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The Kinetics of Alcoholic Fermentation by Two Yeast Strains in High Sugar Concentration Media

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Abstract

Over the last two decades, most of Italian vines have produced grapes with higher sugar to total acid ratios, greater concentrations of phenols and aromatic compounds and greater potential wine quality. As a consequence, the musts obtained by these grapes are more difficult to process because of the risk of slowing or stuck of fermentation. With the aim of describing the time evolution of the sugars bioconversion during alcoholic fermentation, the kinetics of the D-glucose and D-fructose degradations, promoted by two yeast strains (*Saccharomyces cerevisiae* (strain C) e *Saccharomyces bayanus* (strain B)), was investigated using synthetic media, added or not with ethanol. The concentrations of both the substrates and the products of the sugars conversions, as well as the number of viable cells of yeasts, were determined as a function of the alcoholic fermentation time and the related kinetics constants determined.

If the reaction medium contained high concentrations of both glucose and fructose, the strains showed significant different fermentatory ability. In these conditions a stuck of fermentation occurred and the remaining sugar was only fructose (strain C) or prevailing fructose (strain B).

If the reaction medium contained only glucose as substrate, the strain C seemed more efficient while the kinetics behavior changed completely in presence of only fructose.

On the basis of the information collected using this kinetic approach, it would be possible to develop technical data sheets, specific for each yeast strain, useful to choose the optimal microbial strain as a function of the different operative conditions. Moreover the kinetic constant of hexose conversion could be adopted as bio-markers in selection and breeding of wine yeast strains having a lower tendency for sluggish fructose fermentation.

Keywords: Hexose metabolisation; Stuck of fermentation; *Saccharomyces bayanus; Saccharomyces cerevisiae;* Alcoholic fermentation; Kinetics parameters

Introduction

Several *Saccharomyces* species have been extensively used in wine making, sake making, and brewing processes such as in bioethanol production, despite yeasts are rather sensible to ethanol accumulation in the reaction medium [1]. In fact, in winemaking the number of stuck of fermentations is continuously increasing, particularly in countries characterized by warm climates [2].

As widely reported in literature, a number of stress factors occurring during the process, the lack of micro and macronutrients necessary for yeasts, unsuitable reaction temperatures, too low pH values, the presence of significant concentrations of inhibitors (ethanol, phenols, etc.) in the reaction medium, the development of dangerous microorganisms as well as the alteration of ionic equilibrium can induce a deep modification of the alcoholic fermentation kinetics [1,2].

Over the past two decades, wine producers aimed to produce grapes with increased sugar to total acidity ratios to obtain higher concentrations of phenols and aromatic compounds in order to increase wine quality. As a consequence, the musts obtained by these grapes are more difficult to process because they present unsuitable conditions for yeast reproduction [3,4].

The basis for the decline in fermentation rate is not fully understood. The increase in alcoholic fermentation rate by the addition of selected yeasts strains to the must could result ineffective so the residual sugars were also utilized by contaminating microorganisms able to carry out unwished metabolic pathways. Such an example, in these conditions, some heterofermentative lactic acid bacteria strains could significantly increase volatile acidity inducing a remarkable loss of quality of the alcoholic beverage. Moreover, yeasts lysis, occurring at the end of alcoholic fermentation, determines the solubilisation of cellular contents which can greatly stimulate *Brettanomyces spp.* growth [5].

Although a great number of references, providing a lot of information on the different aspects of alcoholic fermentation, are available in literature, it is still difficult to identify the possible causes of slowing or stuck fermentations even if the change of some compositional parameters (ex: D-glucose/D-fructose ratio, glycerine produced/hexoses converted) or an unusual accumulation of intermediates of sugar catabolism could be assumed as valid signals of a possible deviation from *Saccharomyces* metabolic pathways [6].

The molecular basis of the differential utilization of glucose and fructose, i.e., the glucose/fructose discrepancy in fermentation by *Saccharomyces cerevisiae*, in general, is not known. We have shown previously that different wine yeast strains have strain-specific G/F discrepancies [7,8], and the basis of these differences also is unknown.

In particular, it is interesting to find the reason why alcoholic yeasts preferably metabolise D-glucose rather than D-fructose [2,7-

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11], and investigate the kinetic aspects related to their conversion in order to optimize specific substrate consumption rates. Moreover, this kinetic approach appears to be potentially able to clarify many metabolic aspects related to hexoses conversion with the aim to better control alcoholic fermentation and to avoid that unwished dangerous processes take place.

