novel foods becomes possible [5].

Enzymes are proteins acting as biological catalysts in living organisms. Enzymes are involved in catabolism and synthesis of vital (essential) substances required for the development and functioning of living organisms. All enzymes in a living organism belong to multienzyme systems that usually contain an enzyme component determining the catalytic activity and bioreaction rate in the whole system [4, 5].

Alcohol oxidase (EC 1.1.3.13) is an enzyme catalyzing the oxidation of ethyl alcohol to form acetic aldehyde, which is further metabolized into acetic acid subsequently decomposed to form water and carbon dioxide. A scheme of ethanol degradation by alcohol oxidase resulting in ethanal formation is shown in Figure 1. The enzyme may catalyze the oxidation of methyl, propyl, isobutyl, and amyl alcohols, ethylene glycol, formic acid, and various aldehydes [8].

The aim of the present work was to analyze the process of cultivation of the yeast *Candida boidinii* on a nutrient medium supplemented with cheese whey and to determine the optimal cultivation parameters for the yeast *Candida boidinii*.

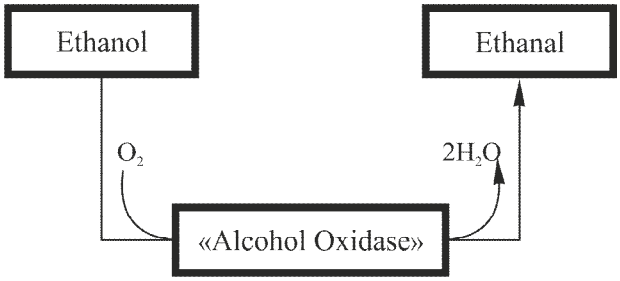

Fig. 1. Reaction of ethanol conversion to ethanal by alcohol oxidase.

OBJECTS AND METHODS OF RESEARCH

Yeast *Candida boidinii*, cheese whey conforming to the Technical Specifications of Belarussia (TU RB) 100098867.131-2001, ethanol (96 vol. %) conforming to GOST (State Standard) 5964-93, cultivation medium containing molasses conforming to GOST (State Standard) 171-81, and yeast autolysate conforming to the Technical Specifications (TU) 9184-003-56588117-08 were used as objects of the present study.

Standard, conventional, and original methods of analysis were used when performing research.

Yeast was grown aseptically under standard cultivation conditions in a batch bioreactor on a molasses medium supplemented with whey. The culture was continuously aerated during the cultivation process. Yeast was separated from the culture fluid by centrifugation and washed with purified water [9].

The specific growth rate was calculated as the yeast growth per hour, per unit of growing biomass.

Yeast biomass was quantitated as the difference between the biomass of microorganisms before and after cultivation.

The content of ash in the yeast biomass was determined by ashing the sample in the presence of an ethanol solution of magnesium acetate, which decomposes easily upon heating, promoting the formation of a large number of pores in the yeast sample.

Fractionation of proteins by molecular weight for further purification was performed using electrophoresis according to the procedure of Laemmli. Electrophoretic fractionation is based on the different mobility of proteins in an external electric field. The sign and magnitude of the electrical charge of a protein molecule are determined by the amino acid composition of the protein, and the mobility of the protein molecule at a given pH, ionic strength, and the strength of the electric current is dependent on its molecular weight and shape of the molecule. If an external electric field is imposed on a protein solution, the charged protein molecules move towards the oppositely charged electrode with a speed proportional to their charge and inversely proportional to the size and the degree of hydration of the particles [10, 11].

Electrophoresis was performed in polyacrylamide gel (PAG) plates immersed in a chamber filled with electrode buffer (0.066 M Tris, 0.19 M glycine, and

0.1% sodium dodecyl sulfate). The solutions to be analyzed were loaded into every well of the gel prepared. Electrophoresis was started at a current of 40 ± 0.2 mA and subsequently the current was increased to 80 ± 0.2 mA. The postelectrophoretic washing and staining of the gel involved consecutive incubations with a fixing solution, a washing solution, and a staining solution; all incubations were performed at 80 ± 2°C for 10 minutes. The gels were then destained in distilled water at a temperature of 25 ± 2°C.

