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Selectivity of Methyl-Fructoside Synthesis with β-Fructofuranosidase

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ABSTRACT

Enzyme synthesis of methyl fructoside was studied using β-fructofuranosidase from *Sacharomyces cerevisiae* and sucrose and methanol as substrates. Taking into account the inhibition and deactivation effects of methanol on the enzyme, a system with 4.9*M* (20%, v/v) methanol was selected. At this alcohol level, 35% of sucrose is converted to fructoside at low or high substrate concentrations. The effect of enzyme concentration, pH, and temperature on both the synthesis and the hydrolysis of the fructoside was investigated. It was found that if the reaction proceeds at pH 6.0, 4°C and/or 0.014 mg/mL (3 U/mL) of β-fructofuranosidase at varying sucrose concentrations, methyl fructoside may be obtained with a minimum loss of the fructoside at the end of the reaction.

Index Entries: Alcoholysis; Methyl-fructoside; β-fructofuranosidase; invertase.

INTRODUCTION

Enzymes are increasingly being used for the synthesis of organic compounds that are difficult to produce by chemical processes. One of the major advances that has been fruitful in this direction is the use of

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enzymes in organic solvents (*1*). In particular, the regiospecifity of biocatalysts in the synthesis of glycosides has received considerable interest owing to the now well-established fact that nucleophiles, other than water, may act as acceptors of the glycoside–enzyme complex usually formed during the enzymatic hydrolysis of di- or oligosaccharides. Several glycosidases have been used for this purpose with a wide variety of alcohols. This is the case for β-galactosidase (*2*), Xylanase (*3–5*), β-fructufuranosidase (*6,7*), and α- and β-glucosidase (*8–10*).

The production of alkyl β-D-fructosides from sucrose using invertase in alcoholic solutions has been the subject of several reports (*6,7,11*). Most of these studies are focused on the effect of alcohols on invertase activity and alkylfructoside yields. The reaction in the presence of methanol proceeds according to the following steps:

$$\text{Sucrose} + H_2O \longrightarrow \beta\text{-D-fructose} + \alpha\text{-D-glucose}$$

$$\text{Sucrose} + \text{Methanol} \longrightarrow \text{Methyl-}\beta\text{-D-fructoside} + \alpha\text{-D-glucose}$$

$$\text{Methyl }\beta\text{-D-fructoside} + H_2O \longrightarrow \text{Methanol} + \beta\text{-D-fructose} \quad (1)$$

This provides a method for protection of the anomeric carbon during the organic synthesis of more complex glycosides.

MATERIALS AND METHODS

β-fructofuranosidase or invertase (EC 3.2.1.26) from *S. cerevisiae* was obtained from Gist-Brocades. Sucrose was purchased from Baker, whereas glucose, fructose, and methyl mannoside were from Sigma (St. Louis, MO). Methanol was obtained from Fisher.

Enzyme activity was measured following the release of reducing sugars in a 60 g/L sucrose solution in acetate buffer, 0.05*M*, pH 4.6, at 40°C. Reducing sugars were monitored with the 2,4-dinitrosalicilic acid reagent (*12*). Samples were taken within the first 10 min from 12-mL volume reactions and, after an appropriate dilution, assayed for glucose and fructose from a standard of equimolecular mixtures of the two sugars. One unit of activity (U) is defined as the amount of enzyme transforming 1 μmol of sucrose/min. The specific activity of the enzyme used was 250 U/mg (dry wt).

The products of alcoholysis were analyzed by high-pressure liquid chromatography (HPLC) using a μ-Bondapak-NH2 column 125 Å, 10 μm (3.9 × 300 mm) from Millipore using acetonitrile/water (75:25) as solvent. Sugars were detected with a refractive index detector from Waters Mod. 410. Methyl fructoside is eluted after 5.4 min, followed by fructose (6.75 min), glucose (7.6 min), and sucrose (9.43 min).

