ISOLATION AND CHARACTERIZATION OF OENOLOGICAL YEAST

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Abstract

Alcoholic fermentation is carried out by all microorganisms presented in must, which vary by technological characteristics of interest to oenology. So far, in Albania the fermentation is spontaneous and not directed, resulting in an absence of a standard product. The object of the present study is the isolation, identification and determination of yeasts, isolated in two phases of fermentation and in four different types of musts from different variety of grapes, with the purpose of selecting autochthonous yeasts for a directed fermentation. In this study 14 strains were isolated, with P.D.A. and Y.M.A. mediums. After isolation, purity controls were made to the strains through cultivation and re-cultivation in Petri dish, and also, by running a stereomicroscopic and microscopic examination. Classification and identification of yeast strains in genus and species were based on macro-morphological characteristics of the colonies. Then, morphological characteristics of the cell were observed as an important taxonomic indicator. For the determination of the physiological and biochemical characteristics of yeasts, the assimilation of sugars and the fermenting ability of yeast were used. The property of the assimilation of sugars is used for the differentiation of species which is closely related to the must fermentation. The fermenting ability of yeast strains was determined by the release of CO_2 in the Durham tubes, placed upside-down inside the test tubes filled with the must containing 251 gr/L sugar. At the end were isolated and identified 9 strains of the genus *Saccharomyces*, 1 strain of the genus *Schizosaccharomyce*s, 2 strains of the genus *Brettanomyces*, 2 strains of the genus *Kloeckera*. These isolated species will serve to further work towards their selection based on their fermenting technological characteristics.

Keywords: *oenology, yeast, isolation, fermentation.*

Introduction

Development and modernization of agricultural, bio–industrial and nutritive production, welfare of a myriad of industry fermentative processes food and beverages, necessarily require a quality product and standard. In this context, winery and oenology requires firstly the choice of the structure varieties in relation of ecological factors. Secondly, creating appropriate and specific conditions for the fermentation which is realized through optimization of fermentative processes and use of selected yeasts, isolated from autochthonous varieties in different phases of fermentation process (Zambonelli. C, 1998). The work for the isolation and characterization of endemic strains, which can be used to produce products of high quality and with special features, is of paramount importance. Particular attention is drawn to the use of pure cultures of yeasts, which can be used in the processing industry for the production of grape wine, brandy, cognac, alcohol, vinegar, etc (Delfini C, 1995).

The aim of the present work was focused to isolate, select and create a collection of oenological strains with as high fermentative activity as possible.

Materials and methods

The biological material

Freshly harvested grapes (*Vitis vinifera*) of Merlot variety was taken from the area of Durres and Tirana and Cabernet sauvignon variety from the area of Durres. Samples were taken at 3 (three) different phases of fermentation.

• in must before fermentation, in order to obtain various genus and species that have a secondary role in fermentation of wines,

• in the peak of fermentation, on purpose to isolate the responsible yeasts for producing wine.

• and at the end of fermentation.

Then these samples were submitted to isolation process of yeasts strains (Fugelsang, 1997).

The mediums used

The mediums used for cultivation of these strains have been PDA (Potato–dextrose–agar, 42 g/L) and YMA (Must-Agar, 47 g/L).

Isolation procedures

Samples were serially diluted and then were plated in Petri dishes in 3 (three) replicates for each sample. Then the samples were incubated at 25°C for 72 hours. After incubation, the plates were selected with 30–50 colonies. Control of purity was performed by streaking method for cultivating and re–cultivating all isolated strains, accompanied with stereo and microscopic examinations.

Methods for the determination and identification of yeasts

Classification and identification of yeasts was based on the determination of a set of characteristics, based on Lodder and Kreger–Van Rij (1967):

Macromorphological characterizations of yeasts

Macromorphological characteristics of yeasts were determined observing the form, margins, texture and colony color, grown on solid and inclined YM–agar medium.

Morphological characteristics of the cells

The form of cells as an indication closely related with the vegetative reproduction was observed by optic microscope. The observation was carried out in three-days cultures of yeast strains in YM–agar and on the same medium without agar, incubated at 25°C. The microscopic observation was made with the immersion magnification.