To reduce the large number of variables able to influence the kinetics of alcoholic fermentation, the time evolution of different initial concentrations of D-glucose and D-fructose, dissolved in a model solution simulating a must (citrate buffer at pH=3.4 inoculated by two commercial strains of *Saccharomyces spp.*), have been investigated both in presence and in absence of ethanol in the initial reaction medium.

Materials and Methods

The experimental runs were carried out at 27.0 ± 1.5°C using a 500 mL batch reactor. To ensure anaerobic and sterilized conditions the whole experimental apparatus was autoclaved and subjected to three cycles of vacuum following by replacement with nitrogen sterilized by filtration. Thus, the presence of undesired microorganisms and the aerobic utilization of sugars by yeasts were ruled out.

The characteristics of this bioreactor, realized at the Department of Agriculture, Food and Environment of the University of Pisa, were already reported in a previous paper [2]. The fermentation temperature was maintained constant by a heat exchanger, whereas the homogeneity of the reaction medium was ensured by a magnetic stirrer. The bioreactor was initially filled with 250 mL of a citrate buffer aqueous solution (pH 3.4) containing D-glucose and/or D-fructose (at five different concentrations: 556, 833, 1111, 1389, and 1667 mmol·L^{-1}) added or not with 1320 or 1450 mmol·L^{-1} of ethanol, respectively, that was sterilized by filtration. To the reaction medium containing only buffer and sugars (and ethanol when added) about 1.6 g (6.4 g·L^{-1}) of one of the two lyophilized yeasts commercial strains utilized (*S. cerevisiae Actiflore BJL souche p1 or S. bayanus Actiflore Bayanus souche BO213*, Laffort Oenologie) were directly added to ensure a number of colony forming units (CFU) ranging from 1010 to 1011 (Table 1). This addition represented the initial time of all kinetic determinations.

The time evolution of both CFU and concentrations of both reagents (D-glucose and/or D-fructose) and their products (glycerine and ethanol) were evaluated by a total plate count or utilizing specific commercial enzymatic kits (Megazyme), respectively [2].

The identification of the best values to be assigned to the model parameters was carried out by the specific statistical program me BURENL [9] able to identify in a space of j-dimensions (where j is equal to the number of model parameters) the minimum value of the F function, which is given by the sum of squares of differences occurring among experimental (Yi, $_{exper.}$) and calculated ($Y_{i,calc.}$) data:

$$F = \sum_{i=1}^{N} \left(Y_{i,\text{calc.}} - Y_{i,\text{exper.}} \right)^2$$

Where N represents the total number of experimental determinations. The values assumed by the model parameters at the minimum of the F function represent the best values.

To evaluate the kinetic constants related to the time evolution of the hexose under investigation (k_H, overall hexoses kinetic constants, k_F, fructose kinetic constants, k_G, glucose constants) the experimental data concerning hexose (D-glucose and or D-fructose) decrease and both ethanol and glycerine accumulations were used.

Results and Discussion

According to literature, the ability shown by *Saccharomyces spp.* to metabolise the hexoses depends on the temperature and the composition of culture media (sugars level, D-glucose to D-fructose ratio as well as ethanol concentration) [10,11]. Since the rate of a substrate conversion depends on the concentration of the microbial population present in the reaction medium (CFU/L), an high concentration of lyophilized yeasts was initially added to the reaction medium so that the great number of microbial cells could ensure a remarkable conversion of sugars in all the different operating conditions adopted (Table 1). The analytical points describing the decrease of concentrations of the two monosaccharides (D-glucose and D-fructose), and the increase of the production of ethanol and glycerol as a function of fermentation time when initial concentrations of 300 gL^{-1} (1666 mmol L^{-1}) were used, are reported in Figure 1 (Figure 1a=*Saccharomyces cerevisiae;* Figure 1b=*Saccharomyces bayanus*). The kinetic evolution of hexoses conversion was described by a first order equation and the following mathematical form introduced, in the plane (t, [H]):

