Fermentation Kinetics of Different Sugars by Apple Wine Yeast *Saccharomyces cerevisiae*

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ABSTRACT

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A non-linear kinetic model to predict the consumption of different sugars (glucose, fructose and sucrose) as a substrate, during an apple wine yeast fermentation with *Saccharomyces cerevisiae* strain CCTCC M201022 is proposed. This model was used to predict sugar utilization by this yeast beginning at various initial sugar concentrations. After observation of the experimental data, a model based on the logistic equation of yeast growth, growth-associated production of ethanol with a lag time, and consumption of sugars for biomass formation and maintenance, was developed. After experimental model fitting, kinetic parameters in the model were estimated. The experimental verification of the model was performed using flask-scale fermentations, and the model obtained predicted the fermentation performance effectively, using different sugars as the substrate set at various initial sugar concentrations. Based on estimated kinetic parameters and the characteristics of sugar utilization, the yeast examined appeared to be glucophilic. The effects of different sugars with various initial concentrations on the fermentation performance by this yeast were investigated, and some applications of kinetic parameters are discussed.

Key words: Apple wine, fermentation kinetics, sugar, yeast.

INTRODUCTION

Apple wine is a fermented beverage made from fresh or concentrated apple juice. It has had a long tradition in Europe and has taken an important place in the global fruit wine industry[11]. It has become the second largest fruit wine industry with an increasing demand in China. Today the use of selected pure cultures of yeast for fermentation of apple wine as starters, and the technological advances in other parts of the fermented beverage industry have influenced the apple wine making process, however the available information is not sufficient yet to permit a full understanding and control of the process[5,11].

Apple juice contains many sugars, including fructose, glucose, sucrose as well as other carbohydrates, in varying concentrations. Unlike mash, the sugar in the highest amount in apple juice is fructose, up to 70% of the total fermentable sugar of 100~150 g/L, plus glucose and sucrose[5,11]. Like wine yeast, the primary function of apple wine yeast (*Saccharomyces cerevisiae* is the major industrial strain) is to catalyze the rapid, efficient and complete conversion of sugars to alcohol without the development of fermentation off-flavors. However, slow and incomplete alcoholic fermentations of juice (i.e., sluggish or stuck fermentations) are a chronic problem for the fruit wine industry[3]. This can lead to unscheduled loss of tank capacity due to extended processing times and the potential for microbial instability and off-taste of the final product due to residual sugars. In these cases, fructose is the major factor, causing a high residual sugar concentration. In spite of the importance of fructose fermentation for apple wine production, few studies have addressed this subject. Furthermore, other fermentable sugars from cane, beet or hydrolyzed corn syrup are commonly used as adjuncts in apple wine production. The changes in sugar content will affect the fermentation process. Hence the need for a practical investigation into the mechanism of sugar uptake and utilization and the kinetic behavior of fermentation by yeast used in this process is significant.

Over the past 20 years, numerous papers concerning sugar uptake and utilization by yeast have been published[1]. It is stated that the rate of alcohol production by yeast is limited primarily by the rate of sugar uptake, especially the uptake of fructose[16,17]. In general, while both glucose and fructose are utilized simultaneously, glucose is utilized faster than fructose by yeast[7,21], and *S. cerevisiae* appears to be glucophilic, although some strains have a clear preference for fructose[18]. An appropriate evaluation on the discrepancy between the amount of glucose and fructose consumed by wine yeast strains during fermentation might be helpful to solve, at least partially, the problems caused by the slower fermentation of fructose[2]. However, few systematic analyses or quantifications of the preference for glucose or fructose of yeast strains for apple wine have been carried out. More information is required to select and evaluate yeasts to improve fermentation performance.

