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EVOLUTION AT THE MICROFERMENTER LEVEL OF THE GROWTH DYNAMICS OF *Saccharomyces cerevisiae* AND *Starmella bacillaris* YEASTS WITH POTENTIAL FOR USE IN WINEMAKING AT THE PIETROASA WINERY

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Abstract

The grape surface hosts a complex community of yeast Saccharomyces and non-Saccharomyces species responsible for spontaneous alcoholic fermentation in wine industry. The yeast strains used for this study were isolated from 'Tămâioasă Românească' and 'Busuioacă de Bohotin' grape varieties from Pietroasa vineyard, and the isolates were identified through a molecular method. Identification of yeast strains through the BLASTn analysis of the 5.8S-ITS region revealed that PFE5 strain showed the best sequence match to Saccharomyces cerevisiae (98% similarity) and PFE15 strain to Starmerella bacillaris (99.78% similarity), respectively. In this first micro-pilot study, the differences between Sacharomyces and non-Saccharomyces yeasts in batch (for Starmella bacillaris) and fed-batch fermentation system (for S. cerevisae) and how these regimes influence the culture growth were assessed. The applied fed-batch process was capable for producing two times more S. cerevisae yeast biomass than Starmella bacillaris through a batch process. In addition, the yield of S. cerevisiae converting the substrate into biomass was 42.3%, almost double compared to the yield of Starmella bacillaris. Moreover, the cell wet weight (WCW) for S. cerevisae was 32.5 g/L and for Starmella bacillaris 15.35 g/L, respectively. Both yeast biomass will be used at Pietroasa winery for inoculation separately or mixed as co-culture for 'Tămâioasă Românească' and 'Busuioacă de Bohotin' grape juice.

Key words*: fed-batch system, batch system, fermentation, Starmella bacillaris, Saccharomyces cerevisiae.*

INTRODUCTION

The results of the microorganism growing are the results of the interaction between the cells and culture medium. Pressure, temperature, concentrations of nutrients and of various resulting metabolism products influence the multiplication of yeast cells. Measuring the evolution of yeast cultures can be performed in two ways (Luedeking, 1967): by evaluating cells growth, which represents the mass growth of the microorganisms' culture; and by evaluating the multiplication of microorganisms, which represents the increase in the number of yeast cells (at the population level) (Anghel et al., 1993).

It is known that during the exponential growth phase, yeasts divide at a constant rate. When nutrients are depleted and the by-products accumulate, the growth process slows, and the yeast culture enters the stationary growth phase. At this point, the cultivation process can be usually stopped. If the cultivation continues, it will eventually enter the death phase, which is characterized by a decrease in the density of viable cells (Yang & Sha, 2022)

The industrial yeast fermentation process is a typical nonlinear dynamic process that requires control and monitoring to optimize yeast production (Chuo, 2020). Growth control rate for fed-batch fermentation, which is a semi-continuous process, is challenging because the

process evolves non-linearly and is subject to unpredictable perturbations originating from culture metabolism (Brignoli, 2020). In batch cultivation, which is a discontinuous process, all medium nutrients are placed in the bioreactor at the beginning of the culture, except acid or base for pH control and antifoam agents. Fed-batch cultivation is a more productive modification of batch fermentation that is carried out in a semi-open system, and the volume of liquid culture in the bioreactor increases as the culture is systematically added. *S. cerevisiae* can grow both in aerobic and anaerobic and possesses the ability to use different sugars which influence its growth evolution. Both strains (*S. cerevisae* and *Starmella bacillaris*) required nitrogen and carbon sources to grow (Salari & Salari, 2017). Due to its capacity to produce high glycerol and moderate volatile acidity, *Starmerella bacillaris* is a non-*Saccharomyces* yeast that can contribute to improve wine quality when it is was used for grape must fermentation in association with *S. cerevisiae* (Nadai et al., 2021). This paper presents the evolution of the growth curve for *S. cerevisiae* cultivated in a fed-batch system and for *Starmella bacillaris* cultivated in a batch system.

