

THE UTILISATION OF GRAPE MARC FOR OBTAINING FEED PROTEIN WITH ETHANOL CONSUMING YEASTS

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Abstract

The research presented in this work has been directed to achieve an optimal and economic medium, for the protein biosynthesis. The carbon source and the mineral elements are provided by the fresh marc diffusion solution and the source of organic nitrogen and growth factors are provided by proteic wine yeast extract. For the alcoholic fermentation it was used a strain of Saccharomyces ellipsoideus and for obtaining the biomass, a strain of Candida robusta. There were performed two series of experiments in which the alcoholic fermentation progress was monitored as follows: by titration of the carbohydrates, at the beginning and respectively at the end of the fermentation; by determining the optical density of the culture medium and by the determination of alcohol in the fermented solution. Protein biosynthesis was monitored by measurement of the alcohol concentration at the beginning and end of the process, by the determination of the optical density of the culture medium, by the determination of residual sugar, and by determining wet and dry biomass. The wet biomass was used for the protein titration. At the termination of the bioprocess was determined the dry matter in the culture medium.

INTRODUCTION

Grape marc is considered a waste byproduct of the wine industry and high quantities of this secondary product are released in the environment every year [1], becoming eventually a major disposal problem in the waste management of this industry [2]

It is basically used in low value products as fertiliser in the agriculture [3,4]. However, grape marc is a promising source of many compounds [5] including polyphenols, fibres, tannins, tartaric acid, citric acid, anthocyanin and neutral sugars [6] which can be used for their nutritional properties [7].

Our research is targeted on the utilisation of this so called waste in an economical and effective way.

The aim of this study was to achieve an optimal and economic culture medium, for the protein biosynthesis. The carbon source and

the mineral elements are provided by the fresh marc diffusion solution and the source of organic nitrogen and growth factors are provided by proteic wine yeast extract.

MATERIALS AND METHODS

The biological material used in the experiments was the marc resulted from the processing of white wine grapes and wine yeast (yeast sediment from fermentation of white and red wines). The marc was obtained from the continuous press and contained only the skin and seeds, the clusters being previously removed. The marc was sampled immediately after pressing, therefore it was fresh, unfermented, and obtained from healthy grapes. Both wine yeast and the marc were distributed immediately after harvesting in plastic bags and stored in a freezer at -12 °C.

The *Saccharomyces ellipsoideus* strain used in the alcoholic fermentation process was previously isolated from the marc. To obtain the biomass we tested a strain of *Candida robusta*. The experiments were performed in 750 ml Erlenmeyer flasks on a shaker with adjustable speed. For the alcoholic fermentation was used the minimum speed, because this kind of fermentation requires a high consumption of oxygen [8], and the temperature was set to 28 °C.

The alcoholic fermentation progress was monitored as follows: by titration of the carbohydrates, at the beginning and respectively at the end of the fermentation; by determining optical density (OD) of the culture medium and by the determination of alcohol in the fermented solution. Protein biosynthesis was monitored by measurement of the alcohol concentration at the beginning and end of the process, by the determination of OD of the culture medium, by the determination of residual sugar, and by determining wet and dry biomass. The wet biomass was used for the protein titration. At the termination of the bioprocess was determined the dry matter in the culture medium.

Spectrophotometric determination of sugars

The principle of the method - the method of determination is based on the direct reaction of simple reducing doses, with potassium ferricyanide in alkaline environment. The reaction is quantitative (complies the Lambert-Beer law). The excess of ferricyanide is measured at 420 nm compared to the blank as the control.

Reagents and equipment required:

- alkaline reagent, 2% of potassium ferricyanide 0.05N in anhydrous sodium carbonate 53%.
- 30% solution of zinc sulphate.
- 15% solution of potassium ferrocyanide.
- 20% NaOH-solution.
- spectrophotometer.

The method applied involves completing the following stages:

- neutralization of the hydrolyzed solutions with NaOH solution until reaching neutral pH;

- removal of components that may interfere with the color reaction with potassium ferricyanide, by treatment with a mixture of $ZnSO_4 / K_4Fe(CN)_4$ using a volume ratio of 3/5;

- Filtration of the precipitate formed after the previous operation;

- Preparing the dilutions;

- Performing the color reaction by treating a 2 ml sample with 5 ml of color reagent (alkaline reagent of potassium ferricyanide).

By reporting the monosaccharide concentration expressed as g/100ml to the amount of substrate and to the of dry matter content of the solid material, can be obtained the expression of the concentration in g/100g of solid.

