Quantitatively Calorimetric Characterization of Olygalacturonase - Producing Mutants Isolated from *Saccharomyces cerevisiae* Wine Strains

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Abstract

Polygalacturonase producing mutants have been obtained from commercial wine yeast strains by chemical mutagenesis. This kind of treatment can lead to an improvement of a desired property, but, at the same time, to a weakening of some other useful characteristics, as fermentative power, tolerance to acid medium, high concentration of ethanol, etc. 26 positive mutants have been choosen in the screening and they have been tested for their behavior in wine making conditions, as low pH, high content in ethanol, SO_2, malic acid.

The mutants growth rate was compared to the parent one using a calorimetric approach, and they have been divided into two groups: one with similar growth to the parent strains, and the other one with a different behavior. Calorimetry confers rapidity, efficiency and can give a quantitative difference between the tested strains.

Keywords: wine yeast, mutants, calorimetry

Introduction

Genetic improvements of industrial yeast are useful, but hard because industrial yeast has a higher ploidy, which confers a greater genetic stability to their natural medium. The gene which contains a recessive mutation is complemented by normal homozygous gene in a polyploid cell. To express a recessive mutation by the direct mutagenesis in industrial yeast, a highly effective screening procedure makes it possible to isolate homozygote for the gene of interest (Barre et.al, 1993). On the other hand, construction of haploid strains is useful in order to increase the frequency of mutated gene expression. However, the characterization of such mutants under industrial condition is important because they are likely to change their behavior by a lot of chemical treatments. In wine yeast, mutation and selection (often through replica-plating on selective media) appear to be a rational approach when we want to keep a large number of characters and change only one (Pretorius, 2000). The problem is that mutation can lead to a weakening of other features (such as fermentation power, tolerance to ethanol content, SO_2 content, etc).

Because classical method of measuring the growth rate by optical density is not very effective, a calorimetric analysis has been suggested to be useful in the selection of the

mutants (Antoce et al, 1996). The advantage, besides the rapidity, is that the procedure allows precise quantitative determination of the growth parameters.

Previously, we isolated some mutants from wine yeast, which produce a polygalacturonase (PGase) in glucose rich condition. In this paper, these PGase+ mutants are characterized in some wine making conditions by the quantitatively calorimetric procedure.

Material and Methods

Strains and media

Strains used in this study are shown in (**Table 1**). They have been cultivated and tested in GYP medium (2% glucose, 1% yeast extract and 1% peptone) at 30ºC. Dye-SD plate and SD-PGA plate used in haploid selection and halo-formation test, respectively, was SD containing 25 μg/ml aniline blue and 50 μg/ml Bordeaux S and containing 2 % polygalacturonate, respectively. Ethanol and potassium methasulphite concentration used for the tolerance test were 0-20 % and 0-200 ppm, respectively, in GYP with pH 5.0.

Table 1. Yeast strains used

Strains	Property	Resource
Saccharomyces cerevisiae		
KW4	Wine yeast	IBRC*
4s1-4s8	$PGase^+$ mutants from KW4	This work
UvaFerm	Wine yeast	IBRC
Fh1-Fh12	$PGase^+$ mutants from UvaFerm	This work
Saccharomyces bayanus		
EC1118	Wine yeast	IBRC
Es1-Es6	$PGase^+$ mutants from EC1118	This work

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Isolation of the PGase+ mutants

PGase+ mutants were isolated from wine strains according to Radoi et al., as follows. Wine yeasts cultured in GYP for 12 h were treated with 30 μg/ml 1-methyl-benzimidazolecarbamate (Benomyl), and then cultured in GYP for 12 h. Cells were harvested, spread on Dye-containing GYP plate and incubated for 48 h. Dark blue colonies (considered haploid cells) were selected and mutagenized with 25μg/ml ethyl-methanesulphonate (EMS) by Spencers methods (1998). Halo-forming colonies plate were isolated as PGase+ mutants after incubation of mutagenized cells on SD-PGA plate.