Run	$[H]_{t=0}$	$[N]_{t=0}$	EtOH	$[H]_{t=end\ of\ run}$	$100\cdot[G]_{t=end\ of\ run}$ / $_{[G]t=0}$	$100\cdot[F]_{t=end\ of\ run}$ /$_{[F]t=0}$
	(mmol/L)	(CFU/L)		(mmol/L)		
1C	833.3 G + 833.3 F	3.20*10^{11}	no	F = 52.2	--	6.3%
2C	1111.1 G	1.25*10^{11}	no	--	--	--
3C	1111.1 F	1.10*10^{11}	no	--	--	--
4C	1666.6 G	8.92*10^{10}	no	G = 24.89	1.5%	--
5C	1666.6 F	1.38*10^{11}	no	F = 363.30		19.47%
6C	1111.1 G	5.75*10^{10}	yes	G = 188.0	17.2%	--
7C	1111.1 F	5.80*10^{10}	yes	F = 277.7	--	25.13%
8C	555.5 G + 555.5 F	3.10*10^{11}	yes	G = 10.3; F = 176.4	1.9%	35.0%
1B	833.3 G + 833.3 F	8.30*10^{11}	no	G = 21.5; F = 110.10	2.6%	13.3%
2B	1111.1 G	1.68*10^{11}	no	--	--	--
3B	1111.1 F	9.40*10^{10}	no	--	--	--
4B	1666.6 G	8.89*10^{10}	no	G = 60.56	3.6%	--
5B	1666.6 F	1.10*10^{11}	no	F = 73.28	--	4.4%
6B	1111.1 G	9.70*10^{10}	yes	G = 194.3	18.6%	--
7B	1111.1 F	5.10*10^{10}	yes	F = 289.06	--	27.5%
8B	555.5 G + 555.5 F	5.2*10^{10}	yes	G = 34.67; F = 170.94	6.6%	34.9%

Table 1: Values of initial concentration of hexoses $(H)_{t=0}$, yeasts $(N_{t=0})$, final concentrations of hexoses $(H)_{t=endofrin}$ and percentages of residual sugar for the experimental runs performed.

$$[H]_{t=t} = [H]_{t=0} \cdot e^{-kH}$$

Similarly, two exponential equations were used to evaluate the time evolution of glucose (G) and fructose (F) to determine the kinetic when both these hexoses are present in the reaction medium:

$$[G]_{t=t} = [G]_{t=0} \cdot e^{-kG \cdot t}$$

$$[G]_{t=t} = [G]_{t=0} \cdot e^{-kF \cdot t}$$

A good correlation among the calculated data and the experimental ones, regardless of the initial composition of the medium of reaction (Table 2) has been successfully obtained. Therefore, the comparison of kinetic constants, related to *S. cerevisiae* and *S. bayanus* hexose conversion, has provided useful information on the fermentation efficiency of the strains examined, in the different conditions tested (Figure 2).

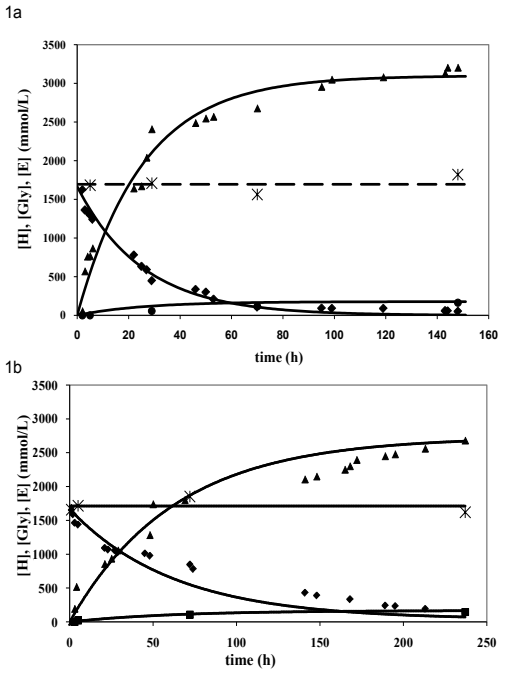

Figure 1: Experimental points (rhombs = hexoses, triangles = ethanol, circles = glycerine, stars= mass balance) and theoretic developments of hexoses conversion as a function of time of fermentation promoted by a *S. cerevisiae* strain (a) or a *S. bayanus* strain (b) in the adopted reaction medium ($[D\text{-glucose}]_{t=0}$=1667 mmol·L^{-1}, $[D\text{-fructose}]_{t=0}$=1667 mmol·L^{-1}).

In the experimental runs characterized by an high concentration both of glucose and fructose (runs 1C, 1B) initially present in the reaction medium, D-fructose conversion became more difficult than D-glucose transformation both for the *S. cerevisiae* strain and for the *S. bayanus* strain, so that D-fructose has not been totally metabolized by yeasts and can be found partially unconverted in the reaction medium (Table 1).