Determination of ethylic alcohol is based on the quantitative reaction of oxidation of ethanol to acetic acid using an excess of oxidizing agent- $K_2Cr_2O_7$ in acid environment. The excess of potassium dichromate which is not included in the oxidation reaction is spectrophotometrically dosed at 590nm. The calculation of the concentration of ethanol in the sample is carried out using a calibration curve determined from absolute ethanol.

Determination of dry mass - was performed by specific treatment of the sample (4500rpm centrifugation for 20 min and washing with distilled water), quantitative prelevation of the biomass and drying at 105°C until reaching constant weight. Calculation of biomass concentration, expressed in dry substance grams takes into account the sample volume after reaching the constant mass. Expression is in g/100 ml medium.

Determination of total nitrogen, respectively of the protein, was performed by the Kjeldhall method [9].

The wet biomass was determined by centrifugation at 4500 rpm for 20 min., washing with distilled water, weighing the sample after the processing and reporting to 100 ml sample.

RESULTS AND DISCUSSIONS

The present study aimed to obtain feed protein by mixed cultivation of a strain of alcoholic fermentation yeast and a strain of biosynthesis protein that consume the released ethanol as the sole source of carbon and energy. An exact quantity of 1 kg of marc was washed with 2 liters of tap water brought to pH 2-3, at a temperature of 70 °C. For the first stage the diffusion lasted for 15 minutes, yielding the diffusion solution A₁ with 3.9 g sugars /100 ml. The diffusion was repeated by using the same parameters, but changing the diffusion time to 30 minutes. The solution obtained was denoted with A₂, containing 3.5g sugars /100 ml. The diffusion solution obtained by mixing A₁ and A₂ was evaluated from chemical point of view, running determinations of total sugars, total nitrogen, and respectively protein, P, K, Ca and Mg. Knowing the volume of the diffusion solution we could calculate the extraction efficiency, total sugars per 100g marc.

Both A₁ and A₂ washing water, combined, filtered and centrifuged, adjusted to pH 4.0, were distributed in 750 ml Erlenmeyer flask, 150 ml per bottle and sterilized at 0.9 atm., 20 minutes. These were further used as culture medium for alcoholic fermentation.

The flasks with 150 ml diffusion solution were inoculated with a 48 hours old yeast culture of *Saccharomiccs ellipsoideus* placed on the shaker at the minimum mixing speed, at a temperature of 28 °C.

The progress of the alcoholic fermentation was monitored by determination of the following parameters: total sugars, alcohol and OD at 0h, 16 h and 24 h of fermentation. The results obtained for these analyzes are summarized in Table 1.

Table 1. Evolution of the alcoholic fermentation in the marc washing solution (experiment I)

Sample	DO λ=600nm	Total sugars (g/100ml)	Alcohol (g/100ml)	pH
0 h	1.35	3.57	-	4.0
16 h	4.575	1.048	1.82	3.5
24 h	4.890	0.13	2.57	3.5

From the data presented in Table 1 it can be observed that the alcoholic fermentation was

quite fast, in 24 hours consuming virtually all the sugar in medium. The amount of 0.13 g sugars/100ml left in the medium can be represented by pentoses which are not fermented by yeasts.

After 24 h of alcoholic fermentation it was started the protein biosynthesis using a 48 h old strain of *Candida robusta*, at an inoculation ratio of 5%. The medium from each fermentation flask was supplemented with (NH₄)₂SO₄ 0.3 g/100 ml considering that other mineral elements such as , K, Ca, Mg, which are absolutely necessary for yeast growth, are already present in the composition of diffusion solution.

Knowing the needs of microorganisms in terms of growth factors, our objective was to study the way that the protein biosynthesis is stimulated by the yeast strain *Candida robusta* by supplementing the culture medium with growth factors originating from wine yeast autolysate.

The wine yeast autolysate was obtained by physico-chemical treatment of the mash (remaining after yeast distillation and used for alcohol recovery) for breaking the cell wall and release of cell contents. The material obtained was centrifuged, and in the supernatant was separated a protein component that was characterized from biochemical point of view, making exact determinations of the following parameters: total nitrogen = 5.4 g/100 d.s., total protein = 33.75 g/100 d.s. and dry substance = 13.70%.

This protein extract served as the source of organic nitrogen and growth factors in all our experiments on biosynthesis protein both for yeast and lactic acid bacteria.

In the vials containing fermentation medium were added varying amounts of protein extracts, thus obtaining the following variants taken into study:

M = control, with no added protein;

3= 5 ml protein extract /100 ml medium;

2= 10 ml protein extract /100 ml medium;

3= 20 ml protein extract /100 ml medium;

4= 30 ml protein extract /100 ml medium;

The Erlenmeyer flasks containing fermentation medium supplemented with ammonium sulfate and with various

concentrations of protein extract were placed on the "back and forth" shaker at a temperature of 30 °C.