Calorimetric quantification of the growth

A multiplex isothermal batch calorimeter having 24 calorimetric units was used to monitor the growth activity of yeasts and their mutants, by detecting the heat evolved during the growth of cultures at 30ºC. Details regarding the apparatus were reported elsewhere (Takahashi, 1996). Apparatus was fabricated by Bio Thermal Analyzer BTA 201H (Nippon Medical and Chemical Instruments Co., Ltd., Osaka, Japan). The temperature difference between reference and samples is transformed into voltage signals by thermopile plates. These signals are amplified and measured at appropriate time intervals and stored on magnetic disks for analysis. The calorimeter presented a sensitivity $A = 14.2 \mu W \mu V^{-1}$ at steady heat effect; the output signal of the apparatus is expressed in voltage units and the corresponding parameter

for converting the amplitude of the signal into the amount of heat volved in the calorimetric units was $\beta = 7.7 mJ\mu V^{-1}$. The reproducibility of the growth thermograms recorded was found to be very good (Antoce et al, 1996). As calorimetric vessel the usual glass vials of 50ml volume (with tight caps) were used.

Experimental procedure started from the overnight preinoculum in GYP basal medium and make appropriate dilution to reach a size of 1 to 3 x 10^6 cells /ml. From the last dilution 1 ml was added on the 5 ml sterilized medium into the 50ml glass vials. Incubation time varies between 24 to 62 hours, until any heat evolution ceased because of the nutrients consumption.

Comparison of the mutants with the parents was done using the calculated growth rate constant Miu (μ) and by the parameter μ_m/μ_i called "specific growth rate". We can define the maximum growth rate constant, μ_m, which corresponds to a culture with no addition of inhibitor, and a growth rate constant μ_i, which is obtained for a culture in the presence of inhibitor at concentration *i*. To obtain the reproducibility of the experiments, 5 vials of each sample have been prepared.

Results and Discussions

Comparison of growth rate between PGase+ mutants and their parents by calorimetric parameters.

Basically, the growth thermograms for wine yeast strains and their mutants look like in the (**Figure1**). Growth thermograms, also called *g(t)* curve, are only the apparent output of the calorimeter. Because the apparatus is isothermal and a permanent heat exchange takes place between the calorimetric unit and the surroundings, the thermograms must be corrected to calculate the actual evolution of the heat. As a result, for each thermogram the actual heat evolution process can be described by a calculated *f(t)* curve following equation (1).

$$f(t) = g(t) + K \int g(t)\, dt \qquad (1)$$

where *K* is the heat conduction constant of the apparatus, calculated according with Takahashi, (1996).

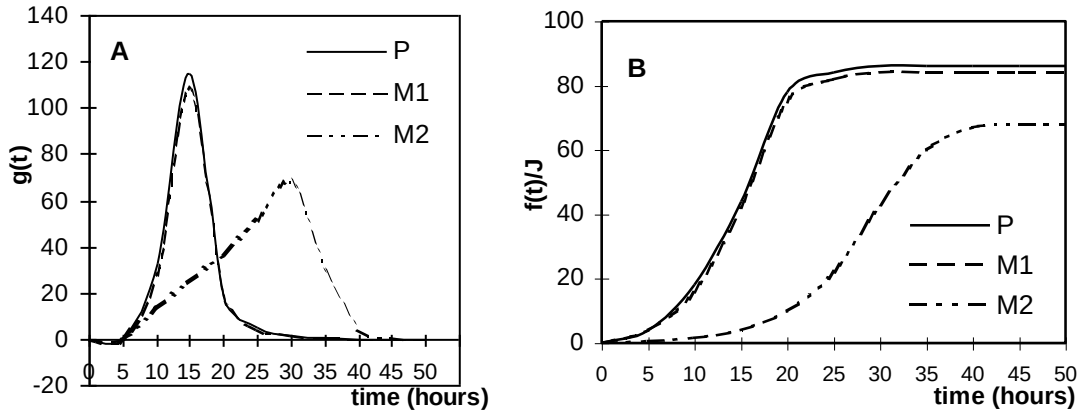

Figure 1. Typical growth thermograms (**A**) and actual heat evolution (**B**) for *S. cerevisaie* wine strains (P) and their mutants (M1 and M2). Theormograms were recorded as calorimetric output. By correction using equation (1) were obtained the actual heat evolution.

