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Microwave Effects on the Enzymatic Hydrolysis of Sucrose

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This work focuses on the experimental study about the influence of microwave radiation (at 2.45 GHz of frequency) on the enzyme catalyzed hydrolysis of sucrose in aqueous solutions, in order to detect possible modifications in the whole reaction rate, and to identify the inhibition effect due to sucrose and/or to the hydrolysis product D-fructose. For comparison purposes, the same experimental conditions were applied both in the presence and in the absence of irradiation. The experimental concentrations were estimated from spectrophotometer acquisitions in the UV wavelengths, and data were fitted with proper kinetic models, taking into account the main reaction pathway and inhibition phenomena. The obtained results show a modification in the reaction kinetics due to the microwave application.

1. Introduction

The basic principles of the invertase-catalyzed hydrolysis reaction of sucrose have been well known for a long time, and this process is commonly applied in the food industry to improve the product taste quality. Furthermore, the hydrolysis of sugars is one of the most important reactions in the process of biomass conversion to bioethanol (Tronci and Pittau, 2015). However, as shown in many scientific works, this reaction can be strongly influenced by inhibition mechanisms, potentially due both to the reagent and to the reaction products (D-glucose and D-fructose), depending on their concentration (Vásquez-Bahena et al., 2004) and on the process conditions (Kulshrestha et al., 2013). Moreover, the different inhibition mechanisms, as well as the elementary steps of the main reaction, involve the formation of intermediates, which consist in a particular interaction between one or more active sites of the enzyme molecule and the substrate or the inhibiting product ones (Combes and Monsan, 1983). Such interactions are mainly electrostatic, thus being able to change as a consequence of an external electric field application (Carta and Desogus, 2010).
Microwave (MW) application is an efficient and valuable technique (Casu et al., 2016), able to influence both chemical (Carta and Desogus, 2013) and biological (Carta et al., 2006) reactions, and useful in biochemical processes (Desogus et al., 2016b), e.g. when conducted in a resonant cavity (Fanti et al., 2016), and in some environmental remediation approaches (Desogus et al., 2016c), e.g. operated in a free space by means of an antenna (Spanu et al., 2016). In the case of microwave application to enzymatic processes, it has been demonstrated that the irradiation can significantly influence the reaction kinetics, even at constant temperature, so bringing to a possible enhancing (Mazinani and Yan, 2016) or dejection (Zhou et al., 2016) in the reaction rate, as well as to a limitation (Mazinani and Yan, 2016) or to a promotion (Zhou et al., 2016) of the inhibition phenomena. From the previous findings, our hypothesis is that the reaction mechanism of the enzymatic hydrolysis of sucrose can be modified by the MW exposition, with a possible enhancing or dejection of the whole reaction rate. If so, it would be useful to quantify such effects, in order to assess the industrial feasibility of applying microwaves to accelerate the process (in the case of a positive effect) or to inhibit it (in the case of a negative effect) when the reaction is undesired (e.g. for control reasons). Moreover, a deeper and quantitative knowledge of kinetics in MW irradiated environments would be helpful in better defining proper reactor models (Desogus et al., 2016a).
Thus, the aim of this work was the experimental study of the invertase-catalyzed hydrolysis of sucrose conducted both in absence and in presence of microwaves at 2.45 GHz of frequency, to assess the influence of both the components concentration and the microwave irradiation. The experimental results have been compared with the theoretical predictions provided by the Haldane (1930) and the Ghose & Tyagi (1979)

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models, with the aim to establish which is the most plausible inhibition mechanism. It was found that the latter one is the most proper to describe the reacting system behaviour. After, a comparison between data in the absence and in the presence of microwaves was accomplished, to quantify the microwave field impact. It was found that microwaves slightly favour each of the elementary reaction steps here considered, but more the inhibition steps than the main pathway ones. Considering the latter aspect, the results here shown are relevant to demonstrate the possibility of modify the process rate by influencing the selectivity of the elementary reactions. However, to assess a more general feasibility of the technique, data about the studied system should be extended.

