Available on-line at http://www.bjbabe.ro
Banat's Journal of Biotechnology
2013, IV(8),

EVALUATION OF SOME YEAST STRAINS TO OBTAIN FORAGE BIOMASS

DOI: 10.7904/2068–4738–IV(8)–48

Steliana RODINO[1,2], Marian BUTU[1,*], Constanta NEGOESCU[3]

[1]*National Institute of Research and Development for Biological Sciences, 060031, Splaiul Independentei 296, Bucharest, Romania, marian_butu@yahoo.com*
[2]*University of Agricultural Sciences and Veterinary Medicine, Bucharest, 011464, Marasti 59, Romania*
[3]*Banat's University of Agricultural Sciences and Veterinary Medicine from Timisoara, 300645, Calea Aradului 119, Romania*

Abstract.The present paper presents the screening of two yeast strains, for the obtainment of forage biomass from grape marc diffusion solution supplemented with wine yeast extract. The yeast strains used were *Saccharomyces ellipsoideus* originating from alcoholic fermentation, and a strain of *Rhodotorula* sp. Protein biosynthesis was monitored by determining the following parameters: alcohol concentration at the beginning and end of the process, the optical density of the culture medium, the residual sugar, and the wet and dry biomass. Better results were obtained for *Rhodotorula* sp. strain, and this can be explained by the presence of glucose which is a much better tolerated carbon source than the ethanol.

Keywords: grape marc, forage protein, wine yeast.

Introduction

The current trend in whole world is to find new utilization for industries secondary products, in order to turn industry waste into profit.

Such a byproduct, released in high quantities at termination of production flux, is the grape marc originating from wine industry [RODINO *et al.*, 2011]. Grape marc is currently used as fertilizer in agriculture [CARMONA *et al.*, 2012; FERRER *et al.*, 2001], or is simply disposed in environment as a waste [BUTU *et al.*, 2011], generating important costs for its management [DEVESA–REY *et al.*, 2011].

In this respect, the aim of present study was evaluation of using yeast strains in order to obtain feed biomass from grape marc diffusion solution supplemented with wine yeast extract.

Material and methods

The biological material used in experimental scheme was marc resulted from processed white wine grapes, wine yeast (yeast sediment from fermentation of white and red wine grapes) and two yeast strains: *Saccharomyces ellipsoideus* and *Rhodotorula sp*.

The marc was obtained from continuous press procedure and was represented only by the skin and kernels, because clusters were separated earlier in the production flux. The marc was collected immediately after pressing, in order to obtain fresh, unfermented material and originating from healthy grapes.

Both wine yeast and marc were distributed immediately in plastic bags and kept in a freezer at –12°C.

The yeast strain used, originating from alcoholic fermentation, was *Saccharomyces ellipsoideus*, isolated from grape marc.

To obtain the biomass we tested a strain of *Rhodotorula* sp. Experiments were performed in 750 mL Erlenmeyer flasks on the shaker with adjustable speed.

For the alcoholic fermentation minimum speed was used, because this kind of fermentation requires a high consumption of oxygen. The temperature was set to 28°C.

Evolution of the alcoholic fermentation was observed by determining sugars in the growing medium at the beginning and at end of fermentation by determining optical

density (OD) of culture and determination of alcohol in fermented solution.

Protein biosynthesis was monitored by determining following parameters: alcohol concentration at beginning and end of process, the OD of culture medium, residual sugar, and the wet and dry biomass. The protein determination was performed from wet biomass. At the end of bioprocess was determined dry substance in culture medium.

Spectrophotometric determination of sugars

The method of determination is based on direct reaction of simple reducing doses with potassium ferricyanide in alkaline medium. The reaction is quantitative (complies Lambert–Beer law), and excess of ferricyanide is determined at 420 nm against blank as the blank.

Reagents and equipment required:

- alkaline reagent, 2% potassium ferricyanide in 0.05N in anhydrous sodium carbonate 53%;
- 30% solution of zinc sulphate;
- 15% solution of potassium ferricyanide;
- 20% NaOH–solution;
- Spectrophotometer;
- The method involves the following steps;
- neutralization of the hydrolyzed solutions with NaOH until reaching a neutral pH;
- removal of components that may interfere with color reaction with potassium ferricyanide by treatment with a mixture of $ZnSO_4/K_4Fe(CN)_4$ in a volume ratio equal to 3/5;
- filtering precipitate formed after previous operation;
- obtaining diluted solutions; and
- actual 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 in g to 100 mL to substrate amount and to dry matter content of solid material, can be obtained concentration in g to 100 g.