Moreover, when ethanol is initially added at reaction medium (runs 6,7,8 C; 6,7,8 B), a remarkable reduction of values assumed by kinetic constant k_H could be pointed out underling the negative effect induced by this compounds on fermentative activity of both strains of yeasts so that about 20% of the initial amount remained unconverted in the reaction medium (Table 1). In particular, when the only metabolized sugar was represented by fructose (runs 7C, 7B), the percentages of unconverted hexose were the highest (Table 1) while they were nearly the same when in the reaction medium was present only D-glucose or D-glucose and D-fructose together (runs 6C, 8C and 6B, 8B).

According to the stoichiometry of alcoholic fermentation, the sum of the analytical data related to the concentrations of unconverted sugars, accumulated glycerine and half of ethanol formed did not vary significantly with time, assuming values very close to the initial concentration of sugar used (see M= molar balance; Figures 1a,1b and 3a,3b). As a consequence, a possible significant accumulation of intermediates can be ruled out and this relation can be written:

$$[H]_{t=0}\text{-}[H]_{t=t} = [0.5\ E]_{t=t} + [Gly]_{t=t;}$$

Dividing both members of this equation by the amount of substrate converted ($[H]_{t=0}$-$[H]_{t=t}$), the following expression can be obtained:

$$1 = [0.5\ E]_{t=t}/([H]_{t=0}\text{-}[H]_{t=t}) + [Gly]_{t=t}/([H]_{t=0}\text{-}[H]_{t=t}) = R_{E,H} + R_{Gly,H}$$

Where $R_{E,H}$ and $R_{Gly,H}$ represent the fraction of hexose converted in ethanol or glycerine and ($[H]_{t=0}$−$[H]_{t=t}$) is the amount of sugars converted.

On this basis, the time evolutions of ethanol and glycerine

Run	$k_H \pm$ i.c. (h^{-1})	r^2	$k_G \pm$ i.c. (h^{-1})	r^2	$k_F \pm$ i.c. (h^{-1})	r^2
1C	0.039 ± 0.011	0.98	0.057 ± 0.005	0.98	0.033 ± 0.002	0.98
2C	0.033 ± 0.150	0.94	--	--	--	--
3C	0.024 ± 0.021	0.98	--	--	--	--
4C	0.012 ± 0.008	0.96	--	--	--	--
5C	0.009 ± 0.001	0.98	--	--	--	--
6C	0.005 ± 0.002	0.98	--	--	--	--
7C	0.009 ± 0.003	0.98	--	--	--	--
8C	0.005 ± 0.001	0.98	0.011 ± 0.003	0.96	0.003 ± 0.001	0.98
1B	0.016 ± 0.004	0.96	0.019 ± 0.009	0.96	0.012 ± 0.003	0.96
2B	0.017 ± 0.034	0.98	--	--	--	--
3B	0.027 ± 0.021	0.96	--	--	--	--
4B	0.008 ± 0.001	0.96	--	--	--	--
5B	0.012 ± 0.001	0.98	--	--	--	--
6B	0.005 ± 0.001	0.98	--	--	--	--
7B	0.004 ± 0.002	0.98	--	--	--	--
8B	0.005 ± 0.001	0.96	0.007 ± 0.005	0.98	0.004 ± 0.001	0.96

Table 2: Values (mean ± c.i.) related to the kinetic constants of conversion of substrates (k_H) obtained for the different experimental runs performed by two strains (C = *S. cerevisiae*; B= *S. bayanus*) analyzed.

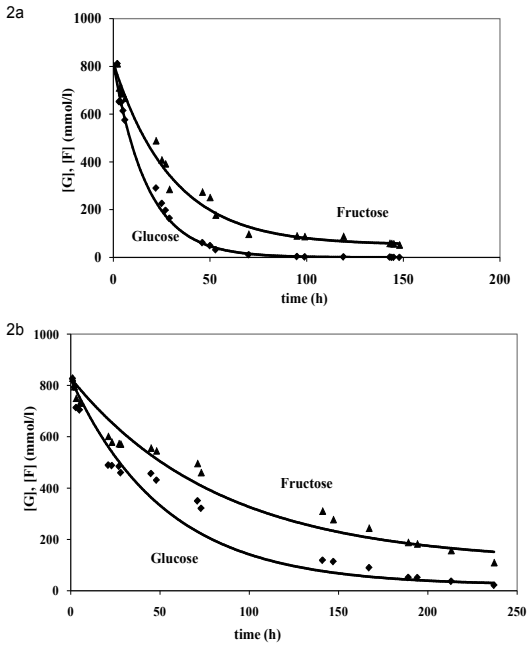

Figure 2: Experimental points (rhombs = glucose. triangles = fructose) and theoretic developments of glucose and fructose conversion as a function of time of fermentation promoted by a *S. cerevisiae* strain (a) or a *S. bayanus* strain (b) in the adopted reaction medium ($[D\text{-glucose}]_{t=0}$=1667 mmol·L^{-1}, $[D\text{-fructose}]_{t=0}$=1667 mmol·L^{-1}).