The obtained *g(t)* curves suggest that PGase+ mutants are composed of two distinct groups. The first group (Group 1) shows the similar thermograms to the parent strains and the second group (Group 2) shows the delayed thermograms than the parent and Group 1 mutants. When *f(t)* curves, reflect the cell growth, were calculated from these thermograms, we are dealing with two mutants, Group 1 having a similar cell growth to the parents and Group 2 having a delayed cell growth than the parents. The lag time of Group 2 deduced from *f(t)* curves (**Figure 1B**) is of approximately 30 minutes. It has been demonstrated (Antoce, 1997) that the *f(t)* curve is in a good relation with the results obtained by colony counting or by the measurement of turbidity, the curves being quite similar.

Table 2. Determined growth rate constant from calorimetrical records for wine yeasts and their Group 1 and Group 2 mutants.

Strain	$\mu_I \pm \mu/n$*	μ_i/μ_m
KW4 (parent)	0.469 ± 0.035	1.000
KW4 (Group 1 mutants)	0.401 ± 0.051	1.005 ± 0.126
KW4 (Group 2 mutants)	0.282 ± 0.025	0.573 ± 0.094
UvaFerm (parent)	0.389 ± 0.037	1.000
UvaFerm (Group 1 mutants)	0.374 ± 0.042	0.961 ± 0.141
UvaFerm (Group 2 mutants)	0.172 ± 0.022	0.442 ± 0.070
EC1118 (parent)	0.381 ± 0.038	1.000
EC1118 (Group 1 mutants)	0.365 ± 0.046	0.958 ± 0.154
EC1118 (Group 2 mutants)	0.237 ± 0.052	0.622 ± 0.150

* □μ/n : standard deviation

Once that we have determined that we have two groups of mutants, we should quantify the difference in growth. For this purpose we used the calculated growth rate constant μ_i. The maximum value of the growth rate, designed μ_m, belongs to the parent strain cultivated in standard conditions (GYP medium at pH 5.5). Thus, the parameter μ_i/μ_m represents the specific growth activity of the yeast cells in the presence of an inhibitor at concentration *i*. **Table 2** shows the correlation between the growth of the parents and their mutants in GYP medium standard condition. Mutants have been grouped by their growth constant rate. Based on the growth rate constant the mutants have been grouped as follows (**Table 3**).

Table 3. Classification of wine yeast mutants using the growth rate constant obtained by calorimetric measurement in GYP medium

Parent	Group 1*	Group 2**
KW4	4s1; 4s2; 4s5	4s3; 4s4; 4s6;4s74s8
UvaFerm	Fh1; Fh2; Fh6;Fh8	Fh3;Fh4;Fh5;Fh7;Fh9;Fh10;Fh11;Fh12
EC1118	Es3; Es6	Es1;Es2;Es4;Es5

*growth rate similar with the parents

**growth rate significant smaller than the parents

Tolerance of mutants against ethanol and sulphite determined by calorimetry

Effects of ethanol content (0%, 5%, 10%, 15% and 20%) and SO_2 content (0, 50, 100, 150 and 200 ppm), on the cells growth have been tested. For every sample two vials were prepared for the calorimetric measurements. (**Figure 2**) shows the profiles of μ_1/μ_m value (growth inhibition rate) in various ethanol contents.