MATERIALS AND METHODS

Grapes from the varieties 'Tămăioasă Românească' and 'Busuioacă de Bohotin' harvested from the Pietroasa Viticulture and Winemaking Research and Development Station, were used for yeast isolation.

Isolation of yeasts from grapes

The grapes were pressed and the skins were left in direct contact with the must in sterile 50 ml tubes of at room temperature for 24 h. Decimal dilutions were made and the culture were grown on Dichloran Rose Bengal Base media for 24-48 h. Pink yeast colonies were isolated on YPG (yeast extract, peptone, glucose, agar) and MP (malt extract, peptone, agar) media.

Identification of yeast isolates by molecular methods

PCR-ITS and sequencing analysis

The extracted DNA of the yeast strains was amplified with ITS1/ITS4 primers. The PCR products were sequenced by Cemia (Greece) in both directions using ITS1/ITS4 primers. The sequenced sequences obtained were aligned and compared with the sequences available in the NCBI database, applying the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Obtaining the maintenance and the preinoculum culture

Maintenance and preinoculum cultures were obtained according to the cultivation method described by Barbulescu et al. (2022).

Inoculum culture and cultivation conditions

Different types of inoculum media were prepared for various purposes.

Nutrients used for liquid inoculum

- YSP is based on yeast extract (Sigma Aldrich), sugar, peptone hy-soy (purchased from WWR Life Science)
- YGP is based on yeast extract (Sigma Aldrich), glucose, peptone hy-soy (WWR Life Science)

The nutrients for YPG and YGP liquid inoculum media were weighed and dissolved in distilled water by shaking. The concentration of glucose and sugar was 8%.

The liquid inoculum media were sterilized through autoclaving for 15 minutes at 115°C.

Both liquids inoculum media were placed in Erlenmeyer flasks of 500 ml with 150 ml of medium and each flask was inoculated with pure preinoculum cultures. Four Erlenmeyer flasks were placed into orbital incubator and were let overnight at 30°C with stirring rate by 240 rpm.

Fermentation bioprocess was performed in bioreactors with total volume 6 L and working volume by 4 L (Figure 1).

The culture media used for fermentation

- sugar, yeast extract (purchased from Sigma Aldrich), KCl (Lach-Ner), $MgSO_4 \times 7H_2O$ (Lach-Ner) for *S. cerevisiae* cultivation.
- Glucose (WWR Life Science), yeast extract (purchased from Sigma Aldrich), KCl (Lach-Ner), $MgSO_4 \times 7H_2O$ (Lach-Ner), for *Starmella bacilaris* cultivation.

The entire 600 mL volume of the liquid inoculum was transferred from one Erlenmeyer flask to a pre-sterilized bottle, and the *S. cerevisiae and Starmella bacillaris* suspensions were pumped into the bioreactor to

reach an inoculation ratio of 10-15% (v/v) for each run.
The bioreactors are specifically designed for microbial applications for obtaining yeasts biomass.
The empty bioreactors were autoclaved at 121°C for 20 min.
Culture fermentation media for each culture were separated sterilized at 1L bottle before to be introduced within the bioreactor.
Antifoam silicone SNAPSIL FD 10 solution (WWR Life Science) was added to the ongoing fermentation medium, if necessary, as excessive antifoam may affect yeasts growth. In both runs presented in this study, the 0.03% (v/v) antifoam silicone was added at the beginning of medium preparation and this was sufficient for the rest of the fermentation process to appropriately control the foaming.
The pH of the fermentation medium from bioreactor was controlled by 5% ammonia solution.
On line parameters for fermentation bioprocess were the following:

- Temperature: 29-30°C;
- Stirring rate: 200-350 rpm;
- Air flow: 1.5-2.5 L/min;
- pH value was maintained by addition of ammonia solution through fermentation, to maintain a pH between 4-4.5.

Total soluble solids (TSS) were used as of-line parameter in fermentation bioprocess.

