# STUDIES ON OBTAINING ACTIVE DRY WINE YEAST USING DIFFERENT NITROGEN SOURCES

**Mihai FRÎNCU[1], Iuliana Diana BĂRBULESCU[1], Mihaela BEGEA[2,3], Răzvan Ionuț TEODORESCU[4], Corina DUMITRACHE[4], Valerica TUDOR[4], Cornel-Daniel BANIȚĂ[4], Ioana Simona MĂRCULESCU[1], Alexandru Ionuț CÎRÎC[2,3], Petre Andrei CĂTĂLIN[1], Carmen-Rodica VRÎNCEANU[4], Tóth TAMÁS[5], György BÁZÁR[5]**

e-mail: barbulescudia@yahoo.com, mihaela.begea@gmail.com

**Abstract**

Most of research on wine microbiology has concentrated on *Saccharomyces* yeasts for development of starter cultures, especially on *Sacch. cerevisiae*. As the importance of the role of *S. cerevisiae* in winemaking has been established, the number of wine yeast strains available in the world market for use as winemaking starters grew in the last years. The upstream process of producing *Sacch. cerevisiae* biomass on a culture medium based on sugar was performed by testing different sources of inorganic and organic nitrogen (yeast extract and monoammonium phosphate) in submerged fermentations using a Biostat B plus bioreactor (4L working volume). The upstream parameters have been monitored on-line (oxygen flow; pH around 4.35; temperature $30^0$C; stirring rate 250 rpm) and off-line (total soluble dry matter; pH). The biomass obtained after the downstream process has been dried through freeze-drying. Through the combination of two carbon sources as yeast extract (0.7%) and monoammonium phosphate 10.71 g/L dry cell weight (DCW) has been obtained, compared with 9.6 g/L DCW in the case of the fermentation without monoammonium phosphate. From the economic reasons, the monoammonium phosphate as an inorganic nitrogen form has been excluded from the experiments. Finally, the higher content of dry yeast biomass (14.43 g/L DCW) was obtained when 11% yeast extract as the only nitrogen source has been added at the fermentation media.

**Key words**: active dry biomass, yeast extract, wine

It is well known that both *Saccharomyces* and *non-Saccharomyce*s yeasts are used to produce wines. The interest in the development of commercial yeasts for aroma has increased a lot, recently. Therefore, many studies in recent years tackled the exploitation of autochthones yeasts for their terroir characteristics. *S. cerevisiae* has a strong performance of alcoholic fermentation in wine process due to its high tolerance to alcohol (Morata, A.; Escott, et al, 2020; Antoce A. O., 2012). Also, several strains of non-*Saccharomyces* yeasts are used in winemaking in co-inoculation with *S. cerevisiae* in order to control and improve the quality and characteristics of wines (Liu, X.Z.et al, 2021 and Yinfeng Li et al, 2022).

Following our previous study that described the production of dry yeast biomass at the laboratory level (Frincu. M. et al, 2022), this paper presents the scale up of the micropilot process to produce active dry wine yeast biomass dedicated to obtain Fetească regală wines using a *Saccharomyces cerevisiae* strain (t14) isolated from the same variety of grapes. Several sources of sugar and nitrogen can be used to obtain yeast biomass. The present study describes a fed-batch fermentation, which used white sugar as carbon source that was dosed throughout fermentation to maximize the volumetric productivity of the bioreactor (Konrad V. Miller et al, 2022), as well as different nitrogen sources.

## MATERIAL AND METHOD

- **Microorganism used for fermentations**

The *Saccharomyces cerevisiae* strain (t14) isolated from Fetească regală grape from Research and Development Station for Viticulture and Wine-making Pietroasa was used for cultivation on synthetic medium.

- **Preinoculum cultures**

[1] Pharmacorp Innovation SRL, Bucharest, Romania
[2] University Politehnica of Bucharest, Romania
[3] ICA Research & Development SRL, Bucharest, Romania
[4] University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania
[5] ADEXGO Ltd., Balatonfüred, Hungary

The preinoculum culture has been obtained using the methodology described for the previous laboratory study (Frincu M. et al, 2022).

- **Culture medium for the preparation of the liquid inoculum**

The ingredients used for the inoculum culture media were sucrose, yeast extract and peptone hy-soy. The ingredients were dissolved and distributed in Erlenmeyer glasses (150 ml/glass), sterilized at 115°C for 20 min and brought to room temperature.