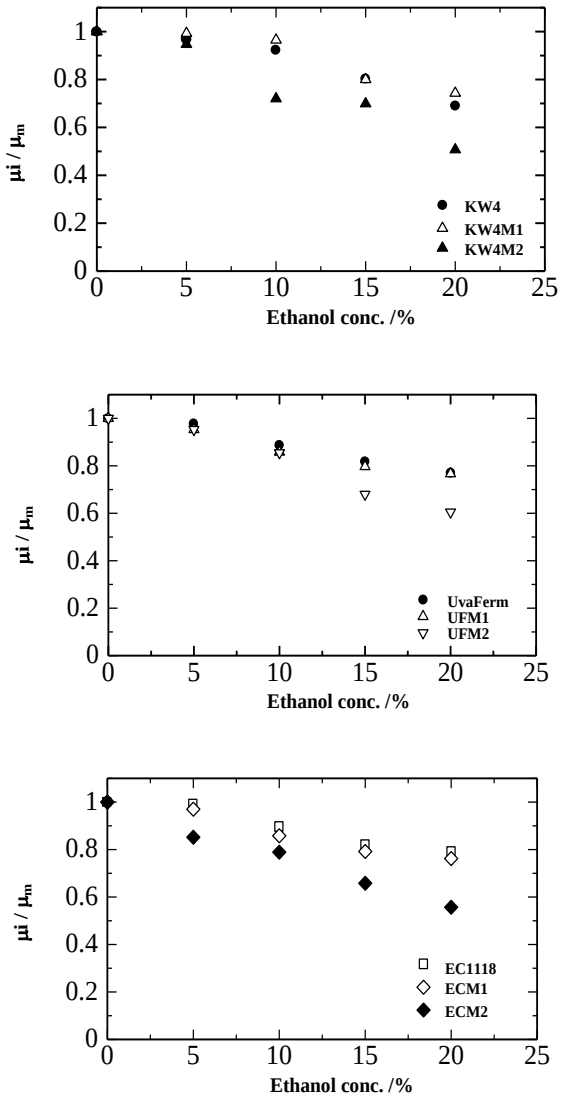

Figure 2. Profiles of μ_1/μ_m value (growth inhibition rate) in various ethanol contents for wine yeast strains and their mutants.

Group 1 mutants were similarly inhibited to their parents but Group 2 mutants proved to be surprisingly more inhibited than their parents. These data suggest that Group 2 mutants are more sensitive to ethanol than their parents, while Group 1 mutants are as sensitive as their parents. The differences of inhibition rate in the content of potassium methasulphite were not recognized in all strains (**Figure 3**). Group 1 and Group 2 mutants contained the similar tolerance to sulphite.

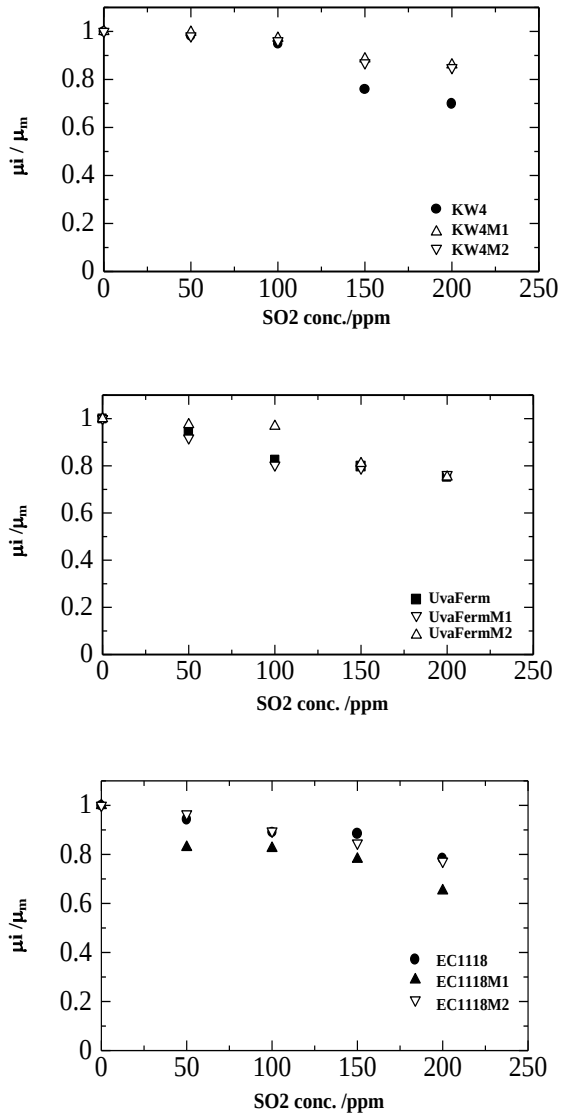

Figure 3. Profiles of μ_1/μ_m value (growth inhibition rate) in various SO_2 contents for wine yeast strains and their mutants.

