Utilization of Mixed Sugars for Alcoholic Fermentation by *Saccharomyces cerevisiae*

Oranut Phowchinda
Department of Agro-Industrial Technology, Faculty of Applied Science,
King Mongkut's Institute of Technology North Bangkok.
Bangkok 10800, Thailand.

Pierre Strehaiano
ENSIGC-INPT, Laboratoire de Genie Chimique, UMR - CNRS 5503,
18 Chemin de la Loge, 31078 Toulouse Cedex 4, France.

Abstract

This work presents the utilization of mixed sugars for alcoholic fermentation by *Saccharomyces cerevisiae*. The composition of fermentable sugar in sweet sorghum and its ratio were studied. Fermentation media composed of single and mixed sugars (saccharose, glucose, fructose) with the ratio similar to sweet sorghum juice were used in the experiments. The results showed behaviour of *S. cerevisiae* on different sugars, kinetics of fermentation process and effects of sugar concentration on cell growth. Some problem of natural materials used in alcohol production was concluded.

Key words : Alcoholic fermentation, Sweet sorghum, Mixed sugars, *Saccharomyces cerevisiae.*

1. Introduction

Many countries are involved in research programs on ethanol production for energy (USA, Brazil, Germany, Italy, France.....). For this purpose, different vegetable materials are able to be used; the sole condition being that the raw materials are rich in single sugar or polymer which is easy to hydrolyze. Table 1 summarizes the different plants used: the precise plant, the kind of sugar, the need for any pretreatment, the nature of microorganism to be used and the expected yield.

Table 1 Comparison of different plants. ADECARBA [1]

Kind of plant	Kind of sugar	Hydrolysis	Microorganism	Productivity m3ethanol/ha.year
Sugar beet	Saccharose	no	*Saccharomyces*	4.1-5
Wheat	Starch	yes	*Saccharomyces*	1.75-2
Maize	Starch	yes	*Saccharomyces*	2-2.5
Potato	Starch	yes	*Saccharomyces*	3-5
Jerusalem Artichoke	Inulin	no yes	*Kluyveromyces* *Saccharomyces*	4.7
Sugar cane	Saccharose	no	*Saccharomyces*	5.3
Sweet sorghum	Saccharose	no	*Saccharomyces*	7

In Thailand, two plants are of interest: sugar cane and sweet sorghum. Sugar cane is already widely cultivated also the problems of cultivation and alcoholic fermentation from juices have been solved. But sweet sorghum is less known.

Table 2 Sugar composition of sorghum stem *(Sorghum bicolor)*.

Variety	Sugar content	Saccharose	Glucose	Fructose	References
Keller	12-20%	-	-	-	Rein B.K., Schulte D.D., Ogden R.L. and Walker C.E. [2]
Keller	35% (dry matter)	55%	24%	21%	Curt M.D., Martinez M. and Fernandez J. [3]
Rio	20%	37.5%	27.6%	34.8%	Eckhoff S.R., Bender D.A., Okos M.R. and Peart R.M. [4]
Wray	14.4%	86%	← ---------14%--------- →		Carlson K.D., Cunningham R.L. and Herman A.I. [5]
Wray	11.1%	-	-	-	Eiland B.R., Clayton J.E. and Bryan W.L. [6]
Wray	43%(dry mater)	80% 50%	←----------20%-------- → (harvest) ← ----------50% ------- → (48 h post harvest)		Hansen R.W. and Rerraris R. [7]
no indication	14%	55%	29%	16%	Samuel W.A. and Lee Y.Y. [8]
no indication	11-15%	60%	33%	7%	Mohite U. and SivaRaman H. [9]
no indication	33%	53%	28%	19%	EL Bassam N. [10]
no indication	44%	56.8%	29.5%	13.6%	Sonnenberg H and Dernedde W. [11]

The possibility of its cultivation in tropical conditions (soil and climate) was recently studied by Soontornchainaksaeng [12]. Our work deals with the problem of the alcoholic fermentation of its juices. The sugar composition of its stems and extracted juices varies a lot depending on the variety and the maturity as shown in table 2. Moreover, the problems of post-harvest conservation must be considered as discussed by Hansen and Ferraris [7] and Eiland et al [6].

In conclusion, the main point is that sugar solution extracted from stems is very heterogenous as far as its sugar composition is concerned: glucose, fructose and saccharose with different ratios and concentrations. So it was assumed that the alcoholic fermentation of such juices by yeasts may be difficult.