Electrophoregrams were analyzed using an UV transilluminator TCP-20M (Vilber Lourmat, the United States) at a wavelength of 312 nm and a Doc-It LS gel documenting system.

A commercial protein marker kit from SibEnzyme consisting of highly purified recombinant proteins of known molecular weight was used to calibrate the electrophoretic gels. Human serum albumin solutions with known concentrations were used to construct a calibration curve for protein quantification.

Protein concentration in the sample was calculated according to the formula:

$$C = (C_p \cdot C_f)/100, \quad (1)$$

where C_p is the total protein content in the sample, g/100 g and C_f is the relative content of the protein fraction in the total protein, g/100 g protein.

The enzyme activity of alcohol oxidase was determined spectrophotometrically by measuring the decrease of optical density at 340 nm. For this purpose, a precise volume of the liquid under investigation, acetic aldehyde, ethyl alcohol (used as substrate), and phosphate buffer necessary for maintaining the required active acidity of the reaction medium were added into a quartz cuvette using a pipette (dispenser). The changes in optical density were monitored during 1 min. The enzyme activity of alcohol oxidase was calculated according to the formula (2) and expressed as U/mg protein:

$$A = \frac{\Delta E_{340} \cdot 3 \cdot 10^9}{6.22 \cdot 10^6 \cdot t \cdot b}, \quad (2)$$

where ΔE_{340} is the optical density measured at a wavelength of 340 nm; 3 is the sample volume in the spectrophotometric cuvette, ml; 10^9 is the factor for conversion of mol to nanomol; $6.22 \cdot 10^6$ is the molar extinction coefficient; t is the duration of the enzyme activity assay, min; and b is the amount of protein in the sample, mg.

Microfiltration was used to eliminate foreign microflora from the concentrate of the alcohol oxidase preparation. The membranes used were from Merck Millipore and had a pore diameter of 0.22 μm. The filtration was performed at 19 ± 2°C and an underpressure of 0.1–0.3 MPa [12].

Ion exchange chromatography on columns packed with diethylaminoethyl cellulose (DEAE-cellulose) and carboxymethyl cellulose (CM-cellulose) was used to remove extraneous proteins reducing the specific activity of the enzyme from the concentrated alcohol oxidase preparation [13].

The adsorbents were conditioned for ion exchange chromatography according to the following procedure: a suspension of 10 g of adsorbent in dilute hydrochloric acid was prepared and incubated for 1 hour at a temperature of 25 ± 2°C. The adsorbent was subsequently washed with copious amounts of distilled water until the pH of the slurry reached a value of 4.0 ± 0.2. Transfer of the adsorbent to hydrochloric acid and washing with distilled water was repeated several times until a pH value of 7.0 ± 0.2 was reached. Five volumes of sodium hydroxide solution were then added to the sorbent; the suspension was incubated for 1 h at room temperature and rinsed with distilled water until neutral pH was attained.

A chromatographic column was packed with the conditioned ion exchanger and equilibrated with a buffer solution (pH 7.4).

The sample was loaded to the column after almost complete removal of excess eluent; the volume of the sample loaded equaled 3 ml and it was carefully pipetted onto the surface of the sorbent. After the sample was absorbed, the surface was washed twice with 3-ml aliquots of eluent. The elution rate equaled 12 ml/h.

RESULTS AND DISCUSSION

The selection of process parameters of microorganism cultivation in order to increase the yield of the desired product, enable easy separation of the product from the culture fluid and ballast substances, and improve stability during prolonged storage, is an important issue for biotechnological production of biologically active substances used in the manufacturing of functional foods, alcoholic beverages, and soft drinks [9]. Cultivation of the yeast *Candida boidinii* at various process parameters was investigated at the first stage of the present study in order to ensure the maximal yield of alcohol oxidase preparation capable of destroying lower aliphatic alcohols.