As a useful tool, the primary objective of a kinetic model developed for wine fermentation is the prediction of the kinetic behavior of yeast fermentation performance based on the initial characteristics of the juice. The development of the corresponding mathematical models of fermentation kinetics is also important in the research of yeast behavior and metabolic regulation[19]. An appropriate model of fermentation, with the technical, economic and physiological implications would be a powerful instrument to predict and control problem fermentations, and be

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helpful to understand the fermentation process[13]. Several physical and mathematical models for wine fermentation have been reviewed, and many factors leading to problem fermentations, such as nitrogen and oxygen limitation, temperature extremes etc., have been well documented by kinetic methods[4,6,8,10,13]. However, the effects of different sugars, fructose, glucose and sucrose, which are the main sugars found in apple and grape juice, on the kinetic performance of yeast fermentation in apple juice are not clear.

Therefore the objective of this work was to propose an unstructured model for apple wine yeast fermentation kinetics using different sugars as a substrate, in which it is assumed that the microorganism can be represented by one state variable, and the consideration of the incorporation of complicated biochemical knowledge concerning the different regulation mechanisms of the cell is not required. Then the fermentation performance in different initial sugar levels by the apple wine yeast is predicted. Based on kinetic parameters obtained, utilization of different sugar by this yeast were compared, and the yeast kinetic characteristics discussed.

MATERIALS AND METHODS

Yeast strain

One yeast strain, *Saccharomyces cerevisiae* strain CCTCC M201022 (China Center for Type Culture Collection) for apple wine fermentation, selected by this laboratory[22], was used for the experiments.

Medium

In order to investigate the effect of a single sugar on yeast fermentation, a synthetic medium was prepared to simulate an apple juice concentrate, according to the composition of an ‘ideal’ apple juice used for apple wine[5,11]. It contained 20~230 g/L single sugar (glucose, fructose or sucrose, respectively), 1.7 g/L yeast extract, 1.7 g/L $(NH_4)_2SO_4$, 0.9 g/L quinate, 3.0 g/L malic acid, 1.0 g/L lactic acid, and 10 mg/L vitamin D_2 and B_1. The pH was adjusted to 3.5. Titratable acidity to 8.1 as malic acid was 3.95 g/L.

Fermentation conditions

Yeast cells were cultured at 25°C in a pasteurized medium for 24 h, and then a 2% (w/v) inoculum was used to inoculate one litre of medium in a 2 litre Erlenmeyer flask, equipped with fermentation air locks. Fermentation was carried out at 15°C and allowed to continue to complete attenuation. Samples were taken periodically during fermentation for analysis of cell weight, as well as sugar and alcohol concentration. All samples were analyzed in triplicate. The initial sugar concentration was ~100 g/L, and specific details are indicated in the text or figures legends.

Analytical techniques

Biomass concentration was calculated from the absorbance measured at 600 nm and a calibration was carried out in which the absorbance and dry weight of yeast were correlated[8]. All samples were taken after suspending the yeast and washing sufficiently. The yeast was resuspended in 0.05% EDTA-Na solution to retard flocculation when measuring biomass. Dilutions were made as necessary to obtain an optical density between 0.2 and 0.8. Sugars and ethanol were determined according to Ough and Amerine[15].

Model simulation

Model simulations and data fitting were performed using the SAS software System for Windows version 8.01 (SAS Institute, Cary, NC). Model parameters are described in the text.

Model development

Kinetic model. Among the numerous models developed, the majority of the models are biochemically knowledge based models[13], which consist of a set of mathematical equations describing the phenomena occurring during wine fermentation. The main advantage of this type of model is that they account for biological phenomena. The model parameters with some biological significance can be obtained, but their structures may be strongly nonlinear, complex and difficult to verify and validate, and can pose problems in terms of parameter identification. In the development of non-linear modeling techniques, there has been an increase in the use of sigmoidal shaped growth models in the predictive microbiology field to forecast fermentation process. The details of non-linear modeling have been described by Speers et al.[20]

In general, the fermentation kinetic model can be subdivided into a growth model, a substrate model, and a product model. There are three different equations derived to describe the kinetic behavior of the concentration of yeast cells, the sugars (glucose, fructose or sucrose, respectively), and ethanol in this study.

Among the many models describing the growth kinetics of microorganisms (e.g. Monod growth model), the model structures were chosen. The classical semi-empirical Monod type models cannot fit processes of fermentation well in many cases, although there are many modified types. Recently the logistic model, as a sigmoidal shaped model, has been a most popular one due to its “goodness of fit”[20] and has been widely used in describing the growth of microorganism[9,13]. Usually, the logistic model was used to show the self-regression made by the increase of cell concentration common in batch-fermentation. In this paper, we develop an equation based on the logistic model with growth-associated production of ethanol.