Indeed, in *Saccharomyces cerevisiae,* these different sugars do not penetrate into the Substrates enter the cell by three different mechanisms which are simple diffusion, facilitate diffusion and active transport. The two last ways use enzymes called permeases. For *Saccharomyces cerevisiae,* the carriers used may be constitutive or inductible. According to Eddy [13], it is known that the glucose carrier is constitutive while the galactose carrier is inductive. Busturia and Lagunas [14] showed that glucose penetration by facilitated diffusion used two different carriers, one of high affinity, the other of low affinity. Fructose crosses over the membrane using the same carriers as the glucose but their affinity is lower. Kinetic studies of Bisson and Fraenkel [15] and Bisson [16] established that the low affinity system is constitutive; it is very efficient during the growth phase and less efficient during the stationary phase. The high affinity system is repressed by high glucose concentration. However, the role of each of these carriers does not seem clear and for Ramos et al. [17], these two carriers are interconvertive depending on the metabolic conditions of the cell.

As far as the disaccharides are concerned, it has been shown that maltose uses an inductible carrier [18] and is hydrolyzed in the cell;conversly, saccharose is hydrolyzed outside the cell. From a kinetic point of view, it is usually accepted that the sugars are used sequentially when mixed in the medium: glucose, fructose, saccharose and maltose (the glucose may inhibit the permease allowing the transport of maltose).

So, the aim of this work was to investigate the consumption of different sugars by *Saccharomyces cerevisiae* under two different conditions: when they are in pure solution and when they are mixed. This may provide some information about the behaviour of sweet sorghum juice fermentation for ethanol production.

2. Materials and methods

2.1 Microorganism: the used yeast strain was *Saccharomyces cerevisiae* K1 produced by Lallemand Inc. (Canada).

cell by using the same transport mechanism.

2.2 Culture media: the synthetic medium was made of KH_2PO_4 : 5g/L, $(NH_4)_2$ SO_4 : 2 g/L, Mg SO_4. $7H_2O$:0.4 g/L, Yeast extract : 1 g/L.
The different sugars tested in pure solutions were: glucose, fructose and saccharose at a concentration of 50 g/L. When mixed, different percentages and concentrations were tested as indicated below. The initial pH was adjusted to 4 by using orthophosphoric acid.

2.3 Culture conditions: the experiments were performed in a 2 liters fermentor (Setric Genie Industriel). Temperature was 30°C. No aeration was provided for the cultures. Inocula were incubated on 150 mL of the medium at 30°C, 15 h., and added to reach 3x 10^6 cells/mL.

2.4 Analytical determinations:
Biomass was measured turbidimetrically at 620 nm, and was calibrated to cell dry weight determination.

For sugar determination, two methods were used. The anthrone method [19] was used for the total sugar measurement. For the specific determination of the different sugars when mixed, the HPLC method was used (spectra Physics apparatus, with an Aminex HPX 87 C column and Interaction GC 801 precolumn, water as eluent).

Ethanol was analysed by gas chromatography (Chrompack 437 A) with a Poraplot Q column, propanol-1 (1%) as internal standard and a flame ionization detector.

3. Results and discussion

3.1 Behaviour of *Saccharomyces cerevisiae* K1 on glucose (50 g/L)

As glucose is the most often used substrate, a preliminary study was made using this sugar. The initial level was 50 g/L. The kinetics of sugar consumption, growth and ethanol production are shown in figure (1).It is clear that there is not a problem concerning the sugar consumption. It is also possible to analyze the relation between ethanol production

(v_p = 1/X .dp/ dt) and growth (μ = 1/X . dX /dt), as illustrated by figure (2). Calculating the values of the parameters of the Luedeking and case, the activities of ethanol production and growth are quite linked with: v_p = 4.75 μ + 0.4. This low value (0.4) of the parameter shows that the part of production due to stationary phase cells is negligible.

Piret's equation we are able to demonstrate that, in this

3.2 Effect of the kind of sugar used

The three different sugars constituting the juice of sweet sorghum (glucose, fructose, saccharose) were tested in pure solutions. In all cases, the initial sugar content was 50 g/L. Figure (3) illustrates the behaviour of the sugar concentration during the fermentation

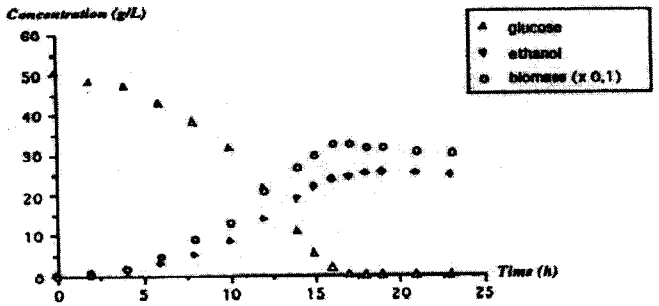

Figure 1 : Glucose, Ethanol and Biomass concentrations versus time for *Saccharomyces cerevisiae* K1.