Yeast are chemoorganoheterotrophic organisms that use organic compounds as a source of both energy and carbon. They need oxygen to breathe; however, most species are capable of deriving energy from the process of fermentation in the absence of oxygen. Pasteur showed that the presence of oxygen in the culture medium stimulates the breathing of yeasts and the production of alcohol and carbonic acid, which in turn accelerate the growth of the yeast [14].

Yeast cells are quite demanding with regard to cultivation conditions and the composition of the culture medium. Raw materials of vegetable, animal, mineral, and chemical origin were used for the preparation of the yeast culture media. Carbohydrates (sucrose, lactose, and glucose) were used as the energy source in the present study, and the inorganic compound dibasic ammonium phosphate, as well as other ammonium salts, was used as the nitrogen source, notwithstanding the ability of the yeast to metabolize nitrates. The natural components of the culture medium were represented by molasses and yeast autolysate. All the components included in the nutrient medium for yeast cultivation were carefully examined for biochemical suitability. Dairy (cheese) whey was added to the

culture medium for *Candida boidinii* yeast as an additional source of organic and mineral nutrients.

Comparative analysis of quantitative and qualitative composition of organic and inorganic substances found in cheese whey showed that this by-product of dairy industry is not inferior to molasses and can be used as an additional supplement for the cultivation of *Candida boidinii* yeast. Active synthesis of essential amino acids, proteins, and enzymes during the growth of the yeast *Candida boidinii* was detected, and the qualitative and quantitative composition of these substances was shown to depend on the composition and type of culture medium and supplements used in the cultivation of microorganisms.

The duration of yeast cultivation reportedly depends on the mode of cultivation, especially on nutrient, water, and air feed rate, and ranges from 12 to 18 hours [8]. The present study involved the assessment of the effect of culture medium supplementation with whey on the duration of the process. The process was carried out at a temperature of (30 ± 2°C) in a medium with an active acidity of 4.5–5.5 and whey content of 60%. The results of the experiments are shown in Table 1.

Table 1. Kinetics of cultivation of yeast *Candida boidinii*

Parameter	Control (18 h)	Duration of the process, h				
		12	14	16	18	20
Biomass yield, g/g	0.57± ±0.02	0.87± ±0.05	0.93± ±0.05	1.05± ±0.06	1.31± ±0.07	1.20± ±0.06
Yeast biomass accumulation, g/l	18.70± ±1.10	26.98± ±1.62	28.13± ±1.68	31.28± ±1.87	37.30± ±2.24	34.47± ±2.07
Specific growth rate, h^{-1}	0.008± ±0.0006	0.011± ±0.0006	0.013± ±0.0007	0.015± ±0.0007	0.016± ±0.0008	0.016± ±0.0008
Specific activity of alcohol oxidase, U/mg protein	18.51± ±1.11	9.11± ±0.54	18.65± ±1.12	28.41± ±1.70	39.82± ±2.39	39.79± ±2.38
Composition of yeast biomass, %:						
protein content by mass	39.0±2.34	41.5±2.4	42.8±2.5	43.4±2.6	42.0±2.5	34.2±2.0
Ash content in dry matter,%	9.3±0.73	9.2±0.73	9.3±0.73	9.3±0.73	9.3±0.73	9.1±0.5

Most of the intracellular proteins and enzymes are less stable than the extracellular ones due to the absence of disulfide bonds; the reducing properties of the intracellular medium render these bonds unnecessary. Cell wall disruption with the maximal efficiency possible is necessary for enzyme extraction from the cells. A large number of cell wall disruption techniques are currently used in biotechnology. Yeast cell walls are known to have a high strength, and therefore a strong mechanical impact is required to disrupt them: however, destruction of the target products, i.e. the inactivation of the enzyme systems isolated, must be avoided.

