OPTIMIZATION AND MATHEMATICAL MODELING STEPS FOR DETERMINING KINETICS OF PECTIN ENZYMATIC HYDROLYSIS

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Bioethanol is one of the renewable fuels, which could effectively substitute fossil fuels in the automotive sector. An alternative potential feedstock for bioethanol production is Citrus Peel Waste (CPW). One novel method to utilize CPW is to produce fuel-grade ethanol from the sugars and polysaccharides in it. Pectin is the main polysaccharide contained in CPW.
In the present work the enzymatic hydrolysis was studied for commercial citrus pectin in order to obtain a sound kinetic rate equation. A program of batch hydrolysis experiments has been set up with the use of Pectinex Ultra SP L as free enzyme. According to the Michaelis–Menten approach, the kinetic parameters, i.e., K'_M and r_{max} have been determined from the initial reaction rate data. Three linearization methods, i.e., Lineweaver-Burk, Langmuir and Eadie-Hofstee, have been employed to compare and minimize the associated experimental error. A further improvement was achieved in the parameter estimation by using a non-linear regression method in Matlab®, which adopts the Gauss-Newton algorithm with Levenberg-Marquardt modifications for global convergence.
The kinetic rate equation well fits the present experimental data in all of the four cases. When comparing the kinetic rate curves predicted by the linear and non-linear fitting methods with the experimental points, the outcome of the non-linear fitting appears to be the best one.

1. INTRODUCTION

During the past century the net carbon dioxide production has increased exponentially because of the tremendous expansion in the transportation sector resulting in notable changes in the Earth's ecosystem. Since the majority of CO_2 is produced by the transportation sector, it would be convenient that this segment utilizes alternative fuels like those derived from biomass (Kadar et al., 2004). Ethanol is one of the candidate fuels that could substitute fossil fuels.
An alternative potential feedstock for bioethanol production is given by Citrus Peel Waste (CPW). The processing of citrus fruit into citrus juice products generates enormous amounts of byproducts. Approximately 40-60% of the fruit weight ends in waste peel.
One alternative method to utilize CPW is to produce fuel-grade ethanol from the sugars and polysaccharides contained in it. Several studies have been conducted that have used enzymatic treatments to hydrolyze pectin, hemicellulose and cellulose to monomer sugars followed by fermentation to produce ethanol from galacturonic acid, xylose and glucose, respectively (Wilkins et al., 2007; Wilkins, 2009).
The main polysaccharide of CPW is pectin (Rivas et al., 2008). Pectin is basically a linear polysaccharide. In each sample of pectin, parameters such as the molecular weight or the content of special subunits will change from molecule to molecule. Pectins are consisting of a backbone of α-(1-4)-linked galacturonic acid residues,

partially esterified (methyl groups: 7.5–75.5%) forming long "smooth" regions, which may be interrupted by "hairy" regions that contain mainly neutral sugars (Fig. 1).

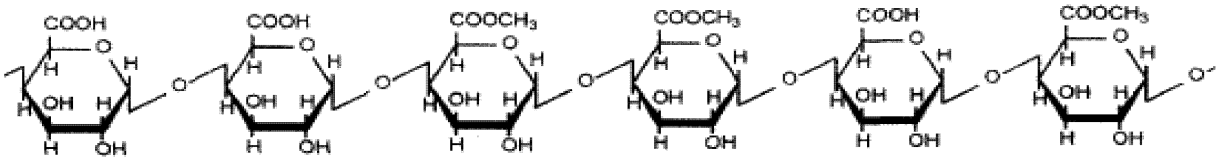

Fig. 1: "Smooth" region structure: polymer of galacturonic acid

Upon hydrolysis, pectin decomposes mainly to its monomer, i.e., the galacturonic acid, and, to a lesser extent, to other hexoses, pentoses and some organic acids.

There is neither detailed modeling nor very general kinetic rate equation for pectin hydrolysis. Baciu and Jordening (2004) took into consideration beet sugar pectin and used Pectinex 100 l hydrolysis enzyme in their experiments. Belafi-Bako et al. (2007) used Pectin substrate (low esterification degree, LM-5CS) from Polding Ltd. (Budapest, Hungary) and adopted purified PG (polygalacturonase, EC 3.2.1.15) as hydrolysis enzyme.