Determination of ethylic alcohol is based on quantitative reaction of oxidation of ethanol to acetic acid using an excess of oxidizing agent, $K_2Cr_2O_7$, in the acid medium.

Excess potassium dichromate which is not included in oxidation reaction [ANNICCHIARICO *et al.*, 2011; ZIEBELL *et al.*, 2010] is determined spectrophotometrically at 590 nm. The calculation of concentration of ethanol in sample is carried out using a calibration curve determined for absolute ethanol.

Determination of dry biomass was performed by specific treatment of sample (centrifugation at 4500 rpm for 20 min., followed by washing with distilled water), quantitative prelevation of biomass and drying at 105°C to constant weight. Calculation of biomass concentration, expressed in dry substance grams takes into account volumes of sample after reaching constant mass. The value is expressed in g to 100 mL medium.

Determination of total nitrogen and *of protein* was realized by the Kjeldhall method. [JUNG *et al.*, 2003, RASMUSSEN *et al.*, 2011; BUTNARIU *et al.*, 2012; STEF *et al.*, 2013; BUTNARIU *et al.*, 2013].

The wet biomass was determined after centrifugation at 4500 rpm for 20 min., followed by washing with distilled water, weighing obtained sample and reporting to 100 mL of original sample.

Results

The protein biosynthesis was started with the strain of *Rhodotorula* sp yeast in two different experimental protocols, as follows:

A–on diffusion solution fermented with *Saccharomyces ellipsoideus*;

B–on diffusion solution was inoculated in same time *Saccharomyces ellipsoideus* for alcohol fermentation and *Rhodotorula* sp. for protein biosynthesis.

Experimental Protocol A.

In 750 mL Erlenmeyer flasks was deposited an exact quantity of 50 mL fermented solution and 50 mL distilled water. The culture medium was supplemented with 0.3g% ammonium

Available on-line at http://www.bjbabe.ro
Banat's Journal of Biotechnology
2013, IV(8),

sulphate and protein extract from wine yeast. Inoculation was made with a 48 h old culture of *Rhodotorula* sp. with an inoculation ratio of 5 %. There were thus obtained following working variants:

M–control sample, with no additional protein extract;

A_1–sample containing 5 mL % protein extract added;

A_2–sample containing 10 mL % protein extract added.

The Erlenmeyer flasks were put on the shaker at maximum agitation, at 30°C, biosynthesis duration 48 h. The evolution of bioprocess was monitored by measurement of OD, (Table 1) total sugars and alcohol, wet biomass, dry substance of fermentation medium, and protein from wet biomass.

Table 1.

The evolution of protein biosynthesis with *Rhodotorula* sp yeast strain in fermented diffusion solution (protocol A)–OD development at λ=600nm

Sample	0h	16h	20h	24h*	44h	48h
M	2.970	8.475	8.875	6.200	9.250	9.700
A_1	3.030	8.925	9.425	6.950	9.775	11.500
A_2	2.750	8.340	8.450	6.550	9.175	10.700

* supplemented

Was also determined biosynthesis yield, g dry matter reported to g alcohol consumed respectively. Results of these determinations are summarized in Table 2. Analyzing data presented in table can be noticed rapid evolution of growth of *Rhodotorula* sp. culture.

Table 2.

The evolution of protein biosynthesis with *Rhodotorula* sp yeast strain in fermented diffusion solution (protocol A)

Sample	0h		48h					
	Alcohol (g to 100 mL)	Sugars (g to 100 mL)	Alcohol (g to 100 mL)	Sugars (g to 100 mL)	Wet biomass (g to 100mL)	Dry matter (g to 100mL)	Yield (gd.m./g alcohol)	Protein (g to 100d.m.)
M	0.51	0.19	0.021	0.048	2.763	1.84	1.48	40.08
A1	0.51	0.19	0.010	0.085	2.916	2.01	2.01	41.11
A2	0.51	0.19	0.013	0.068	3.280	2.10	2.10	42.86

At 20 h of biosynthesis culture was already in stationary phase of growth. At 24 h OD was recording a significant decline (Figure 1). After solution was added a portion of fermented solution, which brought an additional, OD of culture, for all variants, revealed a significant increase.