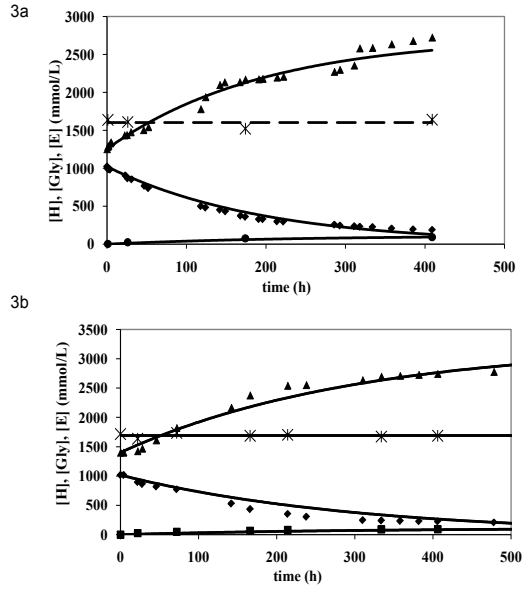

Figure 3: Experimental points (rhombs = hexoses, triangles = ethanol, circles = glycerine, stars= mass balance) and theoretic developments of hexoses conversion as a function of time of fermentation promoted by a *S. cerevisiae* strain (a) or a *S. bayanus* strain (b) in the adopted reaction medium ($[D\text{-glucose}]_{t=0}$ = 1111 mmol L^{-1} plus 255.7 ml L^{-1} ethanol; b) $[D\text{-fructose}]_{t=0}$ = 1111 mmol L^{-1} plus 306.7 ml L^{-1} ethanol.

concentrations can be described as a function of hexoses converted:

$$[E]_{t=t} = 2R_{E,H}\cdot[H]_{t=0}\cdot(1-e^{-kH\cdot t})$$

Runs	$R_{Gly,H}$	$R_{E,H}$
1C ÷ 5C	0.12 ± 0.02	0.92 ± 0.05
6C ÷ 8C	0.10 ± 0.01	0.92 ± 0.05
1B ÷ 5B	0.13 ± 0.05	0.86 ± 0.02
6B ÷ 8B	0.10 ± 0.01	0.88 ± 0.03

Table 3: Mean values and confidence intervals of the fraction of hexoses converted in glycerine ($R_{Gly,H}$) and in ethanol ($R_{E,H}$).

$$[Gly]_{t=t} = R_{Gly,H}\cdot[H]_{t=0}\cdot(1-e^{-kH\cdot t})$$

The mean values of $R_{E,H}$ and $R_{Gly,H}$ assumed similar values for runs carried out both in presence and absence of ethanol in the initial reaction medium (Table 3). This experimental evidence show that the reaction involved in the rate determining step is one of those coming first the hexose cleavage to two triose phosphates regardless the initial concentration of ethanol in the reaction medium. The ratio between the kinetic constant related to time evolution of D-glucose and D-fructose in experimental runs characterized by an high initial concentration of ethanol assumed a similar value (k_G/k_F ~ 3:1) both for *S. cerevisiae* strain than for *S. bayanus* one. This value was lower than that related to the runs carrying out in absence of ethanol in the initial reaction medium (k_G/k_F ~1.6:1), showing that this alcohol induced a sensible reduction of fructose conversion so that a great amount of this sugar can be found unconverted in the reaction medium (> 25%) both using strain C and strain B (Table 1).

Conclusion

The kinetics constants related to hexoses conversion (glucose and fructose) of two yeasts strains have been calculated as a function of the different operating conditions adopted. On the basis of the information collected using this kinetic approach, it would be possible to develop technical data sheets, specific for each yeast strain, useful to choose the optimal microbial strain as a function of the different operative conditions characterizing several biochemical processes (ex: wine making, sake making, brewing processes and bioethanol production). Moreover the kinetic constant of hexose conversion could be adopted as bio-markers in selection and breeding of wine yeast strains having a lower tendency for sluggish fructose fermentation.

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