Figure 1. Biotechnological process

Determination of yeast cell growth
For the analysis of the growth curve of the microorganisms identified and used in the fermentation, samples were taken every 4 hours until the exponential growth phase and then every 2 hours.
Post fermentation process
The fermented medium was centrifuged at 4000-4500 rpm for 5 min and washed 2-4 times with sterile distilled water (Barbulescu et al., 2022).
Yeast viability was determination was performed according to the method described by Barbulescu et al., 2021).

Establishing the growth curve by determining the turbidity
To establish the growth curve through turbidity determination, samples were taken from fermentations, dilutions of 1:50 were made and read on a spectrophotometer at a wavelength of 570 nm, expressing their turbidity. The higher the turbidity, the higher the concentration of yeast in the solution (Groposila et al., 2020).
Determination of the Wet Cell Weight (WCW) - the fermented medium was centrifuged at 4000-4500 rpm for 5 min, washed with sterile distilled water and weighed.

The yield of transformation of the substrate into biomass

Following Monod's (1942) study, it was observed that the dry weight of cells produced was proportional to the amount of carbon source consumed, as long as this was the limiting factor (all other components were in excess).

The proportionality factor, called substrate yield consumed to obtain one gram (mole) of biomass can be calculated with the following formula:

$$Y\frac{X}{S} = \frac{\Delta X}{-\Delta S}\ [g/g]$$

were:

$\Delta X = Biomass\ concentration(\frac{g}{l})$;

$\Delta S = Substrate\ concentration\ \left(\frac{g}{l}\right)$,

Y = yield of transformation of the substrate into biomass.

The substrate yield can vary depending on the specific growth rate of the microorganism.

For the exponential growth phase, a deterministic kinetic model was used based on an exponential growth of the biomass and a constant consumption yield of the carbon source.

RESULTS AND DISCUSSIONS

Macroscopic aspect of the yeast strains was evaluated based on the decimal dilution (Figure 2).

Molecular identification of yeast strains through the BLASTn analysis of the 5.8S-ITS region revealed that PFE5 strain showed the best sequence match to *Saccharomyces cerevisiae* (98% similarity) and PFE15 strain to *Starmerella bacillaris* (99.78% similarity), respectively.

For all yeasts cells, the growing can be characterized by a few steps: increase in cell size, cell division and separation from the mother cell (Slater, 1984). Through these phases, two aspects can be assessed: growth (in biomass) and multiplication (increase in the number of yeast cells).

The system used in the experiments to assess the dynamics of *Starmella bacillaris* cell proliferation was a discontinued one (batch system at bioreactors).

Graphical representation of cell concentrations (expressed as cell count, optical density, or dry matter) over time (Monod, 1949) depicts the phases of the yeast growth cycle (Figures 3-6).

The boundaries of the phases of the growth cycle are usually considered at the points where the slopes of the growth curve change.

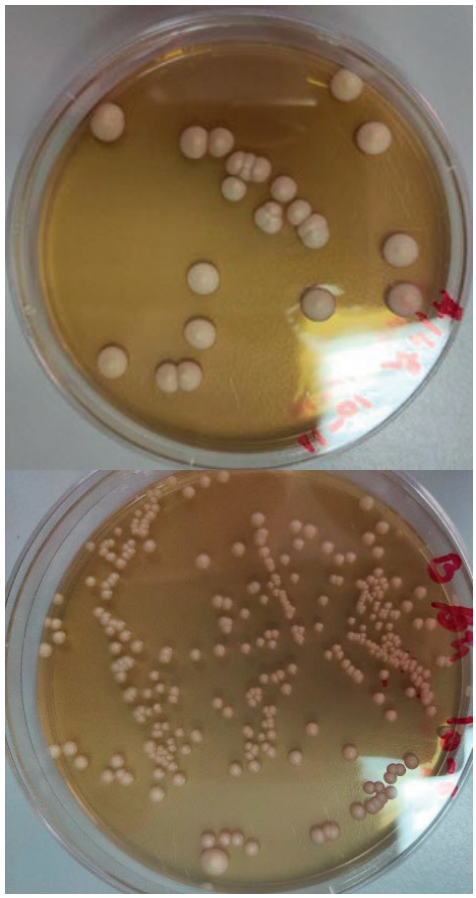

Figure 2. Colonies of *S. cerevisiae* and *Starmella bacillaris*

During the fed-batch process, the feeding with substrate and supplements is performed constantly during cultivation, which prevents nutrients from becoming a limiting factor.