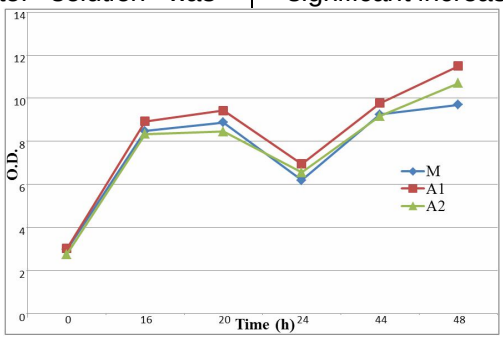

Figure 1. Time evolution of protein biosynthesis–Protocol A

At 48 h, DO values for approximately 11.0 for samples A_1 and A_2, samples containing protein, higher values than those of control (9.7). At end

of biosynthesis alcohol in medium had very low values, for all variants tested thus indicating the affinity of *Rhodotorula* sp. to nutrient substrate–alcohol.

Unconsumed sugars are in small quantities. These may include some sugars to which the yeast strain has no affinity. Wet biomass resulting by protein biosynthesis was in high quantity for both variants A_1 and A_2: (2.92 and 3.28, respectively) with added protein compared to control of 2.76 g %.

More conclusive results on how addition of yeast extract influenced protein biosynthesis are given by data on dry matter to 100 mL culture medium, where two versions A_1 and A_2 recorded higher values 2.01 and respectively 2.10 g to 100 mL compared to control 1.48 g to 100 mL [LI *et al.*, 2013; COMONT *et al.*, 2013].

Moreover, bioprocess yield was higher in samples supplemented by protein extract 2.01 and 2.10 g d.m. reported to g consumed alcohol, compared to 1.48 d d.m. / g. alcohol for control sample. From wet biomass determinations were made for protein content. A value of protein content of over 40 to 100 g d.m. for all three samples represents a satisfactory value.

Experimental protocol B.

In next phase of experiment the protocol B was followed, and namely protein biosynthesis with *Rhodotorula* sp. took place simultaneously with alcoholic fermentation of strain of *Saccharomyces ellipsoideus* in diffusion solution. In 750 mL Erlenmeyer flasks were distributed 100 mL unfermented diffusion solution.

The medium was supplemented by 0.3 % ammonium sulphate and protein extract and inoculated with 48 h old culture of *Saccharomyces ellipsoideus* and *Rhodotorula* sp. The variants obtained for further use in experiments were as follows:

M–control sample, with no additional protein extract;

B_1–sample containing 10 mL % protein extract added.

The Erlenmeyer flasks were placed on the shaker, at maximum agitation, at 30°C, for 48 h of biosynthesis.

The evolution of bioprocess was monitored by measurements of OD, total sugars and total alcohol, wet biomass, dry matter of fermentation medium, and protein from wet biomass.

Yield of biosynthesis was also calculated, respectively g d.m. reported to g alcohol consumed. Results of these measurements are summarized in Table 3 and 4.

Table 3.

The evolution of protein biosynthesis in fermented diffusion solution with simultaneously inoculation of *S. ellipsoideus* and *Rhodotorula* sp yeast strain (protocol B)–evolution of OD at λ =600nm

Sample	0h	16h	20h	24h*	44h	48h
M	2.330	9.150	10.025	7.000	10.050	10.900
B_1	3.130	9.975	10.250	7.125	10.550	12.400

Analyzing data in table can be seen even faster progress of culture in this growing medium than in case of previous experiment. The explanation could be that for initiation of protein biosynthesis, strain of *Rhodotorula* sp. consumed glucose existing in growth medium which was more accessible than ethanol.

Table 4.