For cell concentration, X, the logistic model was derived as follows:

$$\frac{dX}{dt} = \mu_m X \left(1 - \frac{X}{X_m}\right), \qquad (1)$$

where μ_m is the maximum specific growth rate with respect to the fermentation conditions, as the form of the Monod relationship. With the following boundary conditions:

$$t = 0, \quad \therefore X = X_0, S = S_0, P = 0$$

By integration of equation 1, the kinetic model can be formulated. The biomass production rate yields the following equation (the logistic equation):

$$X = \frac{X_0 X_{\mathrm{m}} e^{\mu_{\mathrm{m}} t}}{X_{\mathrm{m}} - X_0 + X_0 e^{\mu_{\mathrm{m}} t}}, \qquad (2)$$

This equation shows the relationship of biomass and the fermentation time, which is used to fit the experimental data of biomass concentration. There are two parameters, μ_{m} and X_{m}, in this equation, and they are estimated from the experimental data by the mathematical software, SAS System.

As observed in the present experiment (Fig. 1), ethanol concentration increased proportionally to increasing biomass during the fermentation process; especially in the exponential phase of cell growth, and only a very small proportion of ethanol production was present in the stationary growth phase. A significant relationship was found in our data between the specific ethanol production rate, $dP/(Xdt)$, and the specific growth rate, $\mu\,[dX/(Xdt)]$, which was expected with growth-associated product formation. Hence ethanol production in this model is viewed as a growth-associated relation with biomass[12], and inclusion of a non-growth-associated term in this model was not justified in this case. However, a delay of ethanol production was found compared with the cell growth, and little ethanol was produced during the yeast lag growth phase. Therefore, a parameter of the lag time, Δt, was introduced to describe the delay of ethanol production to cell growth, and the equation of ethanol production rate was modified as Eq. (3),

$$\frac{dP}{dt} = Y_{p/x} \frac{dX}{d(t - \Delta t)}, \qquad (3)$$

This equation can be integrated using two estimated parameters from Eq. (2), μ_{m} and X_{m}, and the model is described by the Eq. (4). After the experimental data of ethanol production was fitted, two parameters in this equation, the yield coefficient $Y_{p/x}$ and Δt, were estimated.

$$P = Y_{p/x}\left[\frac{X_0 X_{\mathrm{m}} e^{\mu_{\mathrm{m}}(t-\Delta t)}}{X_{\mathrm{m}} - X_0 + X_0 e^{\mu_{\mathrm{m}}(t-\Delta t)}} - \frac{X_0 X_{\mathrm{m}} e^{-\mu_{\mathrm{m}}\Delta t}}{X_{\mathrm{m}} - X_0 + X_0 e^{-\mu_{\mathrm{m}}\Delta t}}\right], \qquad (4)$$

For the alcoholic fermentation process, the equation describing the substrate consumption rate takes into account two aspects, the sugar consumption in the formation of biomass and the maintenance of biomass[6,13]. The consumption rate of sugar was described as below:

$$-\frac{dS}{dt} = \frac{1}{Y_{x/s}} \cdot \frac{dX}{dt} + m \cdot X, \qquad (5)$$

Combined with the Eq. (1), Eq. (3) and estimated parameters, this equation can be integrated and the sugar consumption equation can be obtained as Eq. (6). The related parameters were estimated.

$$S = S_0 - \frac{1}{Y_{x/s}}\left[\frac{X_0 X_{\mathrm{m}} e^{\mu_{\mathrm{m}} t}}{X_{\mathrm{m}} - X_0 + X_0 e^{\mu_{\mathrm{m}} t}} - X_0\right] - \frac{X_{\mathrm{m}} m}{\mu_{\mathrm{m}}} \ln \frac{X_{\mathrm{m}} - X_0 + X_0 e^{\mu_{\mathrm{m}} t}}{X_{\mathrm{m}}} \qquad (6)$$

Parameters estimation. The initial values of X_0 and S_0 were fixed by the experimental conditions. The other parameters, such as μ_{m}, X_{m}, Δt, m and some yield coefficients were estimated by the Newton nonlinear regression method of SAS 8.01 system using batch experimental data.