The advantage of this type of feeding during cultivation is that it allows to obtain higher quantities of product overall (Allman, 2020).

A fed-batch culture is a modification of batch fermentation in which medium nutrients are added systematically and therefore this is a semi-continuous process (Magar, 2021).

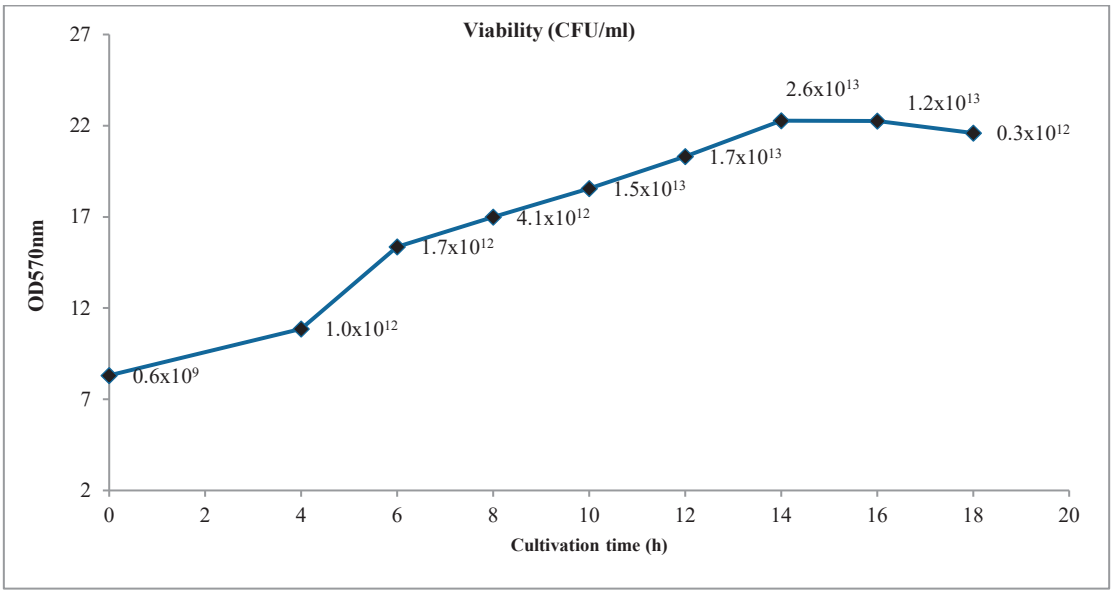

Figure 3. Dynamics of *S. cerevisiae* biomass development in fed-batch system

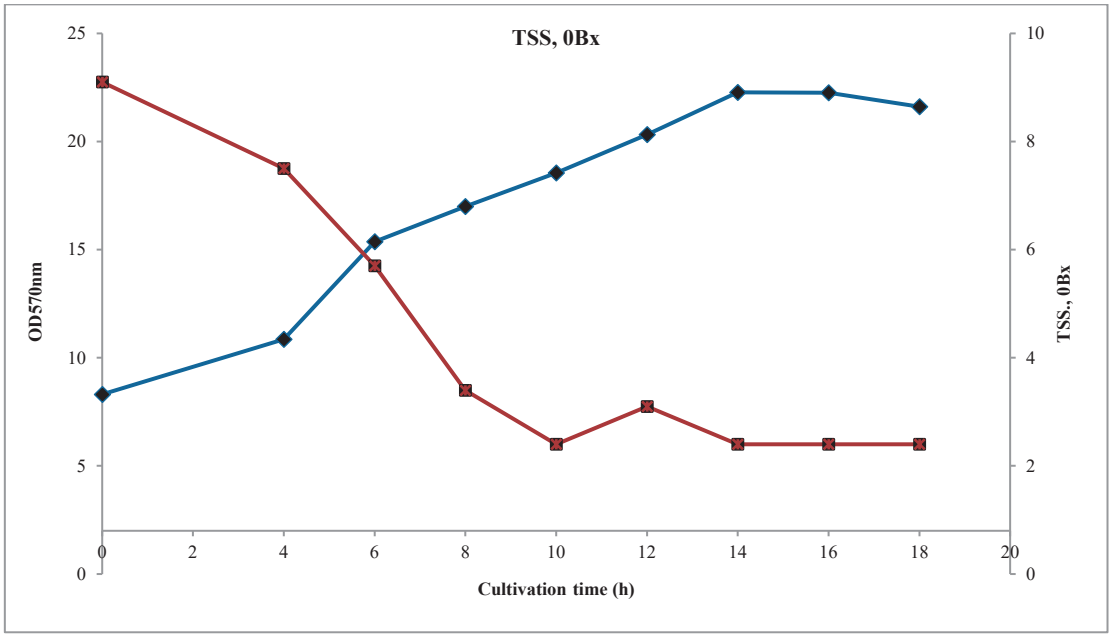

Figure 4. Growth curve in fed-batch fermentation for *S. cerevisiae*

Depending on the evolution of the growth, the fermentation process of the yeast culture evolves in several successive phases.

Fed-batch fermentation for *S. cerevisiae*

The latency phase - also called the induction phase and the lag phase, begins when the cells are transferred to the environment (inoculation or seeding) and ends when they begin to multiply. During this period, the microbial population remains constant. It can vary from a few minutes to a few hours. For *S. cerevisiae* the lag phase is almost 4 h.

The phase of accelerated growth - the period in which the reactions that make up the cellular metabolism continuously increase their speed, tending to the maximum speed that will be reached in the exponential phase. The duration of acceleration phase was between 4-6 h of cultivations.

a) during the lag phase the growth rate is higher than the multiplication rate and, as a result, the cell sizes are larger than any other phase of the cell cycle;

b) duration of the latency phase depends primarily on the characteristics of the microorganism used and the composition of the culture medium.

Exponential growth phase - is characterized by the fact that after a short period of acceleration of the specific growth rate, it

becomes constant and maximum (= max). The max phase was achieved between 12-14 h of cultivation (Anghel et al., 1993).