The evolution of protein biosynthesis in diffusion solution with simultaneously inoculation of *S. ellipsoideus* and *Rhodotorula* sp (protocol B)

Sample	0h	48h					
	Sugars (g to 100mL)	Alcohol (g to 100mL)	Sugars (g to 100mL)	Wet biomass (g to 100 mL)	Dry matter (g to 100mL)	Yield (g d.m./g alcohol	Protein(g to 100 d.m.)
M	3.50	0.98	0.133	2.73	1.88	1.80	40.60
B_1	3.50	1.02	0.096	5.13	2.38	2.38	43.40

Available on-line at http://www.bjbabe.ro
Banat's Journal of Biotechnology
2013, IV(8),

As alcohol formed from fermentation process, culture already developed consumed simultaneously both substrates (glucose /alcohol), at 20 h of cultivation reaching OD values superior to values recorded in previous experiment.

At 24h the OD decreased, but after addition of a portion of fermented medium substantial growth of culture was observed. At 48 h of biosynthesis variant B_1 had a value of OD equal to 12.4, while control had only 10.9 (Figure 2).

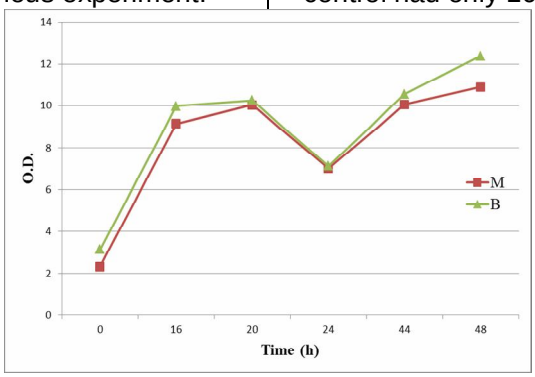

Figure 2. Time evolution of protein Biosynthesis–Protocol B

It can be seen that in both samples M and B_1 unconsumed alcohol was approximately 1 g %, given that total sugars were completely consumed either by *Rhodotorula* sp. strain or *Saccharomyces ellipsoideus*.

The two strains of yeast, competing in the same substrate, probably developed in parallel, depending on capacity of each one to use substrate.

The wet biomass values obtained from two samples were highly differentiated, 5.13 g to 100 mL for sample B_1 and only 2.73 g to 100 mL for the control, M. Differences can be observed also for values of dry substance from biosynthesis medium, 2.38 g to 100 mL for B_1, while value for M only reached 1.88 g to 100 mL [SKLADANKA *et al.*, 2013; WALTER *et al.*, 2012; FU *et al.*, 2012].

Bioprocess yield calculated as g d.m. to g consumed alcohol was 2.4 for B_1 compared to 1.8 for control sample.

The protein content of 43.4 g to 100 d.m of biomass for B_1 sample is also a satisfactory value.

We consider that protein biosynthesis media achieved by using two secondary products of winemaking industry–grape marc and wine yeast–are particularly advantageous media from economical point of view and are also well tolerated and exploited by yeast strains tested.

The results obtained both from point of view of wet biomass as well as biosynthesis efficiency can be improved by optimizing agitation, aeration, and concentration of ethanol, and this could be achieved by cultivation in a bioreactor.

Conclusions

On biosynthesis medium obtained from dilution by a 1:1 ratio fermented grape marc, supplemented with ammonium sulphate and protein extract, the strain of *Rhodotorula* sp. had a rapid growth and after supplementation with a portion of fermented medium OD significantly increased. The yeast extract favorably influenced both OD values, and values obtained for wet biomass and dry substance in the growth medium.

Alcoholic fermentation run concurrently with protein biosynthesis by simultaneous inoculation of yeast *S. ellipsoideus* and *Rhodotorula* sp. in diffusion solution of grape mark supplemented with ammonium sulphate and protein extract lead to obtaining better results for OD value, as well as for wet biomass and dry matter.

Better results recorded for *Rhodotorula* sp. strain in this protocol can be explained by presence of glucose which is a much better tolerated carbon

source than ethanol.
The protein content of 40 g % to 100 g d.m. can be considered satisfactory.