- **Inoculum cultivation conditions**

The submerged cultivation was performed in 500ml Erlenmeyer flasks with 150ml culture medium incubated at 28-30°C on a rotary shaker (200-240 rpm) (Optic Ivymen System, Auxilab, Spain) for 12-18 hours (overnight).

- **Fermentation at micropilot level**

For fermentations, a Biostat B+ from Sartorius and an Applikon bio fermenters were used (Figure 1). The nutrients used for the fermentation process were yeast extract, sugar as carbon source, with or without addition of $NH_4H_2PO_4$, $MgSO_4 \times 7H_2O$, and KCl. The nutrients were dissolved in distilled water and the liquid medium was sterilized at 121°C for 20 min.

- **Cultivation conditions in bioreactors**

A submerged cultivation (discontinuous system organized in batches) in a micro fermenter with a total capacity of 6L and a working volume of 4L was carried on. The culture medium was inoculated with approx. 15% inoculum. The initial pH value was adjusted with alkaline solution, and during the fermentation, the pH was adjusted with alkaline solution as well.

- **Cultivation on-line parameters during fermentations**

The on-line settings of the parameters during the fermentations were the following: stirring rate: 200 - 300 rpm; air flow: 1.5 - 2.5 L/min; initial pH approx. 6.5; temperature: 30°C. In order to avoid the production of foam, silicone oil was used.

- **Cultivation off-line parameters during fermentations**

Along the on-line parameters, some off-line parameters were monitored: total soluble solids (TSS), °Brix; Wet Cellular Weight (WCW), g/L; Dry Cellular Weight (DCW), g/L;

- **Post fermentation process**

Once fermentation ended, the fermented medium was subjected to separation through centrifugation. The fermented medium was centrifuged at 4000-4500 rpm for 5 min and washed 2 times with sterile distilled water.

- **Determination of the DCW and the WCW**

For the determination of the WCW the purified wet biomass was weighed and the resulting purified wet biomass was freeze-dried. For the determination of the DCW, the resulting dried biomass was subjected to protein, moisture and cell viability determination.

- **Yeast viability**

The viability of the yeast biomass was determined through the plate colony-counting method, using YMSP agar (yeast extract, malt extract, sugar, peptone hy-soy-agar) as culture medium (Barbulescu I.D. et al, 2021).

- **Determination of the Moisture and the Crude Protein Content for the Yeast Biomass**

The moisture content was determined by drying at 103°C for 4h.

The crude protein was determined based on the nitrogen content performed by the Kjeldahl method, and then multiplied with a factor of 6.25.

## RESULTS AND DISCUSSIONS

The fed-batch micro-pilot experiments performed in the bioreactor with working volume of 4L have been carried on in a synthetic medium with different nitrogen sources, using a liquid inoculum cultivated on yeast extract – sucrose - peptone (YSP medium) in stirred flasks (*figure 1*). The inoculum medium inoculated with *S. cerevisiae* yeast was fermented for 14 hours and the fermentation results are presented in *figure 2.*


Figure 1 **Biostat B+ and Applikon bio - biotechnological process for obtaining wine yeast biomass**