Enzyme kinetics were determined in the presence and absence of methanol at three sucrose concentrations (50, 100, and 300 g/L) and four enzyme concentrations: 3 U/ml (0.014 mg/mL), 6 U/mL (0.028 mg/mL),

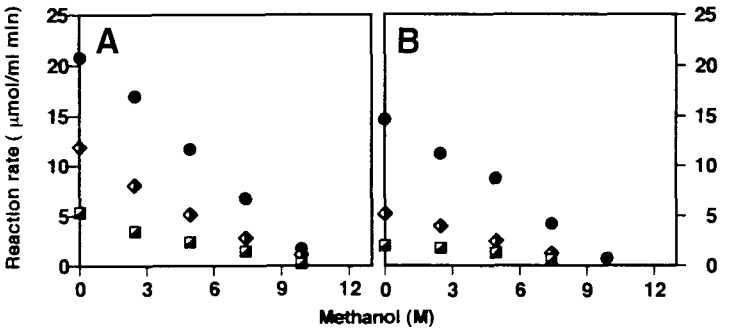

Fig. 1. Effect of methanol concentrations on the initial rate of sucrose conversion (hydrolysis plus alcoholysis) at pH 4.8 and 40°C. Reactions were carried out at varying enzyme concentrations: 0.025 mg/mL (◪); 0.05 mg/mL (◈); and 0.1 mg/mL (●); and two concentrations; 50 g/L (A) and 300 g/l (B).

9 U/mL (0.042 mg/mL) and 12 U/mL (0.056 mg/mL). The effect of pH and temperature was also studied. When required, the reaction was stopped with a 0.32*M* NaOH solution (0.5 mL of sample and 0.5 mL of NaOH solution) and analyzed by HPLC.

RESULTS AND DISCUSSION

Many authors have studied the kinetics of invertase in aqueous solutions. It has been generally recognized as an enzyme following Michaelis Menten kinetics at low sucrose concentrations. There is also agreement on its behavior at high substrate concentrations, and several models have been proposed to describe the inhibitory effect found when excess of sucrose is present. These models are based on medium viscosity, low water activity, and/or modifications to the structure of intra- and intermolecular hydrogen bonds of sucrose (*13–15*). In some of the kinetic models, the production of oligosaccharides resulting from the transferase activity of β-fructofuranosidase has also been considered (*16,17*). The reported K_m values fall within the range of 35–50 m*M* (*6,11,13*), whereas in this work, a value of 32.4 m*M* was found from initial rate experiments (results not shown). Nevertheless, detailed analysis of the kinetics of β-fructofuranosidase in the presence of methanol is scarce. In Fig. 1, the effect of methanol concentration on the initial reaction rate was studied with sucrose at low and high concentrations, and at three different enzyme doses. It may be observed that at 9.9*M* (40% v/v) methanol, the enzyme loses almost all the activity.

This is in agreement with the results reported by Straathof et al. (*6*) and Selisko et al. (*7*). However, activity at higher concentrations of methanol are reported by Ulbrich-Hofmann and Selisko (*11*). From the literature and our results, it seems that the maximum alcohol concentration that the enzyme stands depends on the nature and form of the enzyme

(in whole cells, soluble or immobilized) (results not shown; Selisko et al. [7]). In Fig. 1B, it may also be observed that although the enzyme is inhibited at high substrate concentration, the inhibitory effect of methanol on the enzyme is less severe under this condition (as determined from the slope of the lines). The same effect is not observed when sucrose is partially substituted with another sugar: for instance, sucrose (50 g/L) and maltose (250 g/L). This behavior has already been observed with β-galactosidase (2).

The inhibitory effect of methanol on the rate of reaction has been quantitatively analyzed in the literature, but no attempts to describe it by the common mechanisms of enzyme kinetics have been described. This is probably because of the multiple role of methanol in the reaction, since it acts as substrate in addition to its recognized role as inhibitory and time-dependent deactivation agent in protein aggregation effects.

Methanol as Nucleophile

With a methanol concentration of 4.9*M* (20% v/v), selected from the previous results, the course of the alcoholysis reactions was monitored at three substrate concentrations and four enzyme activities. Although 4.9*M* (20% v/v) is low compared with the optimal values selected by other authors (Straathof et al. [6], who proposed 40–75%), it is well in excess with respect to sucrose and does not significantly affect the enzyme stability (results not shown).