Vegetative reproduction

After inoculation of isolated yeasts in inclined medium, liquid microscopic preparations were prepared. The preparations were observed under microscope with magnification 40x if the multiplication of the yeasts were with budding or division.

Formation of pseudomycelium

In PDA medium the determination of the pseudomycelium formation became through the cultivation on a microscope slide. After 3-4 days of cultivation the back of microscope slides was cleaned from the medium and these slides were observed under a microscope.

Fermentation of sugars

Sugars used for the fermentation tests were: glucose, galactose, saccharose, maltose, lactose and raffinose. The fermenting ability of isolated strains in above sugars was tested through the release of CO_2 in Durham tubes placed upside-down on the mediums with 0.5% yeast extract and 2.0% of sugar, incubated at 25°C. The tubes were controlled each day for a period of up to 5 days (Van der Walt, 1970).

Evaluation of fermentative activity by the strains

Evaluation of fermentative activity by the strains were done in tubes filled with 10 ml musts and Durham tubes placed upside down. After closing the tubes, the mediums were sterilized. The initial air gap produced when the tube is inserted upside down, is lost during sterilization, usually performed at 121°C for 15 minutes. In this sterilized medium were inoculated yeast cells, obtained from selected colonies. They were incubated at 25°C and after 2 (two) days, the examination of cultures became (Van der Walt,1970).

Results and discussion

As described above in the materials and methods, the isolation procedure was carried out enabling the acquisition of 14 pure yeast cultures, as follows:

• The cultivar: Merlot (Durres)

peak of fermentation and 1 (one) at the end of fermentation.

• The cultivar: Cabernet Sauvignon (Durres)

8 (eight) cultures, of which 3 (three) were obtained at the beginning of fermentation, 3 in the peak of fermentation and 2 (two) at the end of fermentation.

• The cultivar: Merlot (Tirana)

2 (two) cultures, which were obtained in the peak of fermentation.

4 (four) cultures, of which 2 (two) were obtained at the beginning of fermentation, 1 (one) in the

The above cultures were labeled as follows:

• From cultivar Merlot (Durres): MS3, MS3', MS (3-5)", MS5, MS5', MS6, MS6 ', MS6''.
• From cultivar Cabernet Sauvignon (Durres): KS1, KS1 ', KS7, KS7'.
• From cultivar Merlot Tirana: MP2, MP4.

Macromorphological characteristics

The appearance of colonies on solid medium in Petri dishes and at inclined medium in tubes was determined, during the isolation. Macro-morphological characteristics of cultures in Petri dishes are presented in the table below.

Table 1. Macromorphological characteristics

Serial Number	Yeasts strains	Images of colonies in Petri dishes
1.	KS1	Round colonies, regular margins, slightly raised, shiny, creamy white color, with less strong consistency. (fig. 1)
2.	KS1′	Round colonies, regular margins, small dimensions, raised, milky color and shiny.(fig. 2)
3.	MP2	Irregular margins, raised mass ruffled on the top like "floral", creamy white color, not shiny, smooth consistency. (fig. 3)
4.	MS3	Round colonies, transparent margins, shiny, small dimensions, milky white color, raised, smooth consistency. (fig. 5)
5.	MS3′	Regular margins, shiny, raised, milky white color, with a little smooth consistency. (fig. 6)
6.	MS(3-5)"	Regular margins, milky white color, raised, shiny, smooth consistency. (fig. 7)
7.	MP4	Round colonies, regular margins, small dimensions, raised, milky white color, shiny, with less strong consistency. (fig. 4)
8.	MS5	Slight oval Colonies, regular and transparent margins, milky white color, shiny, slightly raised, smooth consistency. (fig. 8)
9.	MS5'	Round colonies with two emerged sides, milky color, shiny, slightly raised, smooth consistency. (fig. 9)

10.	MS6	Round colonies, regular margins, small and shiny, cream-colored, slightly raised, smooth consistency. (fig. 10)
11.	MS6’	Round colonies, regular margins, shiny, slightly raised, small dimensions, milky color, with very smooth consistency. (fig. 11)
12.	MS6’’	Round colonies, regular margins, shiny, slightly raised and the small dimensions, milky color, with a little smooth consistency. (fig. 12)
13.	KS7	Irregular margins, raised mass ruffled on the top like “floral”, creamy white color, not shiny, smooth consistency. (fig. 13)
14.	KS7’	Round colonies, regular margins, slightly raised, shiny, creamy white color, with a little smooth consistency. (fig. 14)

The table shows that the most of cultures form colonies in milky white color, rounded in shape, shiny, slightly raised or raised, smooth consistency, with the exception of two cultures (MP2 and KS7) which undergo ruffling on top in the form "floral". Below are the photos of these yeast colonies observed in stereo microscope.