2. Materials and methods

2.1 Chemicals

The reacting solutions were prepared using a buffer solution in distilled water of acetic acid (0.955 cm^3 L^{-1}) and sodium acetate (2.732 g L^{-1}) to maintain a constant pH value of 5. The sugars here used were bought from Sigma-Aldrich®: sucrose (α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) BioXtra, purity ≥ 99.5 %; D-(+)-glucose Sigma, purity ≥ 99.5 %; D-(–)-fructose Sigma, purity ≥ 99 %. The enzyme catalyzing the hydrolysis reaction was invertase (or saccharase), of the hydrolase class, obtained from baker's yeast (*Saccharomyces cerevisiae*), which was also bought from Sigma-Aldrich® (Grade VII, ≥ 300 units/mg solid). Invertase enzyme, previously dissolved in distilled water, was added to the buffer solution in such an amount to always reach a fixed concentration of $5 \cdot 10^{-3}$ g L^{-1}.

2.2 Reaction apparatus and microwave devices

Reaction experiments (see the scheme in Figure 1) were conducted putting the solution in a Schott Duran flask with a volume of 100 mL; the flask content was maintained at constant temperature (40 °C) by a thermostatic bath. The solution was made circulating by a peristaltic pump from the flask into a MW irradiated cylindrical resonant cavity, containing four Plexiglas tubes (in a symmetric position with respect to the cylinder axis) for the liquid passage; the reactor was realized according to the design of a previous work (Fanti et al., 2015). Microwaves were produced at 2.45 GHz by a radio frequency signal generator (Agilent® E4421B), with a power of -7 dBm ($2.000 \cdot 10^{-3}$ W); the signal generator was connected to a power amplifier (Mini-Circuits® ZHL-16W-43+) to have an incident power of about 1 W (having also considered the losses in the cables). The resonance conditions inside the cavity were verified by measuring the reflected signal by a digital spectrum analyzer (Agilent® 8594E). The reagent and products concentrations were derived from UV absorption spectra collected by a programmable UV-Vis spectrophotometer (Varian Cary® 50) equipped with a flow cuvette positioned, in the fluid circuit, after the resonant cavity and before the flask.

2.3 Experimental procedure

The flask was first filled with 50 mL of buffer solution containing an amount of enzyme to give a final concentration of $5 \cdot 10^{-3}$ g L^{-1}, and the peristaltic pump was operated; when all the circuit had been filled by the solution, the spectrophotometer baseline was acquired; after this, other 50 mL of buffer solution containing the sugars, in such an amount to obtain the final desired concentration, were added into the flask. When MW irradiation had to be applied, also the signal generator and the spectrum analyser were started. The duration time of each experimental run was fixed at 5 min.

2.4 UV absorbance acquisition and concentration derivation

The solution absorbance was measured at the wavelength of 222.5 nm; the acquisition was performed throughout the test (5 min) every 0.5 min, so obtaining 11 readings for each run.
Considering that all the sugars (sucrose, D-glucose and D-fructose) absorb in the same wavelength range, and that also the dissolved nitrogen and oxygen do it, the measured (total) absorbance is the sum of the

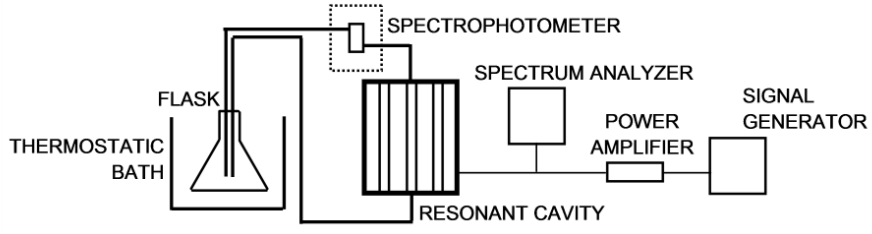

Figure 1: Scheme of the experimental apparatus.

single absorbance of each component. In other terms, considering the Lambert-Beer law, the total absorbance is given by:

$$A_T = A_S + A_G + A_F + A_a = \alpha_S \cdot C_S + \alpha_G \cdot C_G + \alpha_F \cdot C_F + \alpha_a \cdot C_a \quad (1)$$

in which A is the absorbance, C the molar concentration, α is a constant parameter, and the subscripts T, S, G, F and a refer, respectively, to total, sucrose, D-glucose, D-fructose and air (for the purpose of the present work, the dissolved air was considered as a single pseudo component). The latter must be taken into account, considering that its concentration decreases as a consequence of the increasing sugar molar concentration during the reaction (each hydrolysed molecule of sucrose produces one molecule of D-glucose and one molecule of D-fructose). It was chosen to work with air saturated solutions for reproducibility reasons, in such a way that the equilibrium concentration of dissolved gas depends only on the solution composition and not on the degassing conditions or on time. To quantify the air concentration, from the Setchenov equation (Narita et al., 1983), it can be derived that:

$$C_a = C_a^* \cdot \left(1 - {\beta_S}^{-C_S} \cdot {\beta_G}^{-C_G} \cdot {\beta_F}^{-C_F}\right) \quad (2)$$

in which the superscript "*" indicates the reference status (C_S, C_G and C_F equal to zero), and β is a constant parameter. Substituting Eq (2) in Eq (1), the following relationship is obtained:

$$A_T = \alpha_S \cdot C_S + \alpha_G \cdot C_G + \alpha_F \cdot C_F + \alpha_a \cdot C_a^* \cdot \left(1 - {\beta_S}^{-C_S} \cdot {\beta_G}^{-C_G} \cdot {\beta_F}^{-C_F}\right) \quad (3)$$

Thus, using the subscript "0" to denote the initial values, the fractional conversion of sucrose (X) can be used to substitute all the sugar concentrations:

$$A_T = \alpha_S C_{S,0}(1 - X) + \alpha_G\left(C_{G,0} + C_{S,0}X\right) + \alpha_F\left(C_{F,0} + C_{S,0}X\right) + \alpha_a C_a^*\left[1 - {\beta_S}^{-C_{S,0}(1-X)}{\beta_G}^{-\left(C_{G,0}+C_{S,0}X\right)}{\beta_F}^{-\left(C_{F,0}+C_{S,0}X\right)}\right] \quad (4)$$

Eq(4) represents an implicit relationship between the sucrose fractional conversion and the measured absorbance: thus, from the acquisition of the latter, the conversion, and then the concentrations of all the components, can be calculated.

2.5 Kinetic modeling

The substrate inhibition is usually described by the Haldane model (Haldane,1930):

$$\mu_H = \frac{\mu_m C_S}{K_S + C_S + K_I (C_S)^2} \quad (5)$$

in which μ is the reaction rate, the subscripts H and m respectively indicates the Haldane model and the maximum value, K_S is the Michaelis-Menten constant, and K_I is the substrate inhibition constant. In order to consider the combined inhibition effect on the reaction rate due to both substrate and products, Eq(5) can be extended to the Ghose & Tyagi (1979) model, which is reported in Eq(6):

$$\mu_{GT} = \frac{\mu_m C_S}{K_S + C_S + K_I (C_S)^2}\left(1 - \frac{C_F}{K_F}\right) \quad (6)$$

where the subscripts GT and F respectively indicates the Ghose & Tyagi model and D-fructose, and K_F is its inhibition constant, equal to the value of C_F for which $\mu = 0$.

3. Results

To determine the parameters of Eq (7), different experimental runs were performed changing the initial sucrose and D-fructose concentration; moreover, to quantify the influence of MW irradiation on these parameters, the same experimental conditions were adopted in the absence and in the presence of the external electromagnetic field. From the absorbance measurements, obtained as explained in section 2.4, and solving Eq (4) for each reading, X (and so C_S) was determined as a function of time (t); then, the time derivative of C_S, calculated in the first part from the slope of the regression line of C_S vs. t data, was used to estimate the initial reaction rate (μ_0). All the runs were repeated twice.

3.1 Calibration of the absorbance model

To determine X from the spectrophotometer reading (A_T) it was first necessary to find the parameter values to be used in Eq (4): α_S, α_G, α_F, $\alpha_a C_a^*$, β_S, β_G, β_F. This was achieved preparing 30 solutions with variously combined concentrations of sucrose, D-glucose and D-fructose, ranging from 0 to 0.5 mol L^{-1}. The experimental conditions were chosen by resorting to a D-optimal design. The calibration of model in Eq(4) was accomplished by means of a nonlinear regression and the estimated parameter values are reported in Table

1. The determination coefficient R^2 and the mean squared error MSE are equal to 0.9991 and 0.0015, respectively, thus revealing an excellent agreement with the experimental data.