The shaker agitation speed was increased to a maximum in order to ensure proper aeration. Protein biosynthesis duration was 40 h. The evolution of the culture was realized by determinations of the OD at exact time intervals as follows: at 0h, 16h, 20h, 24h, 40h. The alcohol concentration was determined at the beginning and at the end of the biosynthesis. In the final samples was determined the dry substance relative to 100ml medium, thus being able to calculate the efficiency of the bioprocess respectively d.s.g/100 g alcohol consumed.

The results obtained in this experiment are presented in Table 2a.

Table 2a. Evolution of protein biosynthesis by *Candida robusta* yeast on fermented diffusion solution - experiment I, OD evolution at $\lambda = 600\text{nm}$

Sample	0h	16h	20h	24h	40h
M	3.500	5.43	6.061	7.502	7.815
1	4.475	6.19	6.765	8.327	9.675
2	4.875	1.39	7.359	8.096	8.915
3	4.475	5.80	6.677	8.283	9.075
4	4.100	5.79	6.544	8.106	9.605

Table 2b. The evolution of the protein biosynthesis by *Candida robusta* yeast on fermented diffusion solution (experiment I)

Sample		M	1	2	3	4
0h	alcohol (g/100 ml)	2.57	2.57	2.57	2.57	2.57
	alcohol (g/100 ml)	0.16	0.13	0.11	0.13	0.13
40h	wet biomass (g/100 ml)	2.01	2.45	2.35	2.54	2.93
	s.u. (g/100 ml)	2.39	3.11	3.84	4.88	5.31
	efficiency (d.m. g / g consumed alcohol)	0.98	1.27	1.57	2.00	2.17
	protein (g/100 s.u)	41.3	41.1	43.8	41.5	43.6

Analyzing the data provided in the table it can be observed that after 16h of biosynthesis is produced a slow increase in the OD of the culture. This led to the conclusion that due to high concentrations of alcohol in the medium (2.57 g/100ml), the yeasts underwent inhibition, following a very long lag period. Afterwards the OD systematically increased until 40h when the OD value increased over 9

to all four samples containing protein, while the control had a OD value of only 7,815.

The amount of unconsumed alcohol remaining in all samples was very small, being between 0.11 and 0.13 g/100ml. The control had 0.16 g/100ml unused alcohol.

Comparing the values obtained from wet biomass (g/100 ml) we find that in all samples with added protein extract were recorded higher amounts than for the control (2.01 g/100ml). The highest value was achieved in the case of sample 4 (2.93 g/100 ml), representing an increase of 46.5% compared to control.

More significant results were recorded for the dry substance of the culture medium at the end of the biosynthesis. From the data presented in the table it can be observed a progressive increase from sample 1 to sample 4 which recorded a maximum of 5.31 g/100 ml. These progressive increases of the dry matter, together with the small amount (2.39 g/100 ml) obtained for the control, can only be interpreted as a positive response of stimulation of the protein biosynthesis - due to the differentiated addition of protein extract, respectively: 5, 10, 20 and 30 ml of protein extract. Valuable results were obtained in terms of efficiency of the biosynthesis (d.m.g. / g alcohol consumed) that can be observed from the progressively increasing values from 1.27 (sample 1) to 2.17 (sample 4) compared to the control, 0.98 d.m.g / g consumed alcohol, which is explained again by the stimulating effect of the addition of protein extract.

The following experiment was conducted, in terms of alcoholic fermentation, working on the same protocol as the previous one. The Erlenmeyer flask containing 150 ml of diffusion solution were inoculated with a culture of 48 hours old of *Saccharomyces ellipsoideus* yeast, inoculation ratio of 5%. The vials were placed on the shaker with minimum agitation at a temperature of 28 °C. The evaluation of the alcoholic fermentation progress was done by determination of total sugars, alcohol content and OD at 0h and 24h. The results obtained from these analyzes are summarized in Table 3.

Table 3. Evolution of the alcoholic fermentation in the marc washing solution (experiment II)

Sample	DO $\lambda=600\text{nm}$	Total Sugars (g/100 ml)	Alcohol (g/100ml)	pH
0h	1.28	3.57	-	4.0
24h	4.61	0.39	2.01	3.5

By comparing the data in Table 4 with the data contained in Table 2 on the evolution of alcoholic fermentation in the first experiment, can be observed that the values recorded for the OD, final sugars and alcohol are fairly close, the amount of unfermented sugars left being slightly larger (0.39 g/100 ml) compared to the previous experiment (0.13 g/100 ml).

Considering that in the previous experiment due to excessive alcohol concentration at the beginning of the protein biosynthesis the culture had a very long lag period, this time we proceeded to diluting the fermented solution.