Images of colonies, grown in YMA and PDA mediums.

Fig.1

Fig.2

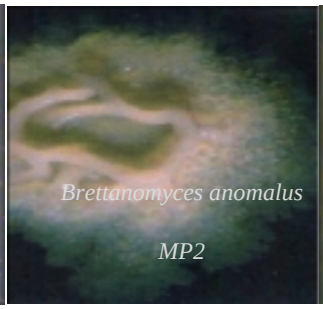


Fig.3

Fig.4

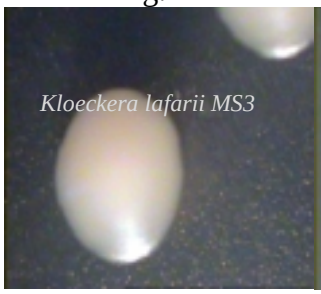


Fig.5

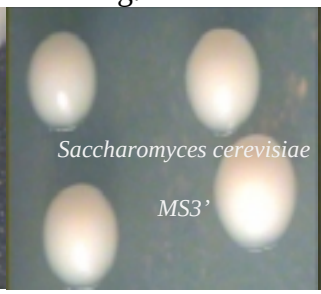


Fig.6

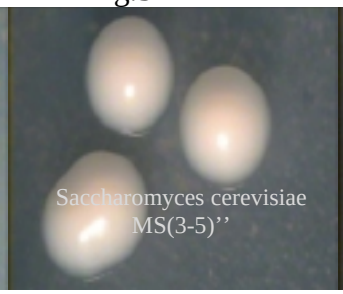


Fig.7

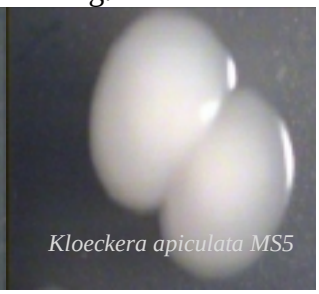


Fig.8

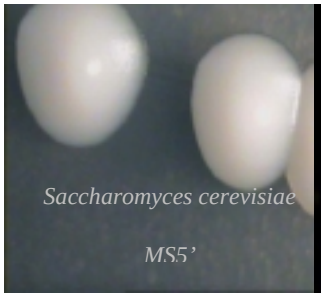


Fig.9

Fig. 10

Fig.11

Fig.12

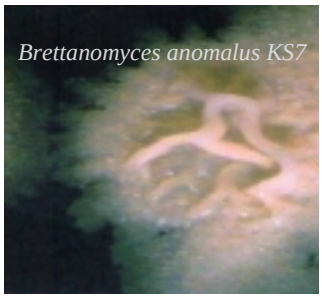


Fig.13

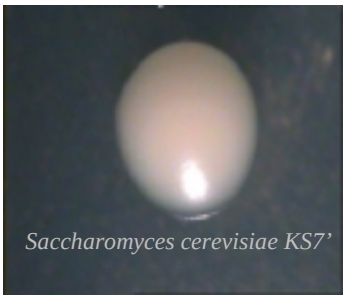


Fig.14

Cells shape and the manner of reproduction.

Shape of the cell is typical for certain genus of yeasts for example the cell with oval or elongated shape belongs to genus of Brettanomyces. For this reason the cells shapes were used for the identification of yeast strains such as KS7, MP2 which belong to the genus Brettanomyces according to their shape. The following enlarged photos will appear identified yeasts cells. Cells shape are closely related to the manner of reproduction. The manner of the reproduction of yeasts is used to determine which genus were reproduced only by budding (like Saccharomyces genus), which ones were reproduced only by division (like Schizosaccharomyces genus) or both forms. The data on cells forms of the selected yeasts and the manners of reproduction are presented in the following table:

Table 2. Cells shape and the manner of reproductive.