In the framework of a more general project aimed at characterizing the enzymatic hydrolysis of citrus pectin and at developing a general kinetic rate equation taking into consideration inhibitory effects, the authors chose commercial Pectinex Ultra SP L as the hydrolysis enzyme for citrus pectin, as it is closer to industrial applications. Actually, Mutlu and coworkers were using Pectinex Ultra SP L in their experiments, but they did not focus on any inhibitory effect. Mutlu et al. (1999) used a viscosimetric method (Brookfield Viscometer model DV-I) for quantitative determination of pectin hydrolysis; Demirel et al. (2003) employed a refractometric method (Refractometer RFM330, Bellinghamt and Stanley, UK) to determine how much pectin was degraded by enzyme. In this way, however, they were not allowed to quantify the actual galacturonic acid production.

In this work the authors discuss how they found the Michaelis-Menten kinetic parameters in the hydrolysis rate equation under the simple case of no inhibition, while minimizing the associated experimental error.

2. MATERIALS AND METHODS

2.1 Materials

Citrus pectin (P9135), Pectinex Ultra SP L enzyme from Aspergillus aculeatus (P2611), its activity being 10462 PGU/mL, Galacturonic acid (48280) and other chemicals (analytical grade) were purchased from Sigma-Aldrich Company (USA).

2.2 Sample preparation and enzymatic hydrolysis

Citrus pectin was dissolved in 50 mL citrate buffer solution with pH 4.8 by using a magnetic stirrer; after adding 200 µL of Pectinex Ultra SP L, it was incubated and agitated in the incubator shaker (Medline Sc. Ltd, MD3000) at 50°C and 150 rpm for 180 minutes. Experiments were carried out in 200 mL erlenmeyeres (shaking flasks). After entering the flask in the shaker and before adding the enzyme, the temperature of flask that contains the buffer solution and substrate must reach 50ºC. The enzymatic degradation and reaction rate were studied by discontinuously sample taking at time intervals of 5, 10, 15, 30, 60, 120 and 180 minutes. For the determination of kinetic parameters, i.e., K'_M and r_{max} in the Michaelis–Menten equation, the release of galacturonic acid was studied by initial reaction rate measurement (the first 30 min of the batch reaction). Samples were inactivated in a boiling water-bath for 15 minutes. After cooling and appropriate dilution, the inactivated samples were filtrated through a 0.45µm nitrocellulose membrane and analyzed by HPLC.

2.3 Standards

As external standards galacturonic acid, di- and tri-galacturonic acid (D4288 and T7407, Sigma-Aldrich) have been used to prepare 0.25, 0.5, 0.75, 1, 1.5 and 2 g/L solution in a citrate buffer in order to find the necessary calibration line for the following analytical method.

2.4 Analytical Method

A HPLC (Waters 2414 Plus) was used with a Shodex SP-0810 column (Showa Denko), a refractive index detector (Waters 2414 Differential Refractometer), a pump (Waters 1525 Binary HPLC Pump), Software (Empower) and sulfuric acid at pH=2.2 and 0.5 mL/min flow rate as eluent at 60˚C.

2.5 Fitting and optimization of the Michaelis-Menten kinetic parameters

For the determination of kinetic constants both linearization procedures and a numerical nonlinear regression method were used.

Concerning to the linearization methods, the Lineweaver-Burk, Langmuir and Eadie-Hofstee transformations of the Michaelis-Menten equation were used for first calculations of the K'_M and the r_{max} values (Bailey and Ollis, 1986).

2.5.1. Lineweaver-Burk method

In this method the variations of 1/r versus 1/S were plotted and the intersection between the regressed line and x axis is equal to $-1/K'_M$ and the intersection between regressed line and y axis is equal to $1/r_{max}$.

$$\frac{1}{r} = \frac{K'_M}{r_{max}} \times \frac{1}{S} + \frac{1}{r_{max}} \qquad (1.)$$

where r is the initial reaction rate and S is the substrate (i.e., pectin) concentration.

2.5.2. Langmuir method

In this method the variations of S/r versus S were plotted; the intersection between regressed line and x axis is equal to $-K'_M$ and the intersection between regressed line and y axis is equal to K'_M / r_{max}.

$$\frac{S}{r} = \frac{1}{r_{max}} \times S + \frac{K'_M}{r_{max}} \qquad (2.)$$

2.5.3. Eadie-Hofstee method

In this method the variations of r versus r/S were plotted; the intersection between regressed line and x axis is equal to r_{max}/K'_M and the intersection between regressed line and y axis is equal to r_{max}.

$$r = -K'_M \frac{r}{S} + r_{max} \qquad (3.)$$

2.5.4. Nonlinear regression method with Matlab®

The Gauss-Newton algorithm is used with Levenberg-Marquardt modifications for global convergence. For implementation of this nonlinear regression three codes, i.e., main code, function and data file, have been written in Matlab®. The Statistics Toolbox provides the function *nlinfit* for finding parameter estimates in nonlinear modeling.