Planetary ball mill PM 400 was used in the present study to destroy the cells of yeast *Candida boidinii*. Destruction of the cell walls in the mill is based on vibration of moving parts. The mechanical strength of yeast and bacterial cells is reportedly different, and therefore selection of the correct parameters for cell wall destruction is necessary. The degree of destruction of the cell walls depends on rotor speed, the duration of exposure to centrifugal force, and process temperature.

Figure 2 illustrates the dynamics of degradation of *Candida boidinii* yeast cells.

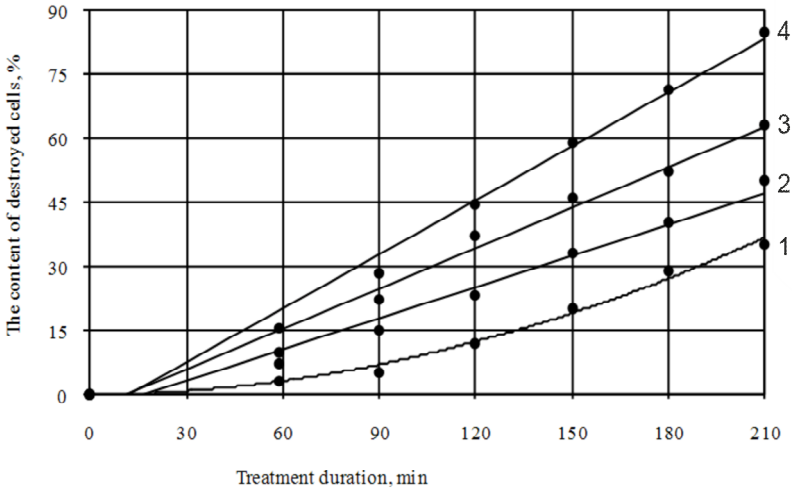

Fig. 2. Dynamics of degradation of yeast *Candida boidinii* cells during treatment with spherical glass beads of approximate size: 1 – 40 mesh; 2 – 60 mesh; 3 – 80 mesh; 4 – 100 mesh.

The use of approximately 60-mesh glass beads resulted in the disruption of 48% of *Candida boidinii* cells, while the use of approximately 80-mesh beads allowed for the increase of this value to 80%, and therefore treatment with approximately 80-mesh glass beads in a planetary mill PM 400 was chosen for cell disruption required for alcohol oxidase production from the yeast *Candida boidinii*.

The structure of the cells is altered and their integrity is lost during the treatment due to the effect of impact and friction forces, as well as Coriolis forces.

Precipitation by organic solvents miscible with water is widely used for the isolation of proteins from raw materials. Adding solvent to the protein extract leads to protein precipitation. The main reasons for this phenomenon are reduction of water activity, protein aggregation induced by the organic solvent, and protein precipitation due to the force of electrostatic attraction being inversely proportional to the dielectric constant of the medium.

The solvent used must be completely miscible with water and have sufficient precipitating capacity; besides, it must not become involved in reactions with proteins. Ethyl and isopropyl alcohols and acetone are most commonly used in protein chemistry for the precipitation of enzymes. Acetone was used in the present study because of its advantages over isopropanol and ethanol, namely, the possibility of using lower concentrations for protein precipitation at low temperatures and higher volatility enabling easy removal of the solvent from the dissolved precipitate under reduced pressure. Consequently, the denaturing effect of acetone is weaker. Dissolved protein precipitates contain residual solvent that must be removed. Removal of solvent and low molecular weight substan-ces is the most important method of concentrating dilute protein solutions; the procedure usually involves the use of semipermeable membranes. The two methods of protein chemistry employing such memb-ranes are dialysis and gel filtration; the former was used in the present study to remove acetone from an alcohol oxidase preparation. The use of special colloi-dal films with ultramicroscopic pores allowed for the retention of high molecular weight protein substances in the concentrate. Ultrafiltration using gas pressure to force the liquid through the membrane was the second method used to concentrate the protein solution. The use of this method resulted in the isolation of two fractions consisting of large macromolecules and smaller macromolecules, respectively. The protein solution was diluted by phosphate buffer several times during concentration because the rate of ultrafiltration decreased concomitantly to the increase of protein concentration in the solution [12, 13].