Serial number	**Yeasts strains**	**Cells shape and the manner of reproductive.**
1.	KS1	Round shape, multilateral budding.
2.	KS1′	The cell has a round shape to oval, multilateral budding.
3.	MP2	Oval or elongated shape, reproducing by budding.
4.	MS3	Lemon – shaped cells. Reproduction with bipolar budding.
5.	MS3′	Round to oval shape. Multilateral budding
6.	MS(3-5)"	The cell has a round shape to oval. Multilateral budding
Serial number	**Yeasts strains**	**Cells shape and the manner of reproductive.**
7.	MP4	Round to oval shape. Multilateral budding
8.	MS5	Lemon-shaped cells. Reproduction of polar and lateral budding.
9.	MS5'	Round to oval shape. Multilateral budding.
10.	MS6	Oval extended shape like a stick, not budding.
11.	MS6'	Round to oval shape. Reproduction multilateral budding.
12.	MS6''	Round to oval shape. Multilateral budding.
13.	KS7	Oval or elongated shape. Reproduction with budding.
14.	KS7'	round to oval with not very regular shape, Multilateral budding

As shown in the table 2, almost all strains are multiplied with budding with the exception of MS6 strain, which multiply with division. The most of the strains are multiplied by multilateral budding, with the exception of MS3 and MS5 strains, which are multiplied with bipolar budding.