An important difference from other reports in the literature is that methanol concentration was selected not only considering its inhibition effect on the initial rate, but also considering its time deactivation effect. A summary of these experiments is shown in Table 1 and an example of such results is illustrated in Fig. 2. Initial rates of hydrolysis and alcoholysis are determined from data obtained during the first 20-30 min of reaction, whereas the rate of fructoside hydrolysis in the specific reaction may be inferred from the concentration data: the fructoside concentration starts to decline when the fructoside hydrolysis rate becomes higher than its rate of synthesis. From the data presented in Table 1, it may be observed that when the maximum concentration of fructoside is reached (designated as t*), the conversion of sucrose to fructoside falls in the range of 30–35% of sucrose in all cases (t* is an obvious function of enzyme and substrate concentration). The initial rates are plotted in Fig. 3, where it may be observed that hydrolysis and alcoholysis reactions have the same dependence on substrate concentration, with inhibition at 300 g/L sucrose. Also, at a given substrate concentration, the initial rate of alcoholysis is in general higher than the initial rate of hydrolysis. Although these results are the average of multiple experiments, it is not conclusive that at a high concentration of sucrose, alcoholysis is favored, as could be expected from the lower water activity. Another important conclusion is that although the initial rate of alcoholysis is in all cases higher than the initial rate of

Table 1
Effect of Sucrose and β-Fructofuranosidase Concentration on the Conditions for Maximum Methyl-Fructoside Production

		Methyl-fructoside[a]				
[E], U/ml	[S], g/L	t* h	[mf], g/L	X mf, %	X hyd., %	XT, %
3	50	2.00	9.72	35.51	38.17	73.68
6	50	1.50	9.71	35.71	46.05	81.76
9	50	1.00	9.22	34.27	47.96	82.23
12	50	0.50	9.40	32.45	33.62	66.07
3	100	4.00	20.04	35.68	46.69	82.37
6	100	2.00	19.45	33.13	45.59	78.72
9	100	2.00	20.23	33.64	56.34	89.98
12	100	2.00	19.53	32.43	37.74	70.17
3	300	21.00	58.50	33.19	35.6	68.79
6	300	15.00	54.80	30.97	46.98	77.95
9	300	10.00	60.35	34.04	39.25	73.29
12	300	5.00	57.76	32.59	36.82	69.41

[a]The values reported correspond to the time needed (t*) to reach the maximum fructoside concentration [mf], and the corresponding sucrose conversions at t*: total (XT), alcoholysis (X mf) and hydrolysis (X hyd.). Reaction conditions: methanol 20% (v/v), pH 4.8, 40°C.

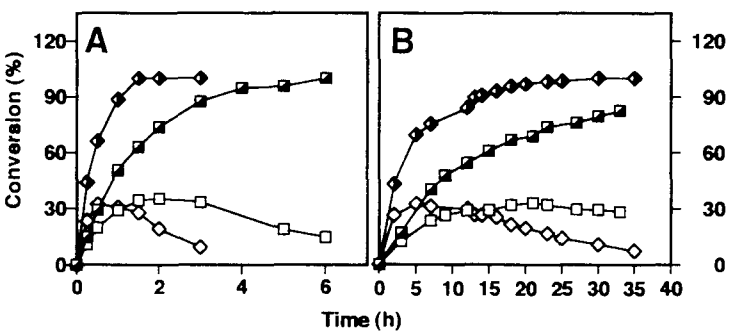

Fig. 2. Illustration of kinetic studies summarized in Table 1. The evolution of total sucrose conversion (-◪-,-◆-) and alcoholysis conversion (-□-,-◇-) is shown as a function of time. The first symbol corresponds to 0.014 mg/mL and the second to 0.056 mg/mL of enzyme. (A) Obtained at 50 g/L sucrose. (B) Obtained at 300 g/L. Other reaction conditions are methanol 4.9*M* (20% v/v), pH 4.8, and 40°C.

