THE SELECTION OF CULTIVATION TECHNIQUES FOR THE PRODUCTION OF FUNGAL ENZYMES ON WINERY SOLID WASTE*

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The wine industry generates significant amounts of waste, the management and disposal of which represents a major environmental problem due to its seasonal character and polluting characteristics. Solid waste generated in wineries in Serbia is mostly used as a biological fertilizer. The production of fungal enzymes could be a potential solution for the valorization of this waste. Fungal enzymes are used in numerous industries, including the detergent industry. The aim of this study was to select the most successful cultivation technique for the production of fungal enzymes by cultivating a wild-type isolate of *Trichoderma* sp. on media that contained solid waste generated in the winery by processing black and white grapes. The success of the bioprocess was evaluated based on the protein content and proteolytic, lipolytic, amylolytic, and cellulolytic activity of the obtained crude enzyme preparations at the temperature conditions of detergent application, i.e. at 30 °C and 60 °C. The submerged cultivation technique with external mixing and spontaneous aeration proved to be the cultivation technique that obtained the highest protein content in the crude enzyme preparations. The submerged cultivation technique with intensive aeration proved to be the most appropriate for achieving the highest enzyme activity of the crude enzyme preparations. On the other side, the solid-state cultivation technique with intensive aeration resulted in the lowest protein content and the lowest enzymatic activity of crude enzyme preparations. Liquid medium cultivation techniques have proven to be more successful when compared to solid-state techniques.

Keywords: biotechnological production, fungal enzymes, *Trichoderma* sp., cultivation techniques, valorization of winery waste

Introduction

In the world fruit market, grapes are one of the most produced, with up to one-third of the grapes produced being used for wine production. During wine production, significant amounts of solid organic waste are generated, such as grape pomace (marc) and stalks, which must be disposed of properly [1]. In the process of making wine, about one-quarter of the grape mass is converted into grape pomace, which is primarily made up of the stalks, skin, ruptured pulp cells, and seeds that are left over after the crushing and pressing processes that are used to obtain the stum or grape juice [2]. According to the Statistical Office of the Republic of Serbia, 162,481 tons of grapes were produced in the Republic of Serbia in 2022 [3], the processing of which generates significant amounts of waste that must be properly disposed of.

Grape pomace has a very complex composition. Unfermented white grape pomace includes residual sugars, whereas black grape pomace contains residual sugars, as well as some alcohol (ethanol) [4]. The amount of residual sugars and alcohol in the grape pomace varies greatly depending on the fruit's origin, as well as the procedures and techniques used in crushing the grapes and producing the wine [5]. Thanks to these organic substances, grape pomace can serve as a very good substrate in biotechnological production, among which is the biotechnological production of fungal enzymes [6,7].

Different microorganisms, including bacteria and fungi, as well as plants and animals, can produce enzymes. The majority of the enzymes used in industrial manufacturing are synthesized by very few microbial species, namely fungi, and bacteria, particularly *Aspergillus, Trichoderma,* and *Bacillus* [8].

The usage of specific enzymes as biocatalysts in a large number of industrial processes demonstrates the commercial significance of these enzymes. They have found uses in the agriculture and medical fields, as well as the food and beverage industry, the textile industry, the detergent industry, the paper industry, waste man

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agement, the leather processing industry, the industry of biofuels, and the pharmaceutical industry [9,10]. The majority of industrial enzymes in use today work hydrolytically and are utilized to break down a variety of natural compounds [9]. Major classes of enzymes in detergents include proteases, lipases, amylases, and cellulases [10]. The majority of stains, whose causes can vary (proteins, lipids, starch), can be removed by combining detergents with hot water and vigorous mixing. These circumstances do harm to the laundry and reduce its lifespan, and it is impossible to overlook the financial costs associated with heating the water and washing the laundry for an extended period of time. Detergents that contain enzymes can be used at lower temperatures and for shorter periods of time while producing more effective stain removal [11].