These data suggest that Group 2 mutants have a half tolerance to ethanol. Tolerance of malic acid (another important compound of wine making) was also tested, but sensitivity of both groups of mutants to malic acid was not different.

Effect of culture pH on growth rate constants

The value of pH is another one of the growth conditions which have important effects on yeast growth. Wine is an acid medium, this is the reason why the pH chose varies in the low area (from 3.5 to 6.0). Growth rates constants in various pH cultures are shown in Fig. 4. Values of the growth rate are in agreement with the *g(t)* curve. The group M1 displays a remarkable stability of growth rate constant over the value of pH from wine making, the same as the parent strain. In the case of the M2 group, growth rates show a direct dependence on these mutants to the pH of the medium. The growth thermogram (data not shown) was similar

in parent and both groups of mutants, though total growth of group 2 mutants was lower than parent and group 1.

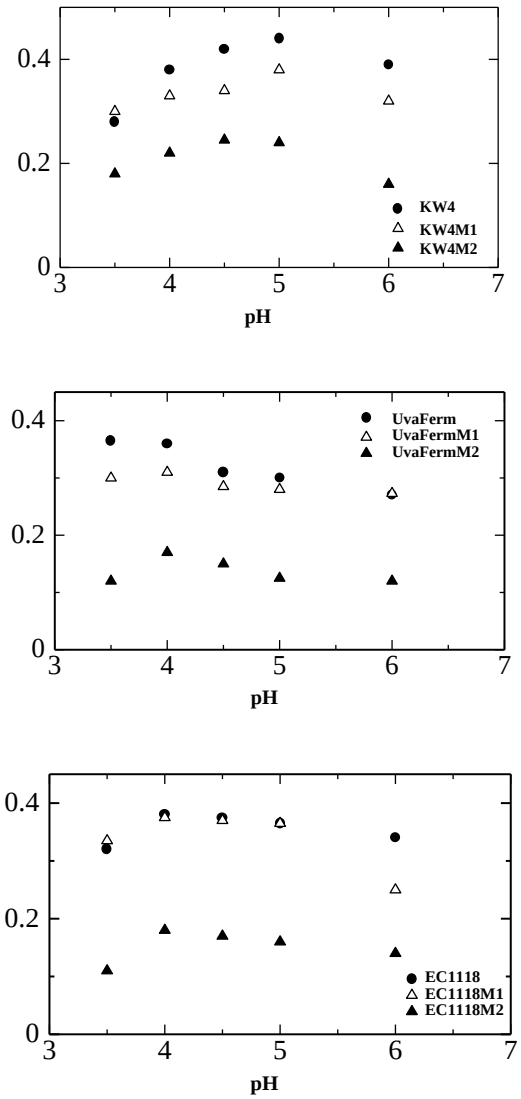

Figure 4. Growth rates constants in various pH cultures for wine yeast strains and their mutants

Discussion

During wine making the ethanol content increase constantly up to 15-16%, this is why, the strains employed should be resistant, if not, tolerant, to a high level of this component. When ethanol was added aseptically into the vials, a constant decrease in the growth rate could be observed either for parents and mutants. In wine making SO_2 is in undesirable because of its antiseptical and antioxidant properties. It is well know that this substance inhibits the development of microorganisms. We are interested in using tolerant strains to this component up to a concentration of 200ppm. Parents and M1 mutants showed an almost constant growth rate until 100ppm. probably above 100ppm. because the SO_2 led to a decrease of the pH in to the medium the growth rates decrease significantly. The M2 group they is very sensible to the presence of SO_2. Their growth thermograms registered smaller initial slope, very small peaks and significantly longer incubation times.

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