Yeasts cells morphology

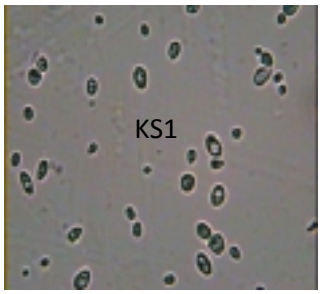


Fig.1

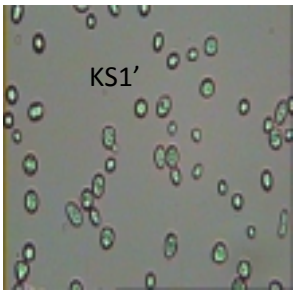


Fig.2

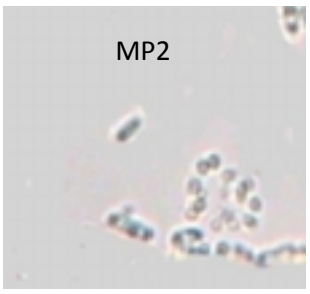


Fig.3

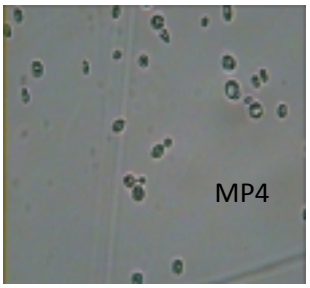


Fig.4

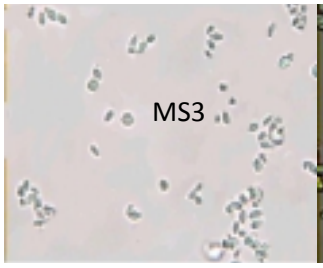


Fig.5

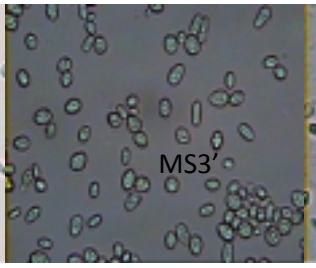


Fig.6

Fig. 7

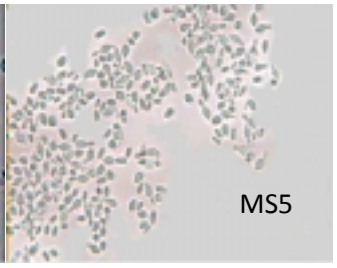


Fig. 8

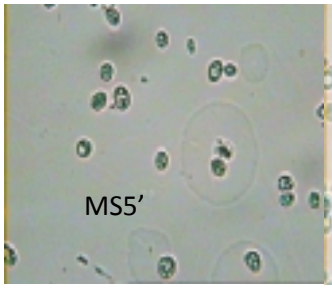


Fig. 9

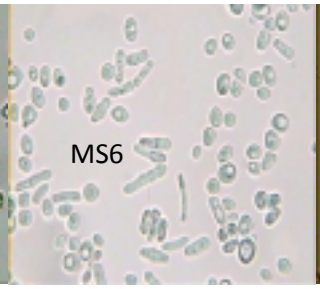


Fig. 10

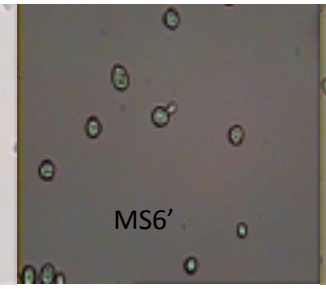


Fig.11

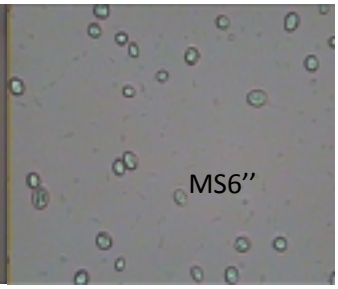


Fig. 12

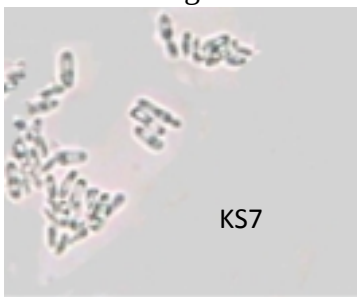


Fig.13

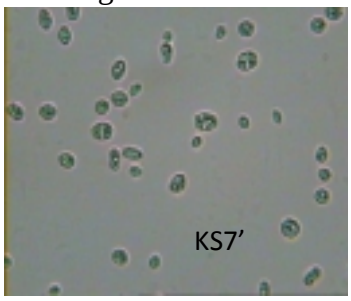


Fig.14

Pseudomycelium formation.

Pseudomicelium are called strings that are formed by prolonged cells, which are generated from reproduction by budding. The following table presents pseudomycelium formative skills of the selected yeasts strains.

Table 3. Pseudomycelium formation.

Serial	Yeasts	Pseudomycelium formation

number	strains	
1.	KS1	Pseudomycelium quite invisible.
2.	KS1′	Good development of pseudomycelium.
3.	MP2	Pseudomycel in long cells shape, branchy and thin
4.	MS3	Vestigial pseudomycelium..
5.	MS3′	Pseudomycelium developed with short branches.
6.	MS(3-5)”	Very good development of pseudomycelium.
7.	MP4	Good development of pseudomycelium.
8.	MS5	Pseudomycelium is not formed.
9.	MS5’	Very good development of pseudomycelium.
10.	MS6	Pseudomycelium is not formed.
11.	MS6’	Very good development pseudomycelium, branchy.
12.	MS6’’	The development of pseudomycel is very good.
13.	KS7	Pseudomycelium in thin cells shape,with long branches.
14.	KS7’	Poor development of pseudomycelium.

As we saw from table 3, genus Saccharomyces showed that it mainly had a very good development of pseudomycelium, while in other genera as Kloeckera, Schizosaccharomyces the formation of pseudomycelium was not noticed.

Fermentation of sugars

Sustainability of the fermentative ability of yeasts strains, made this method used in this study. Yeasts fermentative ability or not, was used to identify the genus and particularly the species. The table below reflects the fermentative ability of yeast to six sugars, for 5 days.

Table 4. Fermentation of sugars

Sugars	Control days	Yeasts strains													
		KS1	KS1’	MP2	MP4	MS3	MS3’	MS(3-5)’’	MS5	MS5’	MS6	MS6’	MS6’’	KS7	KS7’
Glucose	1 day	-	++	-	++++	-	+++	+++	+	+++	-	++	+	-	++++
	2 days	-	++	-	++++	-	+++	+++	++	+++	-	++	+	-	++++
	3 days	-	+	-		-		+++	+++	+++	-	++	+	-	++
	4 days	-		-		-				+++	-			-	
	5 days	-		-		-					-			-	
Saccharose	1 day	-	++	-	++++	-	-	-	+++	++	-	+++	+	-	+++
	2 days	-	++	-	+++	-	-	++	+++	++	-	+++	+	-	++++
	3 days	-		-		-	-	+++		++	-	+++		-	
	4 days	-		-		-	-	+++			-			-	
	5 days	-		-		-	-				-			-	
Ma	1 day	-	+	-	-	-	++	+++	+	+++	-	++	+	-	-