References

1. Annicchiarico, G., Caternolo, G., Rossi, E., Martiniello, P. Effect of manure vs. fertilizer inputs on productivity of forage crop models. *Int J Environ Res Public Health*. **2011**, *8(6)*: 1893–913.
2. Butnariu, M.; Grozea, I.; Antioxidant (Antiradical) Compounds; J Bioequiv Availab.; **2012**, 4(6):xvii-xix. doi:10.4172/jbb.10000e18.
3. Butnariu, M.;Raba, D.; Grozea,I.; Virteiu, A.-M.; Stef, R.; The impact of physical processes and chemical of the antioxidants (Bioactivity compounds); J. Bioequiv.Availab.; **2013**, 5:6; doi:10.4172/jbb.10000e18.
4. Buțu, A.; Butu, M.; Rodino, S.; Studies on the possibility to recover the by–products resulted from the vinification process; *Banat's Journal of Biotechnology*, **2011,** *2*(3):56–63.
5. Carmona, E.; Moreno, M.T.; Avilés, M.; Ordovás, J.; Use of grape marc compost as substrate for vegetable seedlings; *Scientia Horticulturae,* **2012**, *137*:69–74.
6. Comont, D., Winters, A., Gomez, L.D., McQueen-Mason, S.J., Gwynn-Jones, D. Latitudinal variation in ambient UV-B radiation is an important determinant of Lolium perenne forage production, quality, and digestibility. *J Exp Bot*. **2013**, *64(8)*: 2193–204.
7. Devesa–Rey, R.; Vecino, X.; Varela–Alende, J.L.; Barral, M.T.; Cruz, J.M.; Moldes, A.B.; Valorization of winery waste vs. the costs of not recycling; *Waste Management*; **2011**, *31*(11):2327–2335.
8. Ferrer, J.; Páez, G.; Mármol, Z.; Ramones, E.; Chandler, C.; Marín, M.; Ferrer, A.; Agronomic use of biotechnologically processed grape wastes; *Bioresource Technology*; **2001**, *76*(1):39–44.
9. Fu, C., Sunkar, R., Zhou, C., Shen, H., Zhang, J.Y., Matts, J., Wolf, J., Mann, D.G., Stewart, C.N. Jr, Tang, Y, Wang, Z,Y. Overexpression of miR156 in switchgrass (*Panicum virgatum* L.) results in various morphological alterations and leads to improved biomass production. *Plant Biotechnol J*. **2012**, *10(4)*: 443–52.
10. Jung, S.; Rickert, D. A.; Deak, N. A.; Aldin, E. D; Recknor, J.; Johnson, L. A; Murphy, P. A.; Comparison of Kjeldahl and Dumas methods for determining protein contents of soybean products; *Journal of the American Oil Chemists' Society*, **2003,** *80*(12):1169–1173.
11. Li, Z.P., Liu, H.L., Li, G.Y., Bao, K., Wang, K.Y., Xu, C., Yang, Y.F., Yang, F.H., Wright, A.D. Molecular diversity of rumen bacterial communities from tannin-rich and fiber-rich forage fed domestic *Sika deer* (*Cervus nippon*) in China. *BMC Microbiol*. **2013**, *13*: 151.
12. Rasmussen, S., Parsons, A.J., Jones, C.S. Metabolomics of forage plants: a review.*Ann Bot*. **2012**, *110(6)*: 1281–90.
13. Rodino, S.; Butu, M.; Golea, D.; Butu, A.; Qualitative evaluation of fresh marc–raw material with valuable composition; *Banat's Journal of Biotechnology*, **2011**, *2*(4):18–27.
14. Skladanka, J., Adam, V., Dolezal, P., Nedelnik, J., Kizek, R., Linduskova, H., Mejia, J.E., Nawrath, A. How do grass species, season and ensiling influence mycotoxin content in forage? *Int J Environ Res Public Health*. **2013**, 10(11): 6084–95.
15. Stef, R.; Bostan, B.; Butu, A.; Ortan, A.; Rodino S.; Butu, M.; Comparative characteristics of Lupius perennis L. Under allelochemical sorgoleone stress; Romanian Biotechnological Letters; **2013**; 18(3):8327–8332
16. Walter, A., Studer, B., Kölliker, R. Advanced phenotyping offers opportunities for improved breeding of forage and turf species. *Ann Bot*. **2012**, *110(6)*: 1271–9.
17. Ziebell, A., Gracom, K., Katahira, R., Chen, F., Pu, Y., Ragauskas, A., Dixon, R.A., Davis, M. Increase in 4-coumaryl alcohol units during lignification in alfalfa (Medicago sativa) alters the extractability and molecular weight of lignin. *J Biol Chem*. **2010**, *285(50)*: 38961–8.

Received: August 20, 2013
Accepted: October 13, 2013