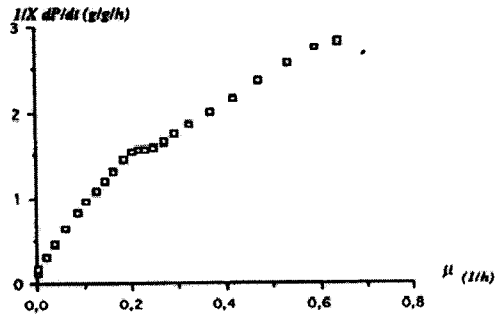

Figure 2 : Relation between the specific growth rate and the specific production rate for *Saccharomyces cerevisiae* K1.

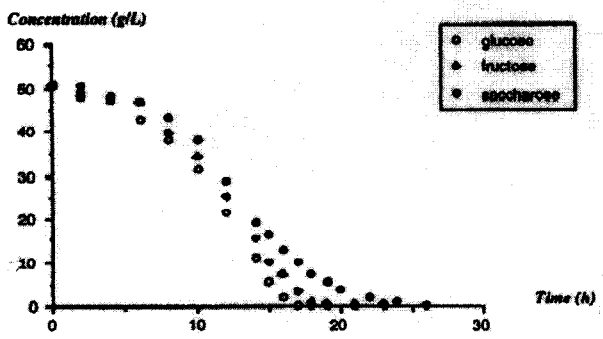

Figure 3 : Consumption of different sugars by *Saccharomyces cerevisiae* K1.

It appears that glucose and fructose have a similar course. The slight difference observed is that the fructose consumption is slower than the glucose consumption. This is in agreement with an observation of Moll [20] and Campagno et al. [21].

Conversly the saccharose course is different. Its consumption rate is weaker, especially at the end of the fermentation (after 17 hours). It allows us to think that the hydrolysis may act as a limiting step. Also, in order to analyse more precisely the behaviour of this sugar, other experiments were performed using the HPLC method to obtain the ratios of glucose, fructose (issued from the hydrolysis of the saccharose) and non hydrolyzed saccharose. The same initial concentration of sugar was used. Also, to avoid the hydrolysis due to the sterilisation, the medium in this case was treated by sterile filtration. The results are shown in figure (4)

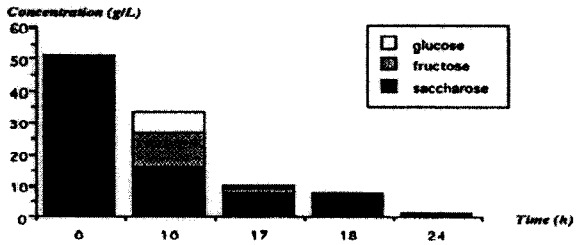

Figure 4 : Ratio of different sugars during growth on saccharose (50 g/L)

It is obvious that the hydrolysis of saccharose takes place very early; we also observe that the glucose level is lower than the fructose level, that confirms the faster use of glucose already shown in figure 3. (The hydrolysis of saccharose leads to equal quantities of glucose and fructose). It is also interesting to note that the glucose is the likely inhibitor of the hydrolysis activity of *Saccharomyces cerevisiae;* in fact, saccharose is hydrolyzed faster when the glucose is exhausted. Finally, by the end of the fermentation (after 15 hours), it seems that a stationary phase is reached: the sugar consumption rate equals the hydrolysis rate. During this phase, no glucose or fructose accumulation is noticed.

As far as the hydrolysis rate is concerned, we can distinguish two phases: during the first phase (in this case 0 to 15 hours) the hydrolysis is fast: 3.5 g/L/h. During the second phase, this rate decreases to an average value of 2.1 g/L/h. Besides, during this phase, the hydrolysis appears to be the limiting step of the reaction. Fontana et al. [22] did the same observation and explained it by a catabolic repression of the invertase (enzyme which catalyses the hydrolysis of saccharose into glucose and fructose) by the glucose acting at a very low level. It is also interesting to note that Essia-Ngang et al. [23] noticed a bad utilization of the saccharose at the end of the fermentation but they did not have any explanation why.