	2 days	-	+	-	+	-	++	+++	+		-	++	+	-	+
	3 days	-	+	-	++++	-			+		-	++		-	++++
	4 days	-		-		-					-			-	
	5 days	-		-		-					-			-	
Galactose	1 day	-	++	-	+++	-	+++	+	+++	+++	-	+++	+	-	+++
	2 days	-	++	-	+++	-	+++	+	+++	+++	-	+++	+	-	+++
	3 days	-	++	-	++	-		+			-	++	+	-	
	4 days	-		-		-					-			-	
	5 days	-		-		-					-			-	
Lactose	1 day	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	2 days	-	-	-	-	-	+	-	-	-	-	-	-	-	+
	3 days	-	-	-	-	-		-	-	-	-	-	-	-	
	4 days	-	-	-	-	-		-	-	-	-	-	-	-	
	5 days	-	-	-	-	-		-	-	-	-	-	-	-	
Raffinose	1 day	-	+	-	+	-	-	+	+	-	-	-	-	-	+
	2 days	-	+	-	+	-	-	++	+	-	-	-	-	-	+
	3 days	-		-		-	-			-	-	-	-	-	
	4 days	-		-		-	-			-	-	-	-	-	
	5 days	-		-		-	-			-	-	-	-	-	

- No fermentation;
+ Weak fermentation;
+ + Medium fermentation;
+ + + Good fermentation;
+ + + + Rapid fermentation;
Empty boxes - Fermentation has finished

From the table it is noticed that strains showed different abilities of the fermentation against these six sugars, excluding strains KS1, MP2, MS3, MS6 and KS7 who did not ferment any sugar. Lactose and rafinose are less fermentable sugars by strains in study. The strains MP4 and KS7' showed good abilities of the fermentation against glucose, saharose, galactose and maltose.

Evaluation of fermentative activity by the strains.

After the identification of the fermentative ability of yeast strains in different sugars, the control prove became about their fermentative ability on the grape must, where this ability was known through the releasing CO_2, which is product of fermentation. The results of this control are shown in the following table:

Table 5. The fermentative ability of the selected strains.

Serial number	Yeasts strains	Activity
1.	KS1	-
2.	KS1′	++
3.	MP2	-
4.	MS3	-
5.	MS3′	+++
6.	MS(3-5)”	+++
7.	MP4	++++

8.	MS5	+++
9.	MS5’	+++
10.	MS6	-
11.	MS6’	+++
12.	MS6’’	+
13.	KS7	-
14.	KS7’	++++

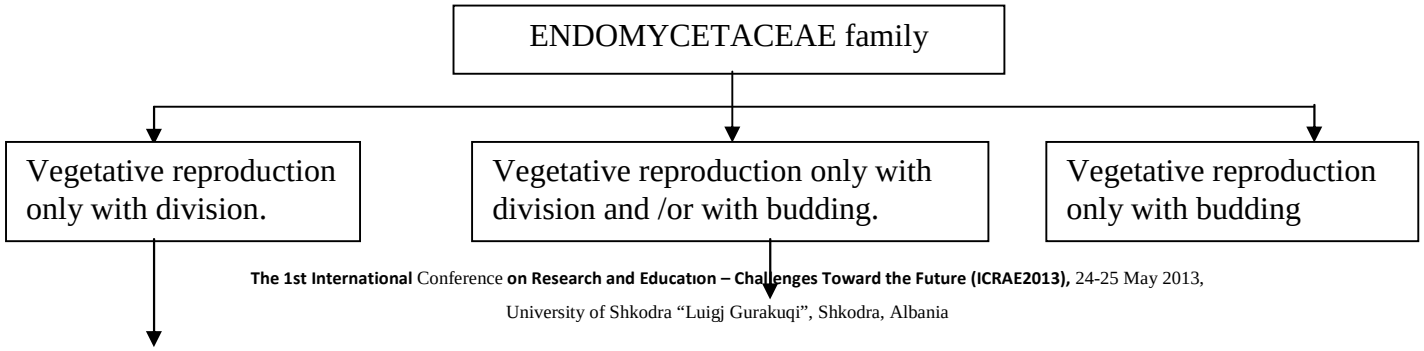
Results: - no fermentative activity
+ weak fermentative activity
+ + medium fermentative activity
+ + + high fermentative activity
+ + + + rapid fermentative activity