Due to simple parameter control and a solid technological foundation for scaling up to the industrial level, submerged cultivation techniques are the primary method for the production of enzymes [12]. As an alternative to these cultivation methods, solid-state cultivation techniques are employed for the production of enzymes because they are less expensive in terms of increased volumetric production [13, 14].

The aim of this study was to select the most successful cultivation technique for the production of fungal enzymes by cultivating a wild-type isolate of *Trichoderma* sp. on media containing solid waste generated in the winery during the processing of black and white grapes. The success of the bioprocess was evaluated based on the protein content and proteolytic, lipolytic, amylolytic, and cellulolytic activity of the obtained crude enzyme preparations at the temperature conditions of detergent application, i.e. at 30 °C and 60 °C.

Material and methods

Producing microorganism

The fungal strain that was used for enzyme production in laboratory conditions was isolated from the natural environment (*Trichoderma* sp.). Producing microorganism has been stored in the Microbial Culture Collection of the Faculty of Technology Novi Sad at 4 °C on an agar slant (Sabouraud Maltose Agar ®, HiMedia, India) and subcultured every six months.

Cultivation media

Sabouraud Maltose Agar (SMA) was used in the initial phase of production. Enzyme production was carried out on media that contained dried winery solid waste (pomace) generated after pressing crushed white grapes and fermented crushed black grapes. Cultivation media for the liquid cultivation techniques contained 15 g/L of solid winery waste as a source of nutrients, and in addition, they also contained $(NH_4)_2SO_4$ and K_2HPO_4 in quantities of 5 g/L and 2 g/L, respectively. Cultivation media for solid-state cultivation techniques was moistened solid winery waste without the addition of other ingredients.

The pH value of these cultivation media was adjusted to 4.5±0.2 and they were then sterilized by autoclaving (121 °C, 2.1 bar, 20 min).

Inoculum preparation

The producing microorganism was subcultured on an agar slant and incubated at 28-30 °C until intensive sporulation. Further, the inoculum preparation procedure has included suspending spores of producing microorganisms in sterile physiological saline in order to obtain a suspension with a concentration of 1.5 x 10^6 spores/mL. The prepared inoculum was used for inoculation in all experiments within this study. Inoculation was performed by adding 10% (v/v) of the inoculum prepared as described above, to a cultivation medium.

Enzyme production

The production of fungal enzymes was carried out through five different cultivation techniques: static emersion cultivation on a liquid medium with spontaneous aeration (Technique 1); submerged cultivation with external mixing and spontaneous aeration (Technique 2); submerged cultivation with intensive aeration with sterile temperate and conditioned air (Technique 3); solid-state cultivation with spontaneous aeration (Technique 4); and solid-state cultivation with intensive aeration with sterile temperate and conditioned air (Technique 5).

The production of fungal enzymes using the mentioned cultivation techniques was carried out under the same conditions, i.e. at a temperature of 28-30 °C for 168 hours in a volume of cultivation medium of 150 mL. Cultivation technique 1 and cultivation technique 4 were carried out in Petri dishes (ϕ 200 mm) by stationary cultivation in a laboratory thermostat under aerobic conditions. Cultivation technique 2 was carried out in an Erlenmeyer flask with a volume of 500 mL under aerobic conditions and 150 rpm (Laboratory shaker KS 4000i control, Ika® Werke, Germany). Cultivation technique 3 was carried out in a 500 mL Drechsel bottle with a supply of sterile tempered and conditioned air with an intensity of 0.5 vvm. Cultivation technique 5 was conducted in a solid-state bioreactor with a supply of sterile tempered and conditioned air with an intensity of 0.5 vvm.

After the end of the biosynthesis, the lost volume in the liquid media was compensated by adding sterile distilled water to the initial volume of 150 mL. The solid media were suspended in 150 mL of sterile physiological saline and the enzyme extraction was performed at a temperature of 28-30 °C with constant stirring at 200 rpm for 30 minutes. The solid and liquid phases of the cultivation media were separated by filtration through qualitative filter paper. The obtained filtrates were used for further analysis as crude enzyme preparations.