In the SAS program, 5 iterative search techniques, including Newton nonlinear regression method, Newton-Gauss, Marguardt, Gradient and Dud technique, were used to minimize the residual sum of squares. All the tech-

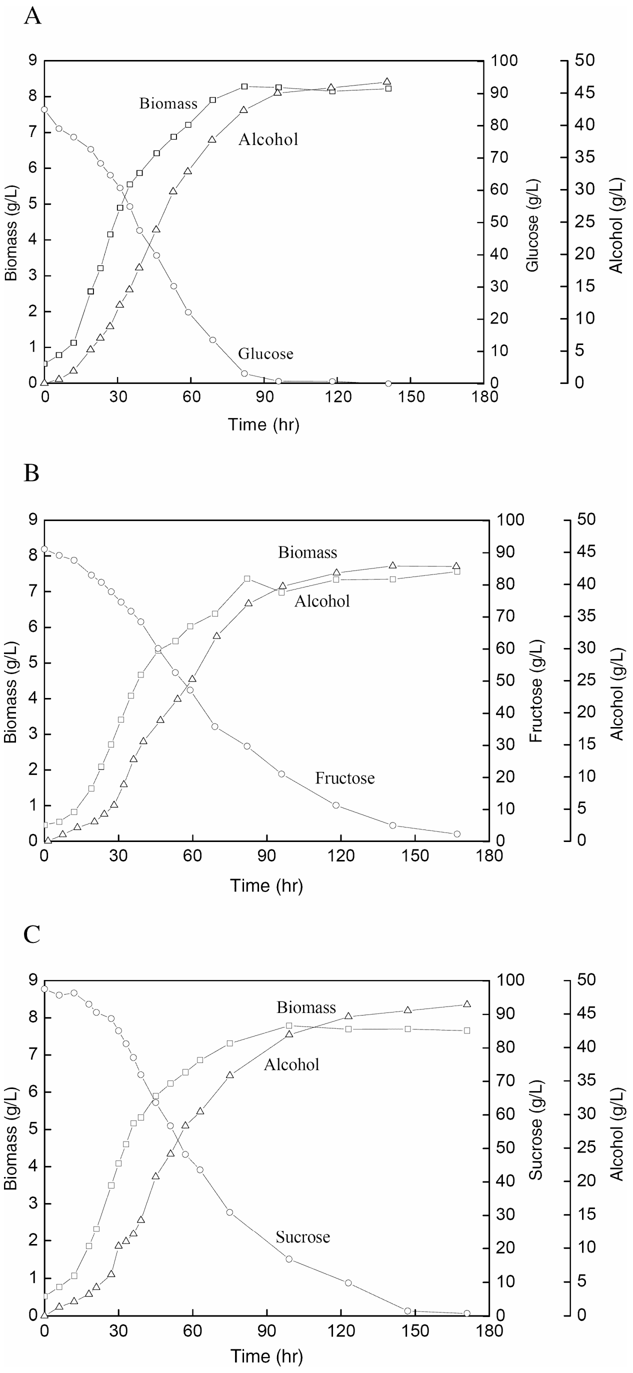

Fig. 1. The fermentation profiles of apple wine yeast using (A) glucose with a determined initial concentration of 85.0 g/L, (B) fructose of 91.1 g/L, and (C) sucrose of 96.6 g/L as a substrate, respectively.

Table I. Kinetic parameters estimated from the experimental data on different sugars.