hydrolysis, the rate of alcoholysis rapidly declines. Therefore, the highest concentration of fructoside is attained (t*) when more substrate has been hydrolyzed, as concluded from the conversions shown in Table 1. These measurements are made from direct quantification of glucose, fructose, and fructoside; the rate of hydrolysis is calculated from the fructose concentration, whereas the rate of alcoholysis is directly determined from the

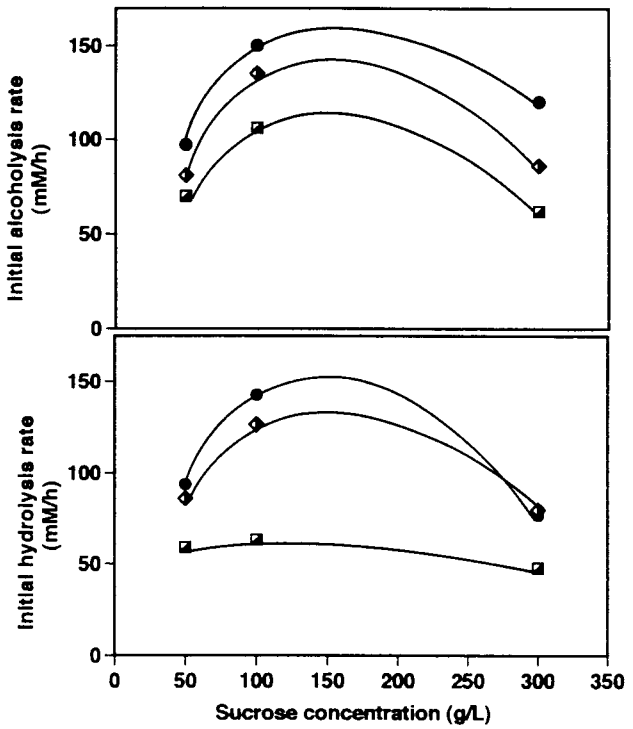

Fig. 3. Effect of sucrose concentration on the initial rate of sucrose hydrolysis and alcoholysis at three different enzyme concentrations: -◪-, 0.028 mg/mL; -◆-, 0.042 mg/mL; -●-, 0.056 mg/mL, in the presence of methanol 4.9*M* (20% v/v), pH 4.8, 40°C.

increase in fructoside concentration. The rate of fructoside hydrolysis was determined when sucrose was exhausted to minimize its rate of synthesis. It is observed that this rate remains almost constant from the moment the methyl-fructoside concentration starts to decline. It is clear from the results shown in Table 1 that, under the conditions studied, this rate is strongly dependent on enzyme activity. In contrast, higher rates of fructoside hydrolysis were obtained from 50 g/L sucrose solutions (around 9.5 g/L of fructoside) than from 300 g/L (around 60 g/L of fructoside), at the four enzyme concentrations used, probably as a result of excess substrate inhibition.

It may be concluded that in aqueous solution, there seems to be a limit for sucrose conversion to fructoside, and although the fructoside is in turn hydrolyzed by the enzyme, this is clearly not the limiting step defining its maximal concentration. At 4.9*M* (20% v/v) methanol, the maximum conversion is the same at the three substrate and four enzyme concentrations. It may be inferred that there is an equilibrium condition limiting the process. Optimal conditions should be chosen aimed at minimizing the loss of fructoside by hydrolysis.

To study the effect of temperature and pH on fructoside synthesis and hydrolysis, a new set of reactions were performed. In Fig. 4 the effect

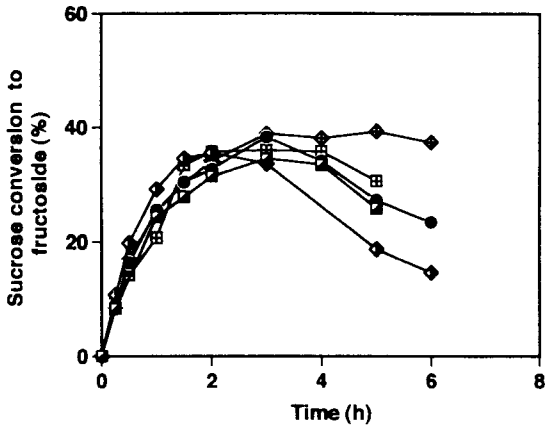

Fig. 4. Effect of pH on the kinetics of fructoside production (alcoholysis) and hydrolysis: -◪-, pH 4.5; -◆-, pH 4.8; -●-, pH 5.2; -⊞-, pH 5.6; -⊗-, pH6; in the presence of methanol 4.9*M* (20% v/v), 50 g/L sucrose, 0.014 mg/mL of enzyme at 40°C.