Protein content evaluation

The content of total proteins in crude enzyme preparations was determined by the modified spectrophotometric method according to Lowry [15, 16] and the ab

sorbance was read at 660 nm, in an optical path of 1 cm, compared to distilled water. Bovine serum albumin standard (BSA®, HiMedia, India) was used to construct the calibration line.

Enzyme activity evaluation

The protease activity of the crude enzyme preparations was determined according to the modified Hagihara method [17, 18] where a 1% solution of casein in a 0.05 M phosphate buffer with a pH of 7.5 was used as a substrate. The casein solution and the crude enzyme preparation (blank: distilled water) were mixed in a ratio of 5:1. After homogenization, hydrolysis was carried out at temperatures of 30 °C and 60 °C for 10 minutes in a thermostatic water bath. After hydrolysis, 0.1 M CCl_3COOH was added and the mixture was homogenized and left for 30 min at 30 °C in a thermostatic water bath and then centrifuged for 20 min at 10,000 rpm at a temperature of 30 °C. The proteolytic activity of the crude enzyme preparations was evaluated based on the content of amino acids, expressed through the content of tyrosine, in supernatants obtained after centrifugation, which was determined by a spectrophotometric method at 275 nm, compared to a blank test. The L-tyrosine standard was used for the construction of the calibration curve.

As a substrate for determining the amylolytic activity of crude enzyme preparations, a 1% solution of starch in distilled water was used, while a 1% solution of carboxymethylcellulose in 0.1 M acetate buffer with a pH of 5.5 was used as a substrate for determining the cellulolytic activity of crude enzyme preparations. Substrate solutions and crude enzyme preparations (blank: distilled water) were mixed in a ratio of 3:1. After homogenization, hydrolysis was carried out at temperatures of 30 °C and 60 °C for 30 minutes (thermostated water bath). The amylase and cellulase activity was evaluated based on the glucose content in the hydrolyzed samples, which was determined by the spectrophotometric method according to Miller for determining the content of reducing substances [19] in which dinitrosalicylic acid was used as a reagent, and the absorbance was read at 540 nm, in an optical path of 1 cm, compared to a blind test. An anhydrous glucose standard was used to construct the calibration line.

As a substrate for determining the lipolytic activity of the crude enzyme preparations, commercial olive oil was used [20]. For this purpose, commercial olive oil, 0.05 M acetate buffer with a pH value of 5.5, distilled water and crude enzyme preparation (blank: distilled water) were mixed in a ratio of 2:4:3:1. Hydrolysis was carried out at temperatures of 30 °C and 60 °C, with constant stirring at 120 rpm, for 30 minutes (laboratory rotary shaker, IKA® KS 4000i control, Germany). The reaction was stopped by adding double of the total volume of a 1:1 mixture of acetone and ethanol. The activity of the lipases was evaluated based on the content of freed fatty acids in the hydrolyzed samples, which was determined by acid-base titration using a solution of 0.05 M sodium hydroxide with phenolphthalein as an indicator. The content of free fatty acids (C, [g/L]), expressed through the content of oleic acid, was calculated using Equation 1.

$$C = \frac{K - K_0}{V_s} \cdot F \cdot 14 \cdot 10^{-3} \quad \text{.....(1)}$$

C - content of free fatty acids, expressed through the content of oleic acid [g/L],

K - volume of sodium hydroxide used for sample titration, [mL],

K_0 - volume of sodium hydroxide used for the titration of the blank sample, [mL],

V_s - sample volume of the crude enzyme preparation, [mL],

F – correction factor of the units from mL to L (1000), [1].

14 - 10^{-3}- oleic acid content equivalent to the consumption of 1 mL of 0.05 M sodium hydroxide, [g/L].