	Glucose	Fructose	Sucrose
μ_m (h^{-1})	0.0945 (0.0023)	0.0800 (0.0019)	0.0887 (0.0017)
X_m (g/L)	8.0366 (0.1164)	7.2270 (0.1101)	7.5215 (0.0933)
$Y_{p/x}$ (g/g)	5.5709 (0.1015)	5.7878 (0.1082)	5.6637 (0.1238)
Δt (h)	15.30 (1.02)	14.59 (1.23)	17.43 (1.21)
$Y_{x/s}$ (g/g)	0.2200 (0.0122)	0.1922 (0.0089)	0.2061 (0.0212)
m (h^{-1})	0.1144 (0.0069)	0.0715 (0.0038)	0.0789 (0.0064)

N.B. Values in brackets denote the approximal standard errors of the estimated parameters using the Newton nonlinear regression method.

niques gave similar results, however the Newton nonlinear regression method tended to give slight better fits as evidenced by examination of the residual sum of squares and F-value.

RESULTS AND DISCUSSION

The fermentation profiles of apple wine yeast on the different sugars are shown in Fig. 1. There were some differences in fermentation performance of this yeast, in cell growth rate, ethanol production rate, and sugar consumption rate, using glucose, fructose or sucrose as sole sugars, respectively. Based on the experimental data of the different sugars, some kinetic parameters, including yeast maximum specific growth rate (μ_m) and maximum biomass concentration (X_m), the yield coefficient ($Y_{p/x}$) and lag time (Δt), $Y_{x/s}$ and maintenance coefficient (m), were estimated by mathematical software with Eq. (2), Eq. (4) and Eq. (6), respectively. The estimated values of the parameters are given in Table I. The estimated values of μ_m agreed with those generally exhibited by yeasts under the present conditions[6,10]. By the method of fermentation kinetics, the effects of the different sugars are described in detail.

Yeast growth kinetics on different sugars

Biomass concentrations on different sugars were fitted by Eq. (2) in Fig. 2, using the estimated parameters. As can be observed from the figure, the model fits well to the experimental results, with high significance for model fitting (all the Pr < 0.0001), and the Correlation Coefficients, r^2, of model fitting are 0.991 for glucose, 0.991 for fructose, and 0.993 for sucrose as a substrate, respectively. In other words, the model is able to predict the growth of yeast in these cases.

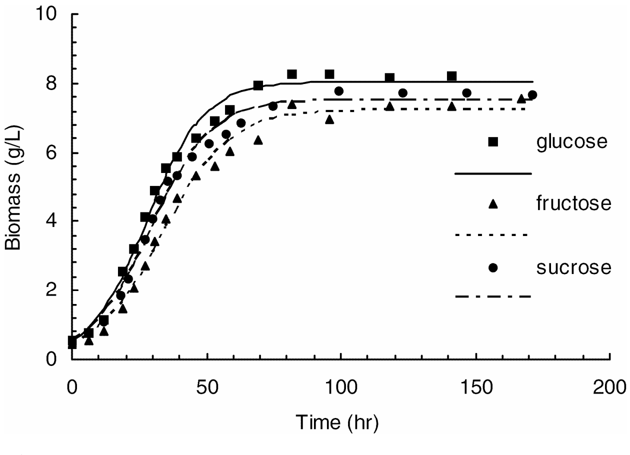

Fig. 2. Experimental data and kinetic model predictions for cell growth, using glucose, fructose or sucrose as the substrate, respectively.

When using glucose as the sole sugar in the medium, the yeast maximum specific growth rate (μ_m) and maximum biomass concentration (X_m) were highest compared with those of other sugars, 0.097 h^{-1} and 8.015 g/L, respectively. On the contrary, the lowest μ_m and X_m were obtained when using fructose as the sole carbon source. Judging from the estimated parameters, it is suggested that with this yeast strain, glucose is more suitable for the production of biomass and this yeast appears to be glucophilic.

Ethanol production kinetics on different sugars

With estimated values of $Y_{p/x}$ and Δt (given in Table I), Eq. (4) was used to fit ethanol production on the different sugars. A high significance for model fitting was verified (all the Pr < 0.0001), and r^2 are 0.990 for glucose, 0.989 for fructose, and 0.985 for sucrose as a substrate, respectively. Experimental data and those predicted by the kinetic model for ethanol production are shown in Fig. 3. There was little difference between the experimental data and the prediction results, and data predictions obtained from the model were reasonable and in some cases very accurate. Hence, the consideration of yeast growth-associated product formation and the introduction of the lag time parameter of ethanol production to cell growth, Δt, were reasonable in the model development.