Table 1: Model parameters for the calculation of the concentrations from the total absorbance

α_S	α_G	α_F	$\alpha_a C_a^*$	β_S	β_G	β_F
$1.01 \cdot 10^{-1}$	$-1.33 \cdot 10^{-2}$	1.05	$-1.96 \cdot 10^{-14}$	$1.43 \cdot 10^{-6}$	$5.12 \cdot 10^{-5}$	$3.01 \cdot 10^{-19}$

3.2 Reference conditions without MW radiation

The first group of experimental runs was conducted measuring μ_0 with only sucrose and invertase in the buffer solution; the data set was obtained for different values of $C_{S,0}$, ranging from 0.03 to 0.40 mol L^{-1}. Then, a second group of runs were performed, having added also D-fructose to the starting solution. Two values of $C_{F,0}$ were chosen and combined with the previous values of $C_{S,0}$: 0.1 and 0.2 mol L^{-1}. Differently from the previous case, the results obtained for $C_{S,0}$ less than 0.15 g L^{-1} were not considered because they revealed to be not significant for the quantification of the effect due to D-fructose. The results of these runs are reported in Figure 2. Thus, with the complete data set, a non-linear regression was performed on the kinetic model reported in Eq (6), so obtaining the parameter values reported in Table 2.

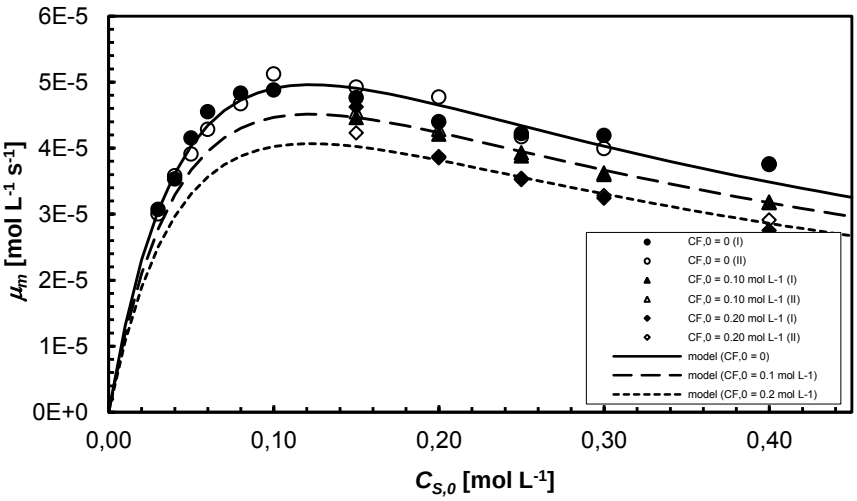

Figure 2: Experimental results of the initial reaction rate as a function of time with: i) only sucrose, and ii) sucrose and D-fructose together in the starting solution, and no MW radiation.

Table 2: Model parameters and statistical indicators for the case of no MW radiation

Parameter	Value	Confidence interval	Mean squared error	Adjusted R^2
μ_m	$1.07 \cdot 10^{-4}$	$1 \cdot 10^{-4} – 1 \cdot 10^{-4}$	$1.48 \cdot 10^{-12}$	0.9990
K_S	$7.08 \cdot 10^{-2}$	$8.52 \cdot 10^{-2} – 1.15 \cdot 10^{-1}$		
K_I	4.73	$2.28 \cdot 10^{-2} – 1.98$		
K	1.11	$8.53 \cdot 10^{-1} – 1.15$		

3.3 Reaction under MW radiation

Analogously to results reported in subsection 3.1, also in presence of MW radiation the experimental runs were divided into two main groups: with only sucrose in the starting solution in the first, and with sucrose and D-fructose in the second case ($C_{S,0}$ levels at 0.1 and 0.2 g L^{-1}). The experimental results are reported in Figure 3. The parameter values of the model in Eq (6) are estimated also in the case of MW irradiation by means of nonlinear regression algorithms and are reported in Table 3.

3.4 Choice of the kinetic model

A visual inspection of the results reported in Figures 2 and 3 reveals that the sucrose inhibition is evident, both in absence and in presence of irradiation. On the other hand, the actual impact of the product inhibition is more questionable, mainly in the second case, for which the differences apparently due to D-fructose concentrations are small. In order to rigorously assess whether the product inhibition is present or not, the

statistical test of the Extra Sum of Squares test was applied. The null hypothesis to be tested is $H_0: \frac{C_F}{K_F} = 0$ and the alternative one is $H_1: \frac{C_F}{K_F} \neq 0$. Model calibration was then performed for the Haldane model that does not consider the product inhibition (hereafter referred as partial model) and the Ghose & Tyagi model (hereafter referred as the full model) (Bates and Watts, 2007). For sake of space the results concerning the MW irradiation are here reported. The sum of square of errors for both partial (SSE_p) and full (SSE_f) models are computed. This allows to evaluate the quantity $SSE_{ex}=SSE_p-SSE_f$ giving the extra sum of squares due to the extra parameters involved in going from the partial to the full model. The resulting ANOVA table for the test is reported in Table 4. Comparing the observed *F-ratio* with the critical value F_α = 4.098, calculated for the *F* distribution with (1,38) degrees of freedom for a significance level of α = 0.05, the null hypothesis is rejected and consequently the product inhibition is necessary to describe the system behaviour. Analogous results were obtained for the no MWs irradiation case. Thus, one can conclude that the Ghose & Tyagi model is a proper choice in both cases (MWs and no MWs).

