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Co-inocula essays of yeasts with “killer” phenotype and sensitive strains of *Saccharomyces cerevisiae* with defects in mannoprotein synthesis. †

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Abstract:

Yeast mannoproteins have been thoroughly studied in recent years due to their contribution to different properties of wines. Our working group has the aim to establish the possible relationship between the structure of the mannoprotein (size and charge) and its effect on the wines. For this, we have different non-transgenic mutants that synthesize mannoproteins with altered but known structures (*mnn* mutants). We have constructed double mutants with each of the available single mutants, thus obtaining a very diverse collection of mannoproteins that differ in size and charge. Since the mutants are unable to complete wine fermentations satisfactorily, an alternative is to grow mutants in the laboratory, purify their mannoproteins and add them later to wines. Another option would be to use co-inocula of the mentioned mutants, together with wine yeasts strains "killer" capable of killing the mutants. In this work, previous experiments have been carried out to determine the behavior of different “killer” wine strains with each one of the defective mutants in mannoprotein synthesis. The yeast *Torulaspora delbrueckii* Killer (Kbarr1) has been proved to be especially suitable for making this type of co-inoculum.

Keywords: mannoprotein; *Saccharomyces cerevisiae*; *mnn* mutants; killer phenotype.

1. Introduction

In recent years, the effect of mannoproteins on wine has been extensively studied mainly due to the high demand for wines aged on lees. This method is mainly used in white wine barrel fermented and natural sparkling wine, among others. This ecological process aims to improve several characteristics of the wine: the quality of the foam, the mouthfeel, the aroma, protein stability and other related features, through the chemical interactions between mannoproteins and wine compounds. The yeasts release some mannoproteins during the fermentation, but mainly during aging, when yeasts undergo autophagy followed by cell death and subsequent autolysis [1, 2].

In our laboratory, we are trying to establish a relationship between charge-size of the mannoproteins and its effects on some aspects of wine. For that purpose, we have used mannan defective single mutants (*mnn*) [3] to make doble mutants with every possible combination of single mutations, thus we can obtain may different mannoprotein structures. However, these strains are not adequate to perform complete wine fermentation so the mannoproteins must be purified from laboratory cultures and added to the wines.

Another way to get mannoproteins from the mutants released to the wine is to design fermentation strategies using co-inocula of a killer strain [4] with high fermentative efficiency and a sensitive *mnn* mutant. Killer strains are very common between yeast used in enology, so they guaranty the completion of fermentation and also, they kill *mnn* mutants, thus accelerating the release of its mannoproteins. In our laboratory, we have a line of research focused on killer strains, [5, 6] which allows us to have a wide collection of killer yeasts.

The aim of this work is to check the effect of every killer strain on every one of the mannan defective mutants under different conditions. The results of these experiments will be very useful to decide the best combinations of strains to be used in main fermentation of wine or second fermentation in CAVA.

2. Materials and methods

Saccharomyces cerevisiae X2180 and single mutants with defects in the structure of mannoproteins *mnn*1, *mnn*2, *mnn*3, *mnn*5, *mnn*6, *mnn*9 y *mnn*10 [3] are from the laboratory collection (Figure 1). Double mutants: *mnn*1*mnn*2, *mnn*1*mnn*6, *mnn*1*mnn*9, *mnn*1*mnn*10, *mnn*2*mnn*6, *mnn*2*mnn*10, and *mnn*6*mnn*10. They are obtained by conjugation of haploid strains and later selection of spores.

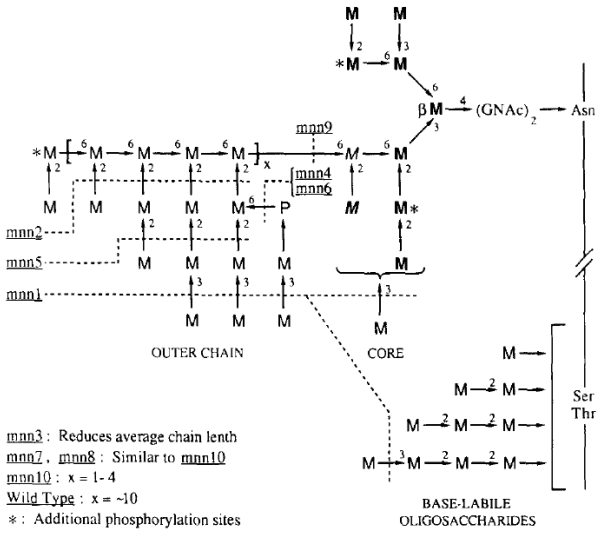

Figure 1. Chemotypes of mannoproteins mutants [3].

Saccharomyces cerevisiae (S.c) killer strains: F166 (K1), E7AR1 (K2), F182 (K28), EX229 (Klus). *Torulaspora delbruekii* (T.d) killer strains: EX1180 (Kbarr1), EX1257 (Kbarr2). As no-killer control EX1180-2K$^-$ (T.d,K^s) and EX33 (S.c, K^s) [5, 6].

All yeasts were grown in YEPD liquid medium at room temperature with agitation until late exponential phase of growth. Then, the mutant yeasts were spread in YEPD-methylene blue plates and killer yeasts drop on top. The plates were checked every day and photographs were taken during the process.