According to the yield coefficient of $Y_{p/x}$, it appeared that fructose and sucrose were more beneficial to ethanol production given the same amount of yeast, with the higher values of $Y_{p/x}$, although they were not as suitable for yeast growth as glucose. Furthermore, the lag times of ethanol production, Δt were similar when fructose and glucose were used as the substrate respectively, but fruc-

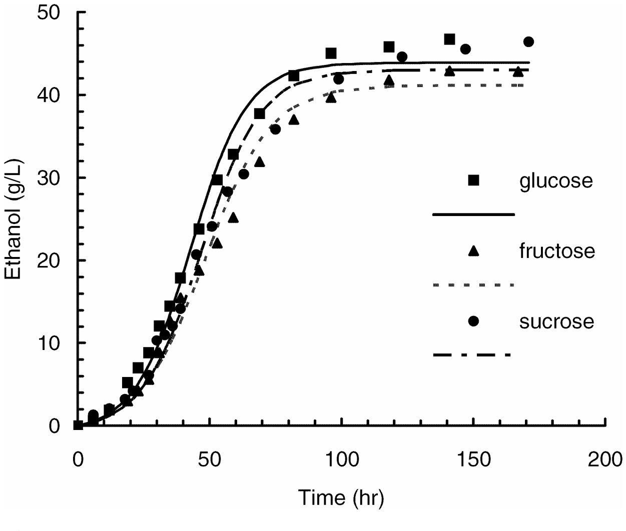

Fig. 3. Experimental data and kinetic model predictions for ethanol production, using glucose, fructose or sucrose as the substrate, respectively.

tose was consumed more slowly for ethanol production than glucose due to slow growth of yeast on fructose. Sucrose was hydrolyzed extracellularly by the action of *S. cerevisiae* invertase producing equimolar glucose and fructose initially[1,14], which was then utilized by the yeast. Therefore, the longest lag time of ethanol production observed on sucrose was understandable in this case. Ethanol in the medium results from the formation of ethanol from sugar metabolism and its excretion into the medium. The parameter of lag time, Δt, reflects this process and indicates fast or slow ethanol production relative to yeast growth.

Substrate consumption kinetics on different sugars

The predicted evolutions of the different sugars by Eq. (6) during the fermentation processes are shown in Fig. 4, together with the experimental data. This figure indicates that the prediction of the model agreed well with the experimental data in all cases. The model was verified with high significance (all the $Pr < 0.001$), and r^2 of model fitting were 0.996 for glucose, 0.994 for fructose, and 0.976 for sucrose as a substrate, respectively.

Clearly, the consumption of glucose was faster than the other two sugars. This phenomenon can be explained by kinetic parameters $Y_{x/s}$ and m. Glucose was more beneficial to biomass production with higher $Y_{x/s}$. Simultaneously, the yeast more readily metabolized glucose, which was reflected by the higher maintenance coefficient (m) on glucose. Due to hydrolysis of sucrose to produce equimolar glucose and fructose, sucrose can be regarded as a mixture of glucose and fructose, and the utilization of sucrose was intermediate between those of glucose and fructose. While fructose was utilized slowly, the utilization of sucrose was more similar to that of fructose, with lower values of $Y_{x/s}$ and m.

Combined with the obtained parameters of μ_m and X_m, it was further suggested by parameters $Y_{x/s}$ and m that the yeast was glucophilic. Although a few yeast strains have a clear preference for fructose[18], most glucophilic fruit wine yeasts utilize fructose more slowly than glucose. It is now generally accepted that the process of sugar uptake represents the major control mechanism for the rate of glycolytic flux under anaerobic conditions[16], and the rate of sugar uptake by yeast is a consequence of the inherent kinetics of the transport process and substrate inhibition (including competitive inhibition of fructose and glucose