Microfiltration, which allows for very efficient elimination of suspensions, large colloids, the majority of bacteria and microorganisms, and viruses from solutions, was used to remove pathogenic micro-organisms from the protein concentrate. Microfiltration cartridges produced by Millipore (pore diameter 0.60, 0.40, and 0.22 μm) were used. Microbiological purity of the resulting protein concentrate was evaluated using culture in petri dishes [15, 16]. The results of the experiments are shown in Table 2.

Table 2. Results of microbiological analysis of alcohol oxidase preparations

Parameter	Standard	Pore diameter, μm		
		0.6	0.4	0.22
Coliform bacteria in 0.1g	not allowed	not found		
Pathogenic microorganisms, including *L. monocytogenes* and *Salmonella* spp. in 25.0 g	not allowed	not found		
St. aureus in 1.0 g	not allowed	not found		
Mesophilic aerobic, CFU/g, not more than	$5 \cdot 10^4$	$8 \cdot 10^6$	$9 \cdot 10^4$	$3 \cdot 10^2$
Facultative anaerobic microorganisms, CFU/g, not more than	$1 \cdot 10^4$	$6 \cdot 10^5$	$8 \cdot 10^3$	$0.7 \cdot 10^2$

Analysis of the microbiological purity of the protein concentrate obtained revealed the inefficiency of Millipore microfilters with pore diameter of 0.60 or 0.40 μm, since colonies of microorganisms developed in the samples when these filters were used. Using Millipore microfiltration elements with a pore diameter of 0.22 μm yielded a concentrated enzyme preparation conforming to the microbiological requirements of the State Pharmacopoeia.

Protein molecules are usually selectively adsorbed on solid phases of different types and therefore adsorption techniques, column chromatography in particular, are widely used for the separation of protein macromolecules. The use of these methods often results in the production of proteins having the highest degree of purification, which is equivalent to the maximum possible increase of specific activity in the case of enzymes. Ion exchangers, potassium phosphate (crystalline or in gel form), and a variety of affinity adsorbents designed for certain types of enzymes are the major protein adsorbents.

Two types of ion exchangers, namely, the cation exchanger carboxymethyl cellulose and the anion exchanger diethylaminoethyl cellulose, are used in protein chemistry. DEAE-cellulose, which is used most frequently, has a complex structure and a complex titration curve. The use of ion exchange chromatography on DEAE cellulose allows for enzyme

purification under mild conditions and the preservation of the properties of the native protein.

Electrophoretic analysis of the fractions revealed the presence of a single band with a molecular weight corresponding to that of alcohol oxidase. The enzyme preparation is subsequently immobilized on an edible carrier, usually a polymer, and can be used in the technology of functional foods.

Thus, the process of cultivation of *Candida boidinii* yeast in a nutrient medium supplemented with cheese whey has been investigated in order to increase the activity of yeast enzyme systems. The following cultivation parameters were found to be optimal for *Candida boidinii* yeast: whey concentration 60%, temperature 30 ± 2°C, pH 4.5–5.5, and cultivation time not less than 18 ± 0.5 h. The technology of isolation and purification of alcohol oxidase from *Candida boidinii* yeast for further use of this enzyme in functional food biotechnology has been developed. Destruction of cell walls of *Candida boidinii* yeast in a PM 400 planetary mill filled with glass beads of approximately 80 mesh in size and enzyme precipitation by a nonpolar solvent were shown to be the optimal parameters of enzyme isolation. The optimum parameters for enzyme purification aimed at increasing the catalytic activity comprised the use of a membrane UPM-67 followed by filtration through a Millipore membrane (pore diameter 0.22 μm), and column chromatography on CM-cellulose and DEAE-cellulose.

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