Results and discussion

In accordance with the defined aim of this research, the selection of the most successful cultivation technique for the production of fungal enzymes on winery solid waste was examined. The choice was made between five cultivation techniques, three of which were carried out on a liquid medium, and two on a solid medium (solid-state cultivation).

By solid-state cultivation techniques (Techniques 4 and 5) and emersion cultivation technique (Technique 1), the producing microorganism formed a rich aerial mycelium with obvious sporulation. By submerged cultivation with spontaneous aeration (Technique 2), the producing microorganism grew in the form of pellets without obvious sporulation, while by submerged cultivation with intensive aeration (Technique 3), the producing microorganism grew abundantly around the air disperser in the form of mycelium without obvious sporulation.

The success of the bioprocess was evaluated based on the protein (enzyme) content and proteolytic, lipolytic, amylolytic, and cellulolytic activity of the obtained crude enzyme preparations at the temperature conditions of detergent application, i.e. at 30 °C and 60 °C.

The enzyme content in the crude enzyme preparations obtained after the bioprocess has been carried out through various cultivation techniques (Techniques 1-5) is expressed through the total protein content, assuming that the initial protein content in the media is equal. The obtained results on the determination of enzyme content in crude enzyme preparations are shown in Table 1.

Table 1. Protein content in crude enzyme preparations

Cultivation technique	Total protein content [mg/mL]	
	White grape pomace	Black grape pomace
Technique 1	0.2904	0.4195
Technique 2	0.4518	0.5808
Technique 3	0.3430	0.3720
Technique 4	0.2782	0.3549
Technique 5	0.1420	0.1940

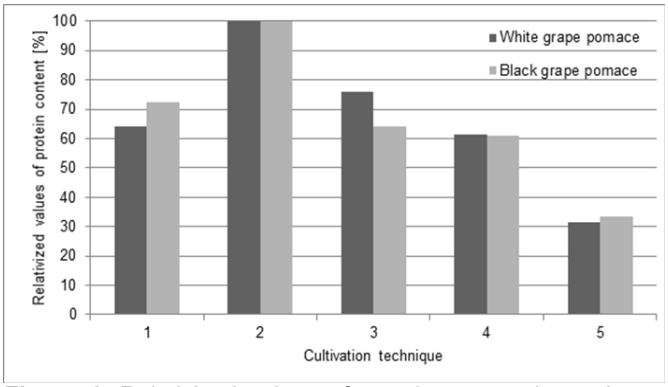

Figure 1. Relativized values of protein content in crude enzyme preparations

The results shown in Table 1 indicate that a slightly higher protein content is obtained in media with black grape pomace than in media with white grape pomace. White grape pomace has an average crude protein content of about 14%, while black grape pomace has a crude protein content of about 16% [21], which is probably the reason why media with black grape pomace has a higher protein (enzyme) content. Regardless of the substrate, the protein content in liquid media ranges from 0.29 to 0.58 mg/mL, which is higher than in solid media, where the content ranges from 0.14 to 0.35 mg/mL.

In order to conduct mutual comparison of the obtained results and to interpret results more easily, the experimental data were relativized. Relativization was done for each of the two substrates used in media formulation in order to represent the influence of the cultivation technique on the success of the bioprocess better. The relativized values of the protein (enzyme) content in crude enzyme preparations are shown in Figure 1.

As can be seen in Figure 1, the cultivation technique that obtains the highest enzyme content in crude enzyme preparations is submerged cultivation with spontaneous aeration and external mixing (Technique 2). Cultivation techniques 1, 3 and 4 proved to be equally successful with each other, with a protein content 30-40% lower than technique 2. Cultivation technique 5 proved to be the least successful, with protein content 70% lower than the most successful technique.

The enzymatic activity of crude enzyme preparations obtained after the bioprocess was carried out through different cultivation techniques (Techniques 1-5) were expressed through the activity of proteolytic, amylolytic, cellulolytic and lipolytic enzymes. The obtained results on the determination of enzymatic activity under the temperature conditions of detergent application in crude enzyme preparations obtained by different cultivation techniques on media containing white grape pomace and black grape pomace are shown in Table 2.