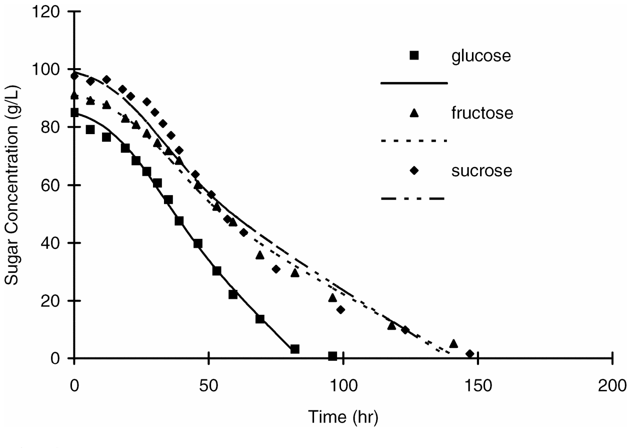

Fig. 4. Experimental data and kinetic model predictions for sugar consumption, using glucose, fructose or sucrose as the substrate, respectively.

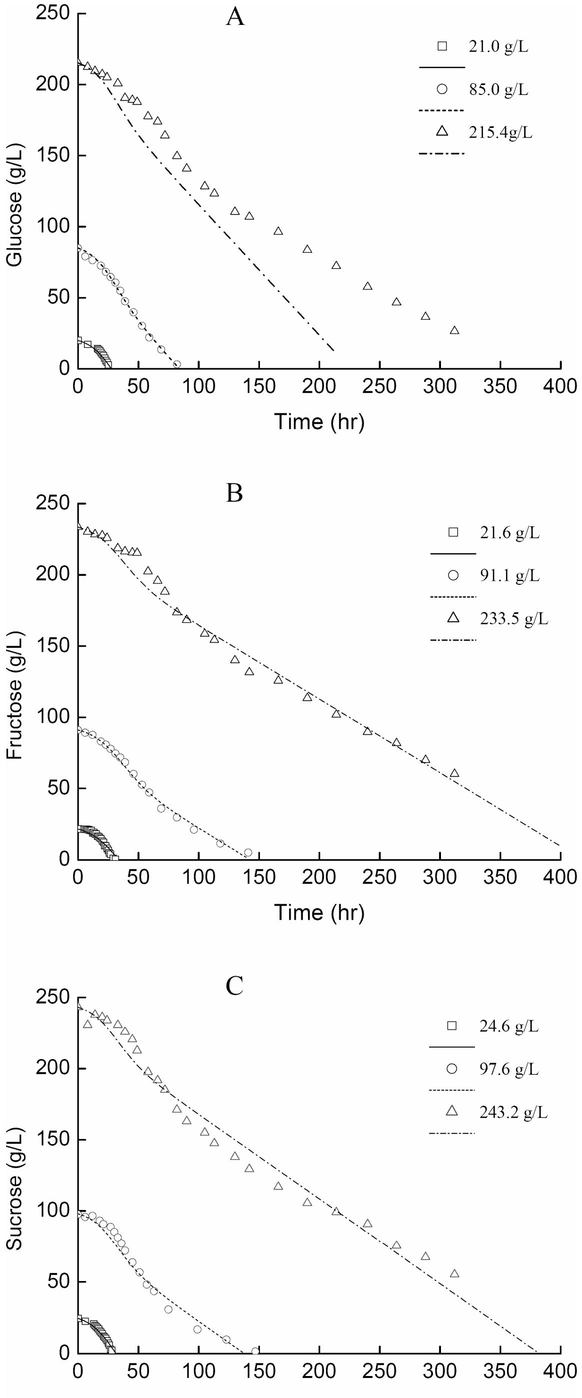

Fig. 5. Model predictions for substrate consumption with various initial sugar levels, using (A) glucose as a substrate with determined initial concentrations of 21.0 g/L, 85.0 g/L, and 215.4 g/L, respectively, (B) fructose of 21.6 g/L, 91.1 g/L, and 233.5 g/L, respectively, and (C) sucrose of 24.6 g/L, 97.6 g/L, and 243.2 g/L, respectively.

in the juice)[3]. Berthels et al.[2] indicated that the cause of the glucose/fructose discrepancy appears to be located in the transport and/or phosphorylation steps of the fermentation pathway, and suggested that determination of the glucose/fructose discrepancy of candidate wine yeast strains should be a standard procedure in strain evaluation and selection.