of pH on the kinetics of fructoside production is shown for reactions carried out at 40°C and 0.014 mg/mL (3 U/mL). Although there is almost no effect of pH on the alcoholysis and hydrolysis rates (*see*, for instance, the similarity in the fructoside production rates during the first hour in Fig. 4), there is a net difference regarding the pH dependence of the fructoside hydrolysis rate. Although the highest rate is observed at pH 4.8, at lower and higher values it is drastically reduced. As already mentioned, the maximum fructoside concentration reached was almost the same in all cases, but shorted times were required at pH 4.8 and delayed at higher (5.2, 5.6, and 6) and lower (4.5) values. The selected value was pH 6.0, where the loss of fructoside by hydrolysis is the lowest.

Finally, the effect of temperature was studied at the highest enzyme concentration 0.056 mg/mL (12 U/mL). The effect of reducing the temperature to 24.5°C and 4°C, both at pH 4.8 and 6.0, was studied to determine if a difference in activation energies might be used to favor alcoholysis. The results at pH 4.8 are shown in Fig. 5 (similar results were obtained at both pH values). As expected, the higher the temperature, the higher the reactions rates. There is a strong effect of temperature on the rate of fructoside hydrolysis: even at the highest enzyme concentration used in the experiment, the hydrolysis of the fructoside is severely reduced. It is interesting to note that although the time needed for total conversion of sucrose is increased fivefold when temperature is reduced from 40 to 4°C, the same fructoside concentration is attained with a negligible hydrolysis rate. This is the consequence of a significant difference in the activation energies of sucrose and fructoside hydrolysis.

As a conclusion, optimal reaction conditions for methyl-fructoside should be chosen from those minimizing its depletion by hydrolysis while maximizing its productivity. It is evident, but not practical, that one alternative is to readjust the reaction conditions once the maximum fructoside

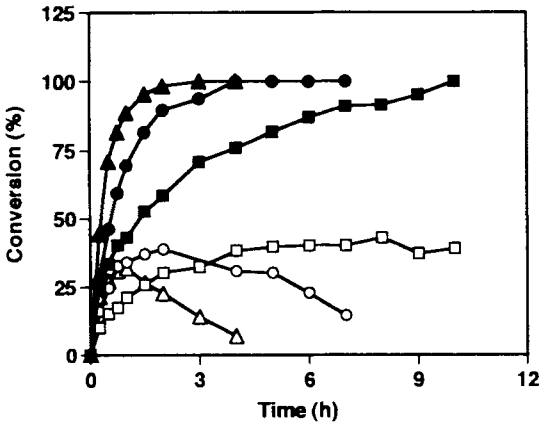

Fig. 5. Effect of temperature on the total sucrose (closed symbols) and methyl-fructoside (open symbols) conversion: 40°C, -▲- and -△-; 24.5°C, -●- and -○-; 4°C, -■- and -□-. Other reaction conditions are pH 4.8, 50 g/L sucrose, and 0.056 mg/mL of enzyme.

concentration is obtained to halt product decomposition. There are no reports in the literature suggesting this alternative. Without cosolvents and with methanol at 20%, conversions obtained of sucrose to fructoside were not higher than 35%, a condition defined by equilibrium conditions and not significantly different from what has already been reported. However, reaction conditions usually employed in the literature are selected from those maximizing initial alcoholysis or hydrolysis rates. Under these conditions, the fructoside is rapidly lost, when sucrose conversion has reached 60–70%. It has been shown in this article that pH and temperature can play an important role in avoiding the product loss: an adequate selection of these parameters, as has been shown in this article, may strongly reduce the fructoside loss by hydrolysis, while maintaining a high productivity.

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