The phase of decelerated growth corresponds to the period when the specific growth rate begins to decrease due to the impoverishment of the environment in essential nutrients.

The decrease in the specific development speed can be fast (in the case of exhaustion of the carbon and/or energy source), or slower (in the case of the accumulation of inhibitory metabolites or the exhaustion of other nutrients). If during the previous phase, the death of microorganisms can be neglected (90% of the cells being viable), during this phase the viability of the cells begins to decrease. After 14 h of cultivation starts the decrease of the cellular concentration and of the viability.

The stationary phase - includes the period in which the microbial population remains constant. Stationarity should not be understood in a static sense, as the growth of microorganisms, but as a dynamic process in which the speed of growth is equal to the speed of death of microorganisms. The stationary phase was achieved for *S. cerevisiae* at 16 h of cultivation.

Accelerated and exponential death phase of microorganisms decrease of microbial population due to drastic decrease in the rate of multiplication (which is lower than the death rate of microorganisms) (Anghel et al., 1993). After 16 hour the cultivation the viability is lower and the dead of microorganisms is visible when the viability is 0.3 $x10^{12}$ with a $D.O_{570nm}$ by 21.60 higher than the 0 h when the $D.O_{570nm}$ was 8.31.

The addition of sugar sterile solution in the fed-batch fermentation with *S. cerevisiae* was 1 g/100 g at 10 hours.

After 14 hours of cultivation, the TSS value clearly indicated that the *S. cerevisiae* yeast is in the stationary phase.

In parallel, a batch fermentation process was carried out using a *Starmella bacillaris* yeast strain to compare its fermentation power with the *S. cerevisiae* yeast strain in the same system.