In the experiments reported in this paper, the focus was on the utilization of single sugars, which eliminated the effect of the competitive inhibition of fructose and glucose. The results suggested that the slow utilization of fructose was due to the nature of this yeast, which was independent as to whether glucose was contained in medium or not. Due to the high fructose content in apple juice, the evaluation and selection of a fructophilic yeast strain, with a high-level expression of hexokinase I (displaying a faster reaction rate in media containing fructose) and some members of the HXT transporter family with a higher affinity towards fructose[3], could be significant to the wine industry. The model developed here and kinetic parameters are potential tools for strain evaluation and selection in this aspect of yeast fermentation performance. Until now, this model has not been used in industrial apple wine fermentation, and further research, such as experiments with different sugar fermentations simultaneously, with varying sugar concentrations, need to be performed.

Model predictions for substrate consumptions with various initial sugar levels

To verify the prediction capability of this model, experiments of sugar consumption with various initial sugar levels were carried out. Predicted curves of sugar consumption, with the experimental data, and the details of various initial sugar levels are shown in Fig. 5. The predictions for sugar consumption by the model are illustrated in this figure, except for the higher initial glucose concentration of 215.4 g/L. When glucose with higher initial concentration was utilized, a discrepancy between experimental data and predicted curves may result due to the inhibitory effect of glucose, which became more clear as the glucose concentration increased[3,17]. As a result, the utilization rate of glucose declined and did not agree with the model. In this case, the model predicted that glucose would be utilized completely in 225 hours, while it took ~370 hours. However, this inhibition was not observed with high concentrations of fructose or sucrose. According to the prediction of the model, high concentrations of fructose (233.5 g/L) or sucrose (243.2 g/L) were consumed completely in ~420 hours and 380 hours, respectively.

These results suggest that the glucose concentration should not be too high in the fermentation medium (apple juice) if the desired fermentation efficiency of the sugar is to be reached, since inhibition of glucose can occur. Furthermore, when fermentable sugars are used as adjuncts in some cases of apple wine production, it is suggested that high levels of glucose could yield this inhibitory effect. Fructose has the caution that most fruit wine yeasts applied in the apple wine industry are glucophilic. Sucrose may be a compromise, if high concentrations of adjunct sugar are required.

CONCLUSIONS

A non-linear kinetic model for different sugar fermentations by one industrial *Saccharomyces cerevisiae* apple wine yeast is proposed, based on the logistic equation of yeast growth, growth-associated production of ethanol with a lag time, and consumption of sugars for biomass formation and maintenance. Some kinetic parameters with physiological significance in the model were estimated by mathematical software. The experimental verification of the model was performed using flask-scale fermentations. The results obtained indicated that the model could predict fermentation performance using different sugars as the substrate with various initial sugar concentrations. Moreover, some kinetic parameters obtained, such as $\mu_{\rm m}$, $X_{\rm m}$, $Y_{x/s}$, m, could be used as indicators for selection or evaluation of potential glucophilic or fructophilic yeast strains. The predictive capability of this model has potential as a useful tool for determining how to address apple wine fermentation issues.

ABBREVIATIONS

X	biomass concentration (g L^{-1})
$X_{\rm m}$	maximum biomass concentration (g L^{-1})
X_0	initial biomass concentration (g L^{-1})
m_s	maintenance coefficient (g sugar/g biomass h)
t	time (h)
P	produced ethanol concentration (g L^{-1})
S	fermentable sugar concentration (g L^{-1})
S_0	initial fermentable sugar concentration (g L^{-1})
$Y_{p/x}$	yield coefficient of ethanol on biomass (g ethanol/g biomass)
$Y_{p/s}$	yield coefficient of ethanol on sugar (g ethanol/g sugar)
$Y_{x/s}$	yield coefficient of biomass on sugar (g biomass/g sugar)
μ	specific growth rate (h^{-1})
$\mu_{\rm m}$	maximum specific growth rate (h^{-1})

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