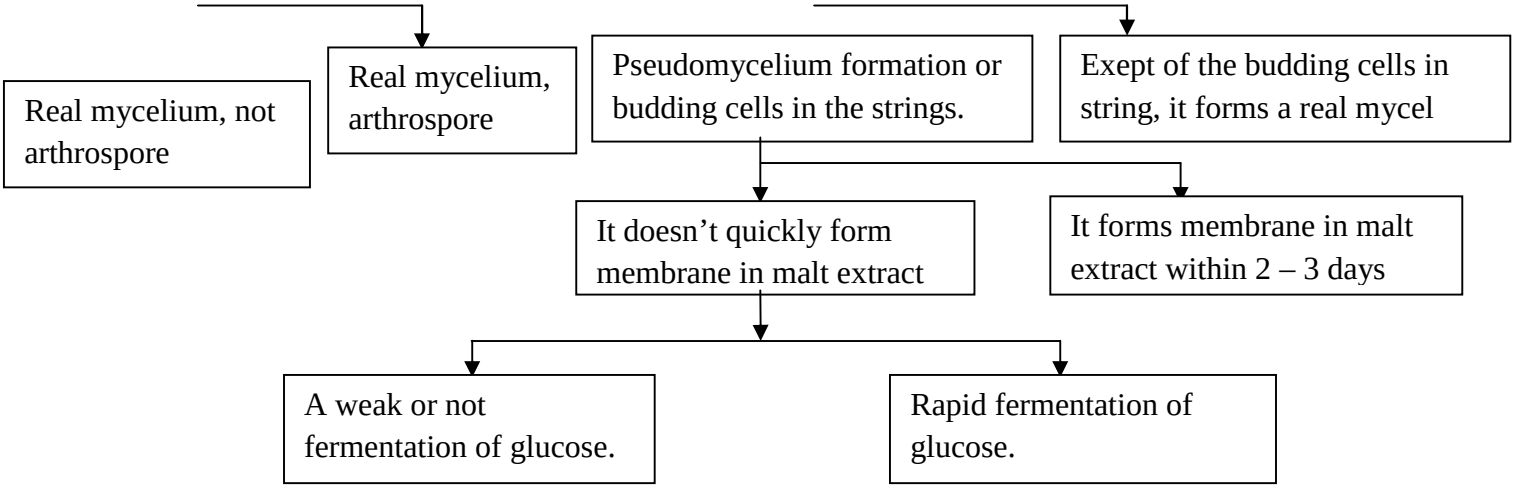
From table 5, we noticed that the strains MP4 and KS7' presented a rapid fermentative activity, while the strains with high fermentative activity were MS3', MS(3-5)", MS5, MS5 'and MS6'. Also it was observed that there were yeasts strains that provide a medium and weak fermentative activity for example KS1' and MS6" yeasts strains. However, there were strains like KS1, MP2, MS3, MS6 and KS7, that had not fermentative activity.

Classification and identification

In our study, for the classification and identification of yeasts, the identification scheme (Treaty Keys Lodder & Kreger Van Rij, 1967) as attended as follows:

Identification scheme of yeasts strains up to genus of ENDOMYCETACEAE family





As a conclusion of the tests carried out by the microscopic and macroscopic observations and all the presented work above, the isolated strains which will be used as biological material, for the selection of oenological strains with better fermentative quality, belongs to the genera and species as follows:

Saccharomyces cerevisiae – KS1', MP4, M3', MS(3–5)'', MS5', MS6', MS6'', KS7'
Saccharomyces fructum – KS1
Brettanomyces anomalus – MP2, KS7
Kloeckera apiculata – MS5
Kloeckera lafarii – MS3

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