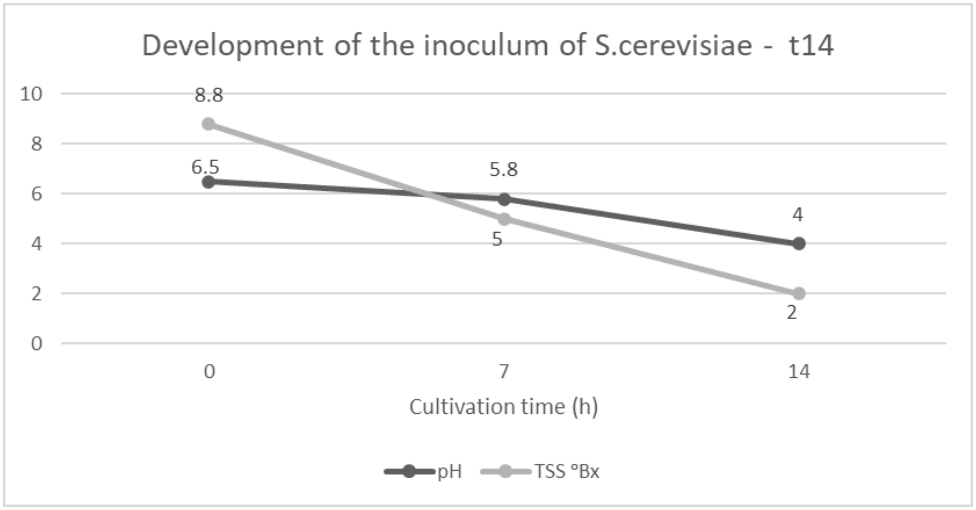


Figure 2 **Development of the inoculum of *Sacch. cerevisiae* – t14**

*Figure 3* presents the overall dynamics of yeast biomass during the fermentation process using a culture medium based on 0.7% yeast extract, sugar as carbon source, addition of salts as $MgSO_4 \times 7H_2O$ and KCl. During fermentation, 40% sterile sugar solution was added gradually, and it can be seen that 9.6 g/L dry active biomass was obtained (figure 7). It is observed that the yeast extract in a percentage of 0.7% led to a consumption of ca. 6°Brix.

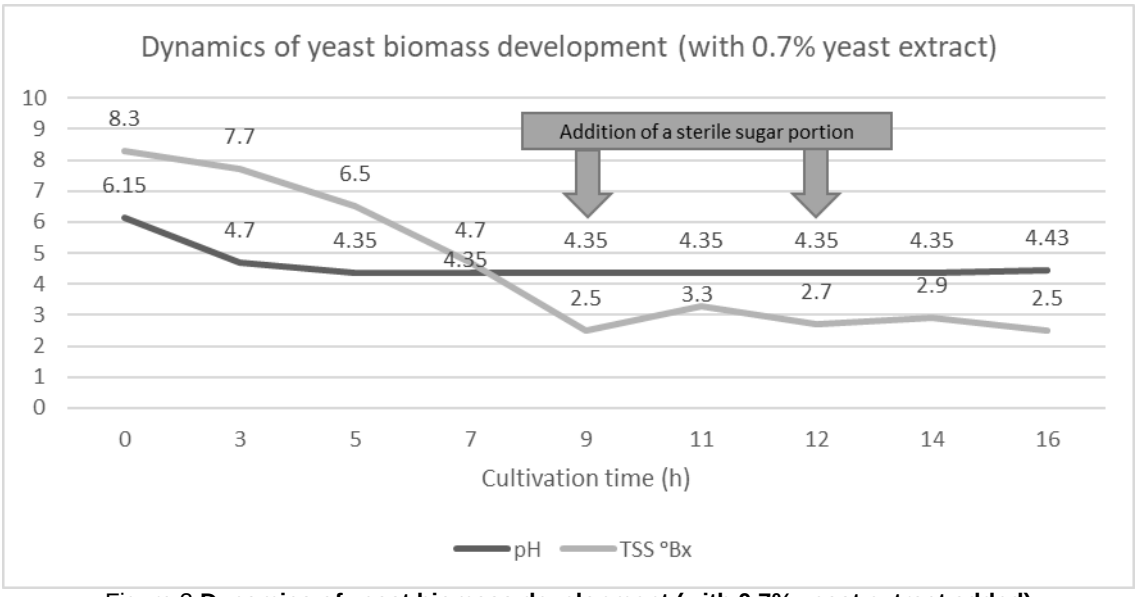


Figure 3 **Dynamics of yeast biomass development (with 0.7% yeast extract added)**

*Figure 4* shows the improvement of the fermentation medium by adding two nitrogen sources: yeast extract 0.7% and monoammonium phosphate 0.05-0.1%. It was observed that although the sugar consumption was approximately the same as in the previous experiment, a higher dry active biomass content of 10.71 g/L was obtained.

*Figure 5* presents the overall dynamics of yeast biomass based on the improvement of the fermentation medium by increasing the content of yeast extract to 1.1% and eliminating the addition of monoammonium phosphate. Although the consumption of sugar was approximately the same as that of the previous experiment, a higher content of dry active biomass of 14.43 g/L was obtained. After 9h of cultivation, a concentration around $2^0$Brix is observed. In addition, it was observed that the higher initial concentration of sugar shortens the lag period to 2 hours from 4-5 hours for the other 2 experiments, and that the dry biomass yield is higher.