3. RESULTS AND DISCUSSION

A series of batch hydrolysis experiments have been carried out for 3, 5 and 8 g/L pectin to find the initial reaction rates. Fig. 2 shows the results in terms time profiles of galacturonic acid concentration till a reaction time of 30 min. From the experimental data, the initial reaction rates r were calculated and extrapolated to zero (Fig. 3).

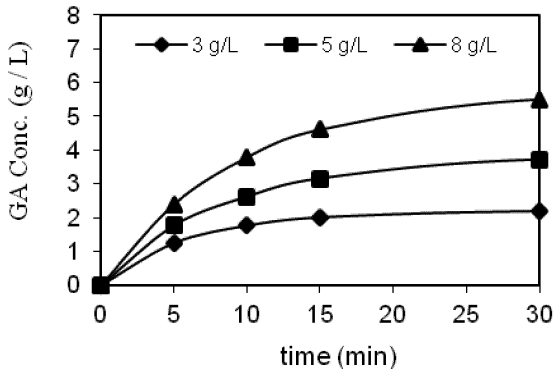

Fig. 2: Progress curves of batch pectin hydrolysis until 30 minutes

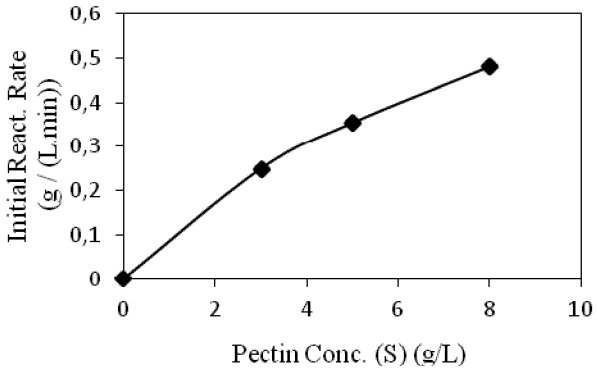

Fig. 3: Initial reaction rates in hydrolysis of pectin

The kinetic parameters have been determined via both linear and nonlinear regression methods in accordance to what was explained above (see Figs. 4 to 7). Table 1 summarizes these results.

Table 1: Michaelis-Menten parameters for enzymatic hydrolysis of pectin (by Pectinex Ultra SP L)

	Lineweaver-Burk	Langmuir	Eadie-Hofstee	Nonlinear regression
r_{max} (g/L.min)	1.05	1.09	1.07	1.10
K_M^r (g/L)	9.68	10.21	9.96	10.42

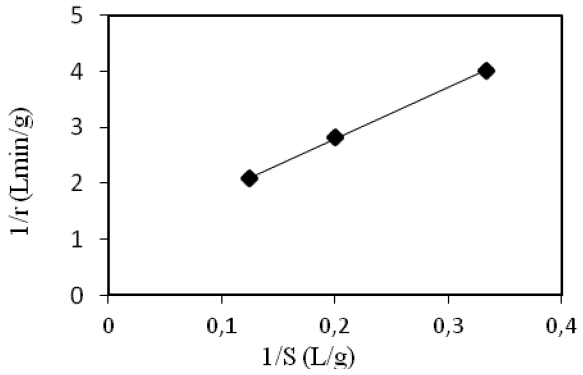

Fig. 4: Lineweaver–Burk linearization

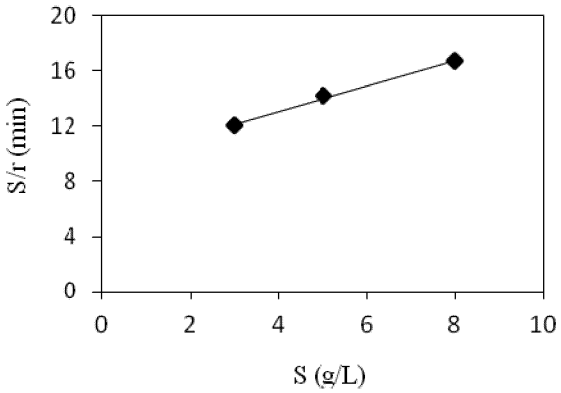

Fig.5 Langmuir linearization

It is interesting to compare the hydrolysis kinetic parameters determined in this work by the Lineweaver-Burk method with those published by Mutlu et al. (1999) or deduced from Demirel et al. (2003), based on the same