Table 2. Enzymatic activity of crude enzyme preparations obtained by different cultivation techniques on media containing white grape pomace and black grape pomace

Substrate	Cultivation technique	Enzymatic activity of crude enzyme preparations after hydrolysis [U/mL]							
		Proteolytic		Amylolytic		Cellulolytic		Lipolytic	
		30 °C	60 °C	30 °C	60 °C	30 °C	60 °C	30 °C	60 °C
White grape pomace	Technique 1	0.20	0.17	0.83	0.92	0.77	0.65	66.91	57.00
	Technique 2	0.23	0.21	1.01	1.03	0.80	0.90	69.39	61.95
	Technique 3	0.27	0.25	1.13	1.21	1.16	1.37	89.21	66.91
	Technique 4	0.12	0.13	0.24	0.33	0.34	0.40	34.69	29.74
	Technique 5	0.09	0.07	0.33	0.42	0.19	0.19	4.96	2.48
Black grape pomace	Technique 1	0.12	0.14	0.93	0.92	0.72	0.78	61.95	69.39
	Technique 2	0.16	0.18	1.12	1.17	0.81	0.86	69.39	69.39
	Technique 3	0.17	0.16	1.16	1.28	1.16	1.18	79.30	89.21
	Technique 4	0.10	0.11	0.22	0.25	0.54	0.64	27.26	14.87
	Technique 5	0.08	0.07	0.31	0.32	0.39	0.44	2.48	2.48

Technique 1 – Emersion cultivation technique

Technique 2 – Submerged cultivation technique with spontaneous aeration and external mixing

Technique 3 – Submerged cultivation technique with intensive aeration

Technique 4 – Solid-state cultivation technique with spontaneous aeration

Technique 5 – Solid-state cultivation with intensive aeration

Table 2 shows that the enzymatic activity of the crude enzyme preparations exhibited similar trends for both media containing white and black grape pomace as substrates. Lipases were shown to be the enzyme class with the highest activity, followed by amylases and cellulases, while proteases showed the lowest activity. The lipolytic activity is several dozen times higher than the others, which corresponds to the results obtained in a similar paper [22]. Since the content of crude lipids (7.4-10.19%) is lower than other constituents of grape pomace [21], it can be concluded that crude proteins (14.48-16.60%) and fibers (28.54-32.28%) from grape pomace are less adoptable by the production microorganisms than crude lipids of grape pomace. Table 2 also shows that the enzymes of the crude enzyme preparations have similar activities at different temperatures, i.e. at 30 °C and at 60 °C. The difference in enzyme activity of the crude enzyme preparations at these two temperatures is about 10% for each enzyme class.

Due to the simplicity of the presentation of the results and the discussion, as well as the similarities mentioned above, the success of the bioprocess based on the enzyme activity of the crude enzyme preparations will be discussed on the example of one substrate and one temperature at which the enzyme activity was tested. In order to conduct mutual comparison of the obtained results and to interpret results more easily, experimental data were relativized. For each enzyme class, relativization was performed individually in order to examine the influence of the cultivation technique on the success of the bioprocess. The relativized values of the enzymatic activity of crude enzyme preparations from bioprocesses conducted on media with white grape pomace as a substrate are shown in Figure 2.