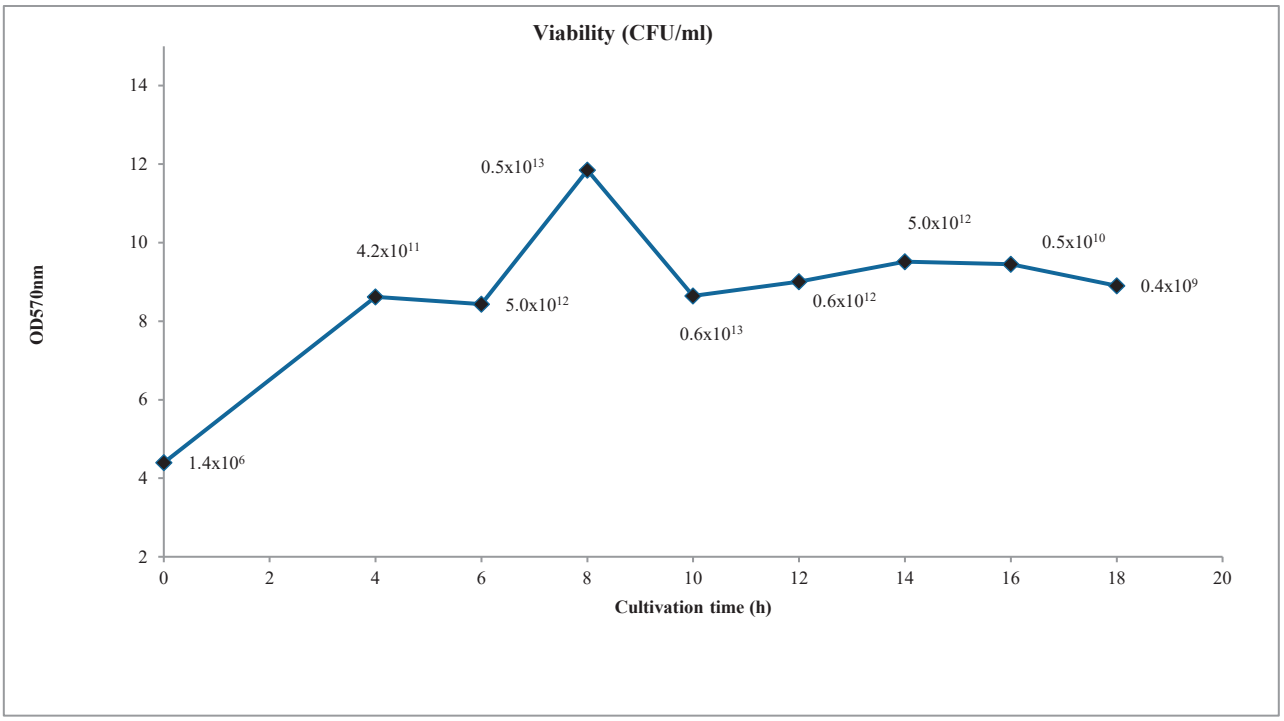

Figure 5. Dynamics of *S. bacillaris* biomass development in batch system

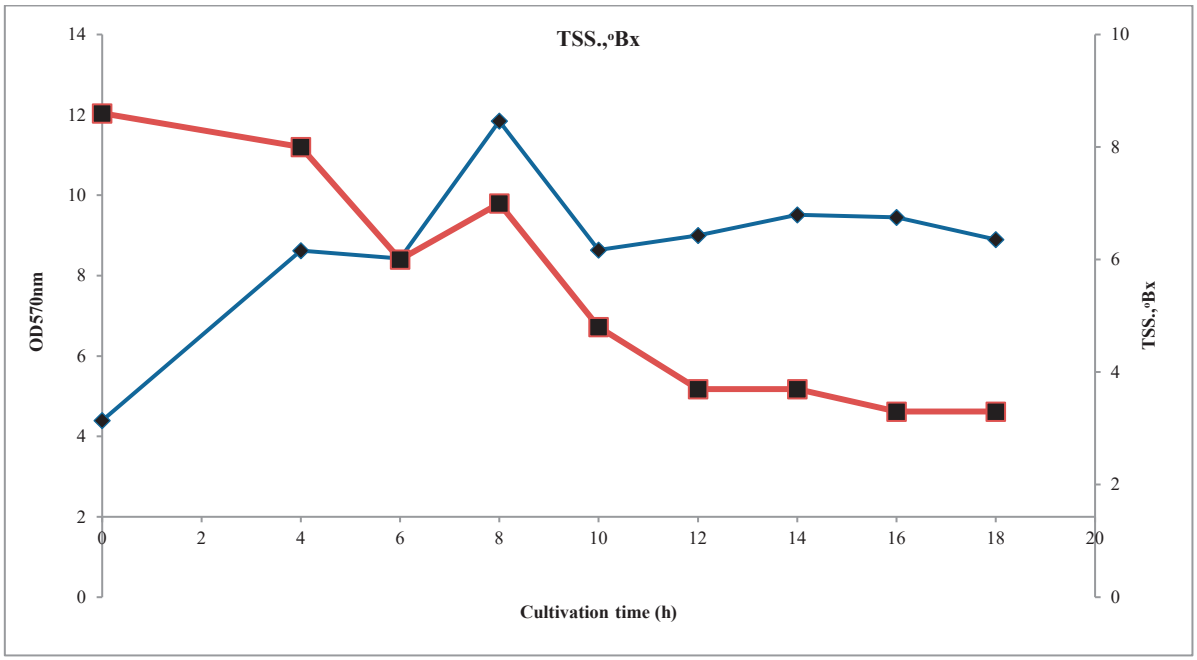

Figure 6. Growth curve for *S. bacillaris* in batch fermentation

After 8 hours of cultivation the decrease in cell concentration and viability begins.

Stationary phase was achieved for *Starmella bacillaris* after 10-12 hours of cultivation.

After 16 hours of cultivation the viability decreased and the death of microorganisms is visible when the viability is 0.5 x10^{10} with a D.O 570nm of 9.45 higher than 0 h when the D.O 570nm was 4.39.

Batch fermentation for *Starmella bacillaris*

WCW (g/L) for fed-batch fermentation of *Starmella bacillaris* was 32.6 g/L yeast biomass compared to the batch fermentation where the yeast biomass was 15.9 g/L. In addition, the amount of biomass of *S. cerevisiae* is double that of *Starmella bacillaris.*

The exponential phase was achieved at 8 h of fermentation.

For the fed-batch cultivation which used *S. cerevisiae strain the* yield of transformation of the substrate into biomass was:

X = 137 g/4 L wet cell weight biomass

S = 323.4 g/4 L

Y = (137/323.4) × 100 = 42.3%

For the batch cultivation which used *Starmella bacillaris strain the* yield of transformation of the substrate into biomass was:

X = 61.71 g/4 L wet weight biomass

S = 212 g/4 L

Y= (61.7/212) × 100 = 29.10%

Even though the biomass yield for fermentation of both yeasts was not substantial, a significant difference can be observed between these two yeast strains in terms of cell wet weight and substrate conversion yield to biomass. For example, the yield in biomass in the case of *S. cerevisiae* was 42.3%, which is almost double in comparison with *Starmella bacillaris* yield (29.10%).

CONCLUSIONS

Experiments developed in order to obtain yeast biomass represent first trials on small scale that will be assessed in order to optimize the bioprocess. The new isolated yeast strains were molecular identified as *Saccharomyces cerevisiae* and *Starmella bacillaris*.

By optimizing the *Starmella bacillaris* fermentation process, it will be possible to improve the batch process, which can be transformed into fed batch that presents several technological advantages (such as higher yield).

The new yeasts biomass will be further studied for the vinification process at Pietroasa Viticulture and Winemaking Research and Development Station. The substrate to biomass conversion yield of *S. cerevisiae* was 42.3%, which is almost double as the yield of *Starmella bacillaris*.

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