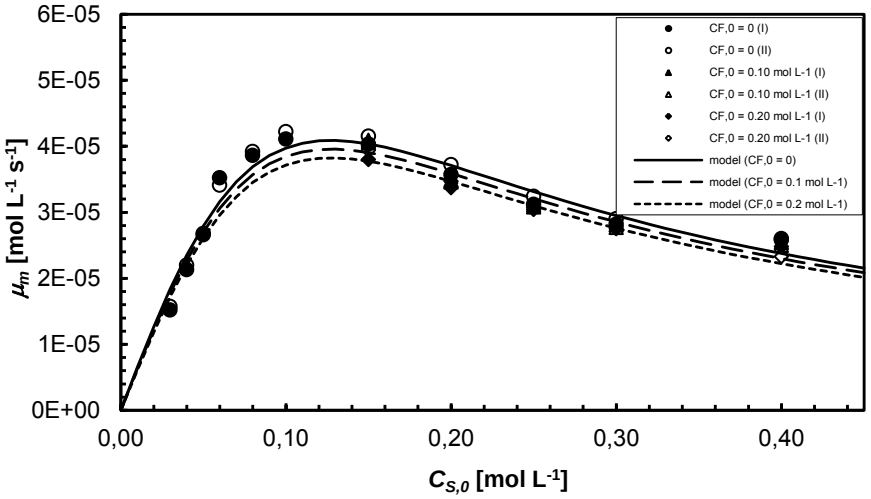

Figure 3: Experimental results of the initial reaction rate as a function of time in the presence of MW radiation.

Table 3: Model parameters and statistical indicators for the case of MW irradiation

Parameter	Value	Confidence interval	Mean Squared Error	Adjusted R^2
μ_m	$3.0\cdot10^{-3}$	$-2.4\cdot10^{-3} – 2.6\cdot10^{-3}$	$3.02\cdot10^{-12}$	0.9985
K_S	4.60	-3.82 – 3.96		
K_I	284.4	-234.6 – 244.11.98		
K	3.09	$-9.49\cdot10^{-1} – 3.17$		

Table 4: ANOVA table of the Extra Sum of Squares test for the case of MW irradiation

Sum of Squared Error	Degrees of freedom	Mean Squared Error	*F*-ratio	p-value
$SSE_{ex} = 1.63\cdot10^{-10}$	1	$MSE_{ex} = 1.63\cdot10^{-103}$	f_0 = 53.97	0.0193
$SSE_f = 1.15\cdot10^{-10}$	38	$MSE_f = 3.02\cdot10^{-10}$		

4. Discussion and Conclusions

First of all, from the experimental results in Figures 2 and 3, the role of sucrose can be easily recognized, as the reaction rate increases with $C_{S,0}$ when the latter is low (up to 0.1 mol L^{-1}) and, after a maximum peak, decreases for increasing $C_{S,0}$: this circumstance clearly evidences the inhibition phenomenon due to the reagent. Also D-fructose produced an inhibiting effect, as μ_m decreases when this component is added to the reacting solution having maintained the same $C_{S,0}$ value.

The comparison between data obtained in the absence and in the presence of MW irradiation confirms the hypothesis of MW effects on the enzymatic reaction mechanism and rate, and allows to evaluate the impact of the electromagnetic field on the enzyme reaction. The general MW effects are a reduction of the reaction rate and a slight enhancing of the inhibition mechanisms. In fact, looking at the model parameters in Tables 2 and 3, the terms with the largest difference between the two conditions are K_S and K_I; in particular, also the ratio

between these parameters and μ_m increases when MWs are applied, so highlighting the bigger weight of inhibition. On the other hand, the very limited increase of K_F denotes a very small effect of MWs on the product inhibition: this phenomenon still appears when the reacting solution is irradiated, but its relative importance on the whole process is much less pronounced (the ratio between K_F and μ_m strongly decreases), so bringing to the conclusion that MWs are able to "inhibit the inhibition" due to D-fructose, and consequently to limit the worsening of performance of the main reaction pathway.

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