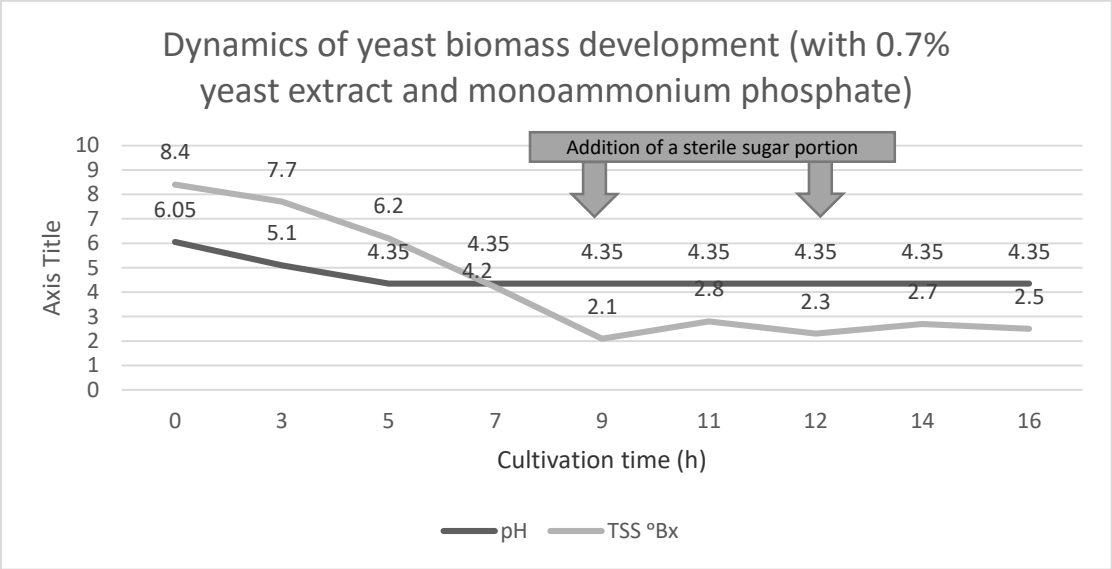


Figure 4 **Dynamics of yeast biomass development (with 0.7% yeast extract and monoammonium phosphate)**