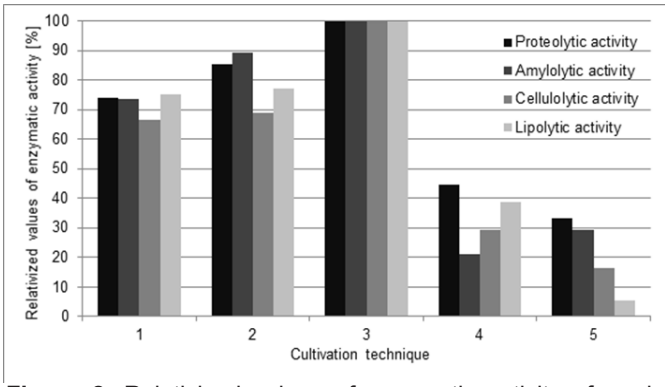

Figure 2. Relativized values of enzymatic activity of crude enzyme preparations from bioprocesses conducted on media with white grape pomace, after hydrolysis at 30 °C

As can be seen in Figure 2, the cultivation technique that proved to be the most successful in terms of enzyme activity is submerged cultivation with intensive aeration (Technique 3). Technique 1 and Technique 2 proved to be equally successful with each other, with enzyme activity 20-30% lower than Technique 3, while solid-state cultivation techniques (Technique 4 and Technique 5) proved to be less successful, with enzyme activity 70% lower than the most successful technique.

Conclusion

Based on the results presented in this paper, it is obvious that solid-state cultivation techniques have proven less successful for the production of fungal enzymes, while submerged cultivation techniques have been much more successful. In order to increase the success of the bioprocess, it is necessary to carry out optimization of process conditions, as well as the composition of the cultivation medium.

The results of this study have great importance from an ecological point of view, considering the fact that the biotechnological production of enzymes on cultivation media containing solid winery waste represents a promising solution for the sustainable valorization of this effluent.

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Izvod

IZBOR TEHNIKE KULTIVACIJE ZA PROIZVODNJU FUNGALNIH ENZIMA NA ČVRSTOM OTPADU VINARIJE

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Industrija vina stvara značajne količine otpada, čije upravljanje i odlaganje predstavlja veliki ekološki problem zbog sezonskog karaktera i zagađujućih karakteristika. Čvrsti otpad koji nastaje u vinarijama u Srbiji najčešće se koristi kao biološko đubrivo. Proizvodnja mikrobnih enzima mogla bi biti potencijalno rešenje za valorizaciju ovog otpada. Među mikroorganizmima, plesni se najčešće koriste kao producenti enzima. Između ostalih plesni, sojevi roda *Trichoderma* se često koriste u proizvodnji enzima. Mikrobni enzimi su našli primenu u brojnim industrijama, uključujući industriju deterdženata. Glavne klase mikrobnih enzima koji ulaze u sastav deterdženata su proteaze, lipaze i amilaze. Cilj ovog istraživanja bio je da se odabere najuspešnija tehnika kultivacije za proizvodnju fungalnih enzima kultivacijom prirodnog izolata *Trichoderma* sp. na medijumima koji su sadržali čvrsti otpad nastao u vinariji preradom crnog i belog grožđa. Uspešnost bioprocesa je procenjena na osnovu ukupnog sadržaja proteina i proteolitičke, lipolitičke, amilolitičke i celulolitičke aktivnosti dobijenih sirovih enzimskih preparata pri temperaturnim uslovima primene deterdženta, odnosno na 30 °C i 60 °C. Submerzna tehnika kultivacije sa eksternim mešanjem i spontanom aeracijom se pokazala kao tehnika kultivacije kojom se dobija najveći sadržaj proteina u sirovim enzimskim preparatima. Submerzna tehnika kultivacije sa intenzivnom aeracijom se pokazala kao tehnika kultivacije kojom se dobija najveća enzimska aktivnost sirovih enzimskih preparata. Solid-state tehnika kultivacije sa intenzivnom aeracijom se pokazala kao tehnika kultivacije kojom se dobija najniži sadržaj proteina i najmanja enzimska aktivnost sirovih enzimskih preparata. Tehnike kultivacije u tečnom medijumu su se pokazale uspešnije od solid-state tehnika kultivacije.

Ključne reči: biotehnološka proizvodnja, fungalni enzimi, *Trichoderma* sp., tehnike kultivacije, valorizacija vinarijskog otpada