3.3 Fermentation on mixed sugars

After this analysis of the sugar consumption on pure sugar media it was necessary to study the behaviour of *Saccharomyces cerevisiae* during the fermentation of a complex medium containing the three different sugars and simulating the composition of sweet sorghum juice.

Different experiments were performed, using different ratios and different total amounts of sugars. Some of the results are plotted on figures 5, 6 and 7.

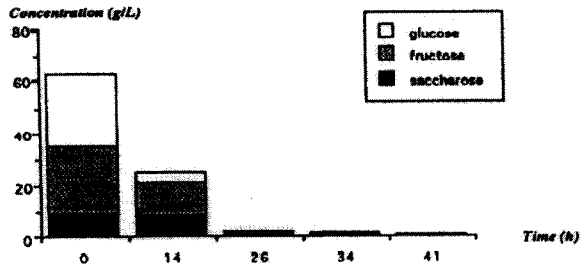

Figure 5 : Ratio of different sugars during a growth on a complex mixture of saccharose, glucose and fructose. (total amount: 60 g/L).

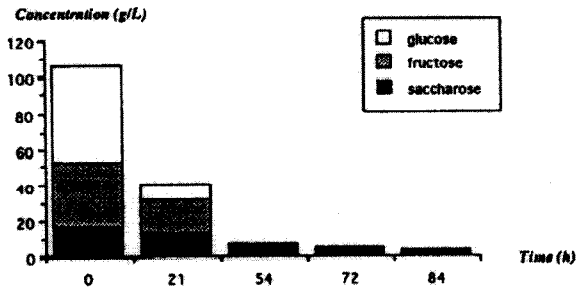

Figure 6 : Ratio of different sugars during a growth on a complex mixture of saccharose, glucose and fructose (total amount: 100g/L).

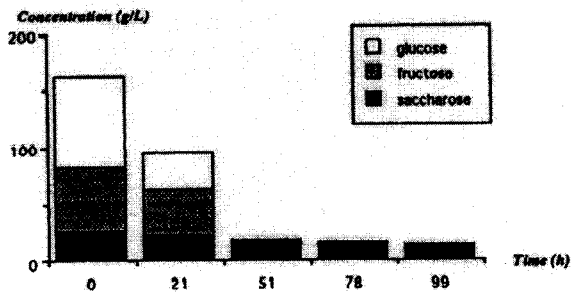

Figure 7 : Ratio of different sugars during a growth on a complex mixture of saccharose, glucose and fructose (total amount : 150 g/L)

The main observations are:

-The glucose is always used before the fructose.

-The two phases of hydrolysis are observed again. The hydrolysis is fast at the beginning of the reaction and becomes slower near the end.

-Moreover, when the initial sugar (saccharose) content is high, the sugar is not totally used and the saccharose level remains at a level of 10 g/L approx. We can observe the same thing in the work of Essia-Ngang et al. [23], but there is no comment. This non utilization of the sugar was never observed on glucose medium for this level of initial sugar [24], but recently Salmon and Mauricio [25] noticed a loss of the activity of the sugar transport during alcoholic fermentation with non-limiting substrate conditions.

From a practical point of view, this last observation is of great interest regarding the sweet sorghum juice fermentation. Indeed, the use of too concentrated juices will not be efficient, leading to a non utilization of all the sugar content. And so, maybe it will be better to operate an hydrolytic pretreatment prior to the fermentation.

4. Conclusion

The use of natural and complex materials (such as juices of beet roots, sugar cane, hydrolyzates or sweet sorghum juices ...) as fermentation media often leads to some problems. In this study it is shown that the behaviour of the yeast *Saccharomyces cerevisiae* on mixed sugars is different on pure and simple sugar. The different sugars constituting the media are not used with the same rates: from a kinetic point of view, the sugars are used sequentially when mixed in the medium: glucose, fructose, saccharose... showing some complex problems of regulation (different affinities, enzymatic repression). Moreover, the hydrolysis of disaccharide appears to be a limiting step in the fermentation reaction.

Also, for industrial purposes, these data should be taken into account to avoid losses of substrates and bad reaction yields. An alternative may be an hydrolytic pretreatment. by steam for example.

Finally we also notice that the bad yields or productivities of the alcoholic fermentation under industrial conditions may also be due to some operating conditions and/or microbial infections as recently showed and discussed by Phowchinda [26] and Phowchinda et al. [27]

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