The killer effect of the yeasts in the patches, was determined by observing the halos generated by the death of the mutants (**figure 2**)

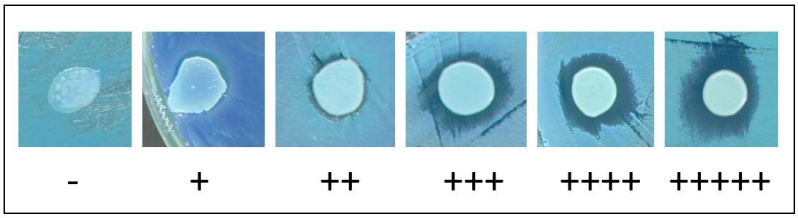

Figure 2. Punctuations according to the halo produced by the death of the mutants due to the toxin.

3. Results and discussion

3.1. The more affected the mannoprotein is, the more sensitive it is to the killer toxin.

In the light of the results, it seems clear that independently of conditions, the sensitivity to the toxin is higher when the mutation leads to a reduction in size of N-linked chains (**table 1**), probably because it facilitates the entry of the toxin or the interaction with its receptor.

pH	T	wt	mnn1	mnn2	mnn3	mnn5	mnn6	mnn9	mnn10
3,5	13ºC	++	+	++	+++	++	++	+++++	++++
	25ºC	+	++	+	++	+	+	++++	++++
4	13ºC	++++	+	+++	+++	++	+++	++	++++
	25ºC	++	++	+++	++	++	+	+++++	++++
4,7	13ºC	++	+	+	+++	+	+	++++	++
	25ºC	+++	+	++	++	+	+	++++	+

Table 1. Kbarr1 killer effect at different pH and temperatures.

3.2. The mnn1 mutation in combination with other mnn improve the killer resistance

The *mnn1* mutant is defective in α1-3 mannose addition. The lack of such mannoses, results in a more exposed negative charge (due to phosphate groups) than wild type. On the contrary, the *mnn6* mutant is defective in the phosphate addition to N-linked chains, which results in a drastic reduction in negative charge to <5% of the wild type. It seems that the more exposed negative charge in mnn1 results in an increment of killer resistance, when compared to wild type.

In some cases, the data showed a level improvement regarding to the single mutation when it is combined with *mnn1* (higher negative charge) and no change in sensitivity with *mnn6*. In **table 2** it is shown the kbarr1 killer effect on representative mutants.

pH/T	mnn1	mnn6	mnn10	mnn1,10	mnn10,6
3.5/13ºC	+	+++	++++	+	++++
4/13ºC	+	++	++++	++	++++
4.7/13ºC	+	+	++	++	+++

Table 2. Killer kbarr1 as representative data. Other data not shown affirm that a simple mutation with *mnn*1 which proportionate higher negative charge improves the toxin resistance, whereas in combination with *mnn6* is more o equal sensitive.

3.3. Kbarr1 a good killer candidate for co-inocula.

Kbarr1 killer toxin, present in *Toluraspora delbruekii* (EX1180 in this work) has been widely studied by our research group [6]. The mechanism seems to be similar to K1 and K2 by interacting with beta-glucan in the cell wall. It makes sense that a yeast with reduced size in N-linked oligosaccharides of mannoprotein could be more sensitive because the toxin reaches the receptor easily.

Table 3 show the results of killer behavior of several killer strains on several single mutants. Kbarr1 and K2 have similar killer efficiency. EX1180 (kbarr1) is a *Torulaspora delbruekii* which has proved to be very interesting concerning final characteristics in the wine.

killer	Strains	wt	mnn1	mnn2	mnn3	mnn5	mnn6	mnn9	mnn10
K1	**F166**	++	+	+	+	+	+	+++	+++
K2	**E7AR1**	++	++	++	+	+	++	++++	+++
K28	**F182**	-	-	-	-	-	-	-	-
klus	**EX229**	-	+	+	+	+	+	+	-
kbarr1	**EX1180**	++	+	++	+++	++	++	+++++	++++
kbarr2	**EX1257**	-	-	-	-	-	-	-	-
no killer	**2K-**	-	-	-	-	-	-	-	-

Table 3. Killer effect of different killer strains on single *mnn* mutants at 13ºC and pH 3.5.

Table 4 shows the results o a similar experiment including double mutants.

killer	Strains	mnn1,6	mnn1,2	mnn2,6	mnn10,6	mnn1,10	mnn1,9	mnn10,2
K1	**F166**	++	+	+	+++	++	+++	+++
k2	**E7AR1**	++	++	++	+++	-	++++	+++
K28	**F182**	-	-	-	-	-	-	-
klus	**EX229**	+	+	+	++	+	+	+
kbarr1	**EX1180**	++	+	+++	++++	+	++++	+
kbarr2	**EX1257**	-	-	-	-	-	-	-
no killer	**2K-**	-	-	-	-	-	-	-

Table 4. Killer effect of different killer in double mutants.

4. Conclusions

a) Once the relation size-charge of mannoprotein and effects will be clarified, it would be possible to choose mannoproteins on demand (size- charge) in order to improve some characteristics of wine like reduce tartaric or protein precipitation, or improving clarification, mouthfeel, aroma…

b) The use of co-inocula with the mutant which synthesize the desired mannoprotein and the appropriate killer strain, will allow direct release of mannoprotein from the mutant, avoiding the step of purification.

c) *Torulaspora delbruekii* EX1180 (Kbarr1) has proved to be a good candidate to be used as the killer counterpart of the co-inoculum.

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