The culture media for the protein biosynthesis was prepared by mixing 25ml of fermented solution and 75ml distilled water. Therefore, the initial alcoholic concentration in the medium was 0.5 g/100 ml.

The medium was supplemented with ammonium sulphate 0.3 g% and protein extract, this time in smaller quantities, achieving the following working variants:

M = control, with no added protein;

1= 2.5 ml protein extract /100ml medium;

2= 5 ml protein extract /100ml medium;

3= 7.5 ml protein extract /100ml medium;

4= 10 ml protein extract /100ml medium.

The fermentation media which were supplemented with ammonium sulfate and protein extract were inoculated with a strain of 48h old *Candida robusta*, at an inoculation ratio of 5%.

The protein biosynthesis occurred at 30 °C on the "back and forth" shaker with high agitation speed in order to ensure the proper aeration. The duration time of the biosynthesis was set to 48h. At 27h after the cultivation in all of the vials was added a portion of 50 ml of fermented and diluted medium (25ml medium + 25ml distilled water).

The evolution of the culture was monitored by measurements of the OD at 0h, 20h, 27h, 27h, after supplementation, 44h and 48h.

There were also observed the determinations of the alcohol concentration at the beginning and at the end of the biosynthesis process, also. In the final samples it was determined the wet biomass, the dry substance reported to 100 ml of medium, thus being able to calculate the efficiency of the bioprocess expressed as g D.M. / g consumed alcohol.

The values of these determinations are presented in Table 4.

Table 4a. Evolution of protein biosynthesis by *Candida robusta* yeast on fermented diffusion solution - experiment II, OD evolution at $\lambda = 600\text{nm}$

	0h	20h	27h	27h*	44h	48h
M	2.15	5.11	6.32	4.82	4.48	4.85
1	2.83	5.93	7.11	5.53	5.31	5.58
2	2.59	6.75	7.82	5.67	5.60	5.96
3	3.02	6.42	7.60	5.86	5.92	6.03
4	2.97	6.40	7.59	5.11	4.82	5.27

*27 h after supplementation with fermented medium

Table 4b. Evolution of protein biosynthesis by *Candida robusta* yeast on fermented diffusion solution - experiment II

Sample		M	1	2	3	4
0h	alcohol (g%ml)	0.5	0.5	0.5	0.5	0.5
	sugars (g%ml)	0.1	0.1	0.1	0.1	0.1
48h	alcohol (g%ml)	0.16	0.13	0.10	0.13	0.15
	sugars (g%ml)	0.018	0.013	0.015	0.019	0.013
	wet biomass (g/% ml)	1.62	1.72	1.93	1.70	1.71
	s.u. (g%ml)	0.82	0.95	1.28	1.25	1.23
	efficiency (g s.u. /g consumed alcohol)	0.98	1.13	1.42	1.43	1.45
	protein (g %s.u)	40.9	41.0	43.2	42.8	42.6

Analysing the data given in the table, it can be seen that at 20h biosynthesis the OD of the culture practically doubled. The increase of the OD at 27h is practically no longer significative. After adding the fermented medium the values of the OD decreased, remaining practically unchanged until the end biosynthesis at 48h.

All samples with added protein extract recorded higher values of OD than the control

sample. Lower values of OD achieved in this experiment can be explained by the fact that by diluting the fermented solution to 1:3 intended for the decreasing of the alcohol concentration, it strongly decreased the mineral ingredients existing in the fermented diffusion solution, probably insufficient to achieve a normal growth of the culture. Actually this is reflected in the values obtained from wet biomass and dry substance /100ml medium values well below those achieved in the previous experiment.

The efficiency of the process was also lower. The only value that as constant was the efficiency of the control 0.98 d.m.g./ g.alcohol consumed, this value being still lower than the ones obtained for the four samples containing added protein extract.

Therefore, it was found that diluting the fermented solution in order to achieve an optimal concentration of alcohol decreases also the quantity of mineral constituents of the diffusion solution, and thus the biosynthesis cannot take place in optimal conditions.

CONCLUSIONS

Reducing the alcohol concentration resulted in shortening the lag period, but the increase was not significant.

By supplementing the medium after 27h with a portion of the fermented solution there were no changes in OD.

The result of the experiment was lower values of OD, as well as those for the wet and dry biomass. This could be explained by the fact that by diluting fermented solution, by a 1:3 ratio, in order to decrease the alcohol concentration, decreased considerably the other minerals constituent of the fermented solution. This concentration of minerals was probably not enough to for a normal growth of the culture.

The efficiency of the process above 1.0 are good, but the process must be optimized to increase the amount of wet biomass that is actually the "harvest" of this biosynthesis process.

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