linearization procedure in all cases. Mutlu et al. (1999) found r_{max}=2.76 g (pectin)/(L.min) and K_M^r =11.37 g/L for free enzyme; from the Demirel et al. (2003) paper it can be argued that r_{max}=4.06 g (pectin)/(L.min) and K_M^r =20.7 g/L for free enzyme again. Actually, the kinetic parameters determined in this work (i.e., r_{max}=1.05 g/(L min) and K_M^r =9.68 g/L in Table 1) are substantially lower. The main explanation is that their results were determined after pectin hydrolysis, but both Mutlu et al. (1999) and Demirel et al. (2003) did not put pectin conversion in a quantitative relationship with the galacturonic acid production. By measuring the viscosity decrease and the refractive index, respectively, of aqueous solutions of their samples, they assessed in fact pectin and not galacturonic acid concentration.

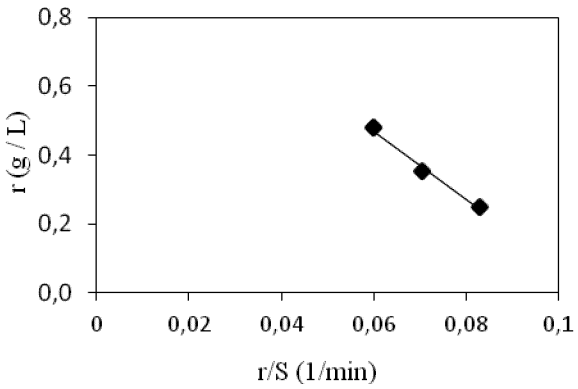

Fig. 6: Eadie-Hofstee linearization

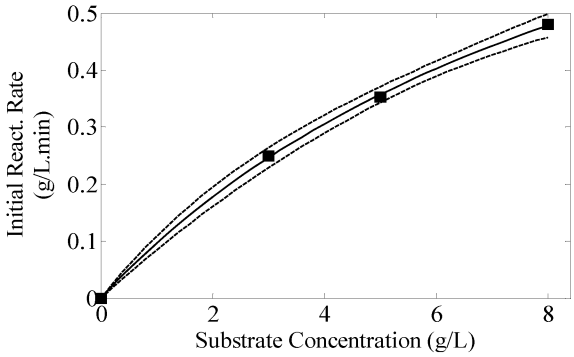

Fig. 7: Nonlinear regression method with Matlab® showing 95% confidence region

When comparing the kinetic rate predictions based on all of the four methods with the experimental data used to generate the regression itself, the nonlinear regression method shows the best performance. This is clearly demonstrated in Table 2 that reports the SSE (Sum of Squared Errors) values exhibited by all of the linear and nonlinear method.

Table 2: Comparison between predicted and experimental initial reaction rates in terms of SSE (Sum of Squared Errors)

	Lineweaver-Burk	Langmuir	Eadie-Hofstee	Nonlinear regression
SSE $[g/(L\ min)]^2$	3.75×10^{-5}	2.75×10^{-5}	2.81×10^{-5}	2.31×10^{-5}

4. CONCLUSIONS

- An experiment program has been set up to obtain a sound and easy-to-use kinetic rate equation for the hydrolysis of citrus pectin by means of a free commercial enzyme. To this end around 60 lab experiments have been globally carried out (including duplicate tests), 707 samples have been taken from partial and final hydrolysis products and about 350 man-hours have been spent in HPLC analyses.
- After adopting the Michaelis-Menten approach to quantify the enzymatic hydrolysis rate equation, the two relevant parameters have been determined by means of linear regressions based on the Lineweaver-Burk, the Langmuir and the Eadie-Hofstee transformations of the original Michaelis-Menten equation. Further, the parameters have been determined by means of a nonlinear regression method implemented in the MATLAB® Statistics Toolbox.

- When comparing the hydrolysis reaction rates predicted on the base of regressions and the experimental data used to generate the regression itself, the nonlinear fitting method shows the best performance.
- As a further work, following the present preliminary results, the kinetic rate equation for enzymatic pectin hydrolysis will be tested and modified to consider more complex and realistic conditions, including all the possible inhibitory activities and the synergistic effects, which the commercial enzymes usually employed in industry exhibit. Moreover, actual CPW will be taken into consideration, its enzymatic hydrolysis will be investigated and the corresponding rate will be checked against the present rate equation for pectin hydrolysis.

5. ACKNOWLEDGEMENTS

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