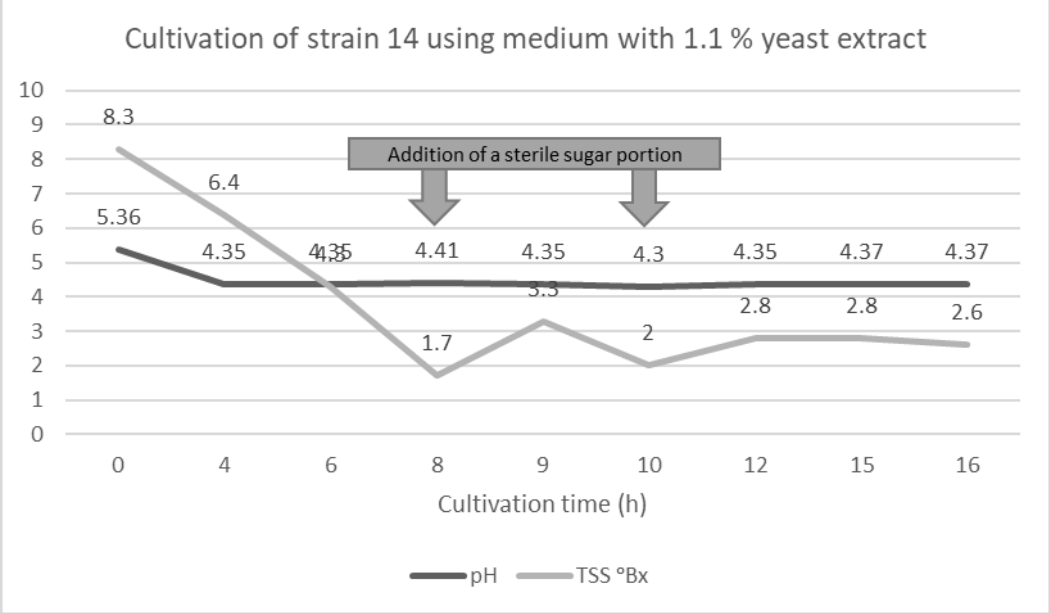


Figure 5 **Cultivation of strain 14 using medium with 1.1% yeast extract added**

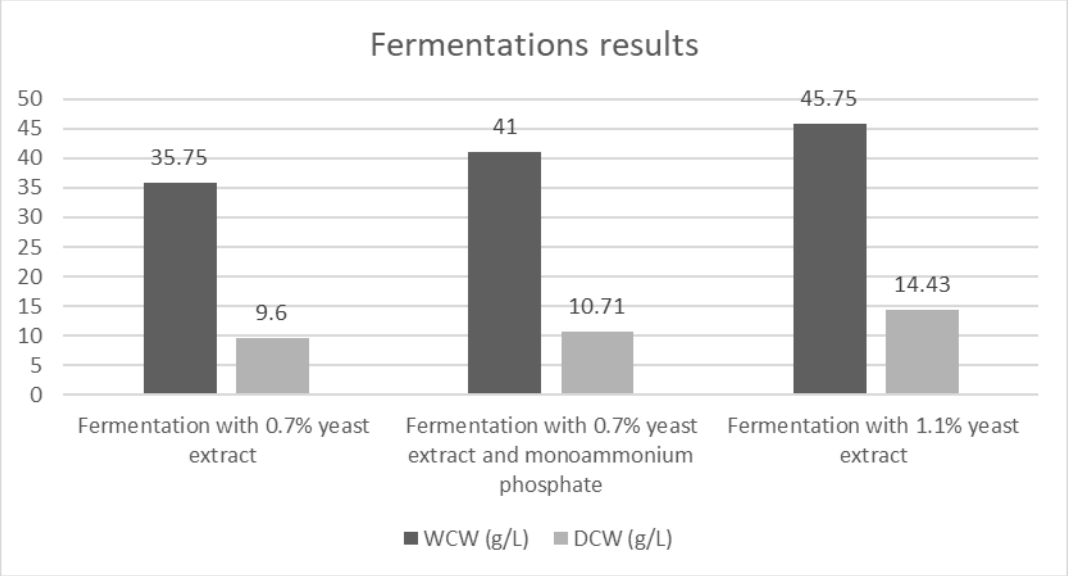


Figure 6 **Fermentation results**

The accumulation of a larger amount of yeast biomass is correlated with a higher rate of substrate consumption. From the data presented in Table 1 and Figure 6, the existence of a correlation between cell viability on the one hand and the growth of the microorganism, sugar consumption and the accumulation of yeast biomass on the other hand can be observed.

The determined values of the dry matter content in the dry biomass were between 93.56% and 94.22%. Based on the analytical determinations, the best technological solution is considered to be when 1.1% yeast extract was used as nitrogen source. The content in dry yeast biomass for the 4L bioreactor was 57.74g, and 14.43 g/L respectively. When 0.7 % yeast extract was used, the content of dry yeast biomass was 9.6 g/L.

Table 1

**The protein content and the viability for the dried yeast biomass**

| Samples | Dry matter (%) | Protein (%) | Protein (% d.m.) | Viability (cfu/ml) |
|---|---|---|---|---|
| Medium with 0.7% yeast extract | 93.56 | 39.01 | 41.70 | $0.78 \times 10^6$ |
| Medium with 1.1% yeast extract | 94.14 | 36.69 | 38.97 | $0.5 \times 10^7$ |
| Medium with 0.7% yeast extract and monoammonium phosphate | 94.22 | 43.73 | 46.41 | $0.5 \times 10^4$ |

It was observed that higher protein content was obtained when two sources of nitrogen were added to the fermentation medium. Because the obtained biomass is not intended to be used in animal feed, but in winemaking, the version with dry biomass obtained using 1.1% yeast extract was selected because it has the highest viability. Therefore, in order to shorten the lag period and increase the yield of the substrate, it is preferable to use a concentration of 1.1% yeast extract to obtain a higher content of biomass, as well as higher biomass viability.

In the Traviña-Muñoz study, the *S. cerevisiae* showed a negative effect at high aeration rates such as 1.5 vvm (volume of air under standard conditions per volume of liquid per minute). The best parameters obtained by Traviña-Muñoz for biomass production were 1 vvm and 200 rpm, obtaining 4.92 g biomass/L.

The total number of viable cells for the active dry yeast is very important for the winemaking process and it is an important criterion of quality for the fermentation activity.

In addition, a rehydration time of 10-15 minutes is satisfactory. In the case the rehydration is not performed correctly, some cellular constituents are leached from the yeast cells into the medium of grape must, leading to reducing the fermentative power of yeasts (J.K. Kraus et all, 1983).

## CONCLUSIONS

When a combination of two carbon sources as sugar and yeast extract (0.7%) and mono-ammonium phosphate as nitrogen source was used, 10.71 g/L dry cell weight (DCW) has been obtained, compared with 9.6 g/L DCW in the case of the fermentation without mono-ammonium phosphate;

When the inorganic nitrogen source was excluded and the organic nitrogen sources was increased from 0.7 to 1.1%, the quantity of dry yeast biomass increased to 14.43 g/L DCW;

Based on the results of this micropilot study, the fermentation technological flow to obtain wine dry yeast biomass to be used for the vinification process of Feteasc ă regală variety was proposed.

## ACKNOWLEGMENTS

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