Hydrolysis of pectin by *Aspergillus niger* polygalacturonase in a membrane bioreactor

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Abstract

Hydrolysis of pectin by a polygalacturonase (PG) enzyme was studied and it was found that strong product inhibition occurs. The process could be described by a competitive mechanism and the inhibition constant determined was $K_I = 3.13$ g/l. A thermostated, ﬂat-sheet membrane bioreactor was applied for the reaction to avoid product inhibition and enhanced (more than 40% higher) productivity was achieved compared to the batch system.

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1. Introduction

Pectolytic enzymes are used for the fruit-processing industry to increase yields, improve liquefaction and clarification (Alkorta, Garbisu, Llama, & Serra, 1997; Kashyap, Vohra, Chopra, & Tewari, 2001; Pilnik & Voragen, 1993), moreover to produce d-galacturonic acid (monomer of pectin), which is an important compound, raw material in the food, pharmaceutical and cosmetic industry to manufacture e.g. vitamin C, or acidifying, tensioactive agents (Jörneding, Baciu, Berensmeyer, & Buchholz, 2002). Hydrolysis of pectin can be carried out by pectinases that are classiﬁed into three main groups:

- pectinesterases—catalysing deesteriﬁcation of the methoxyl group of pectin;
- depolymerising hydrolytic enzymes (including polymethyl-galacturonases and polygalacturonases)—catalysing the hydrolytic cleavage of 1,4-glycosidic bonds;
- lyases—catalysing the cleavage of glycosidic bond by transelimination.

Among the pectin hydrolysing enzymes (endo)polygalacturonases are probably the most important biocatalysts. Polygalacturonase enzymes (PG, E.C. 3.2.1.15.) are able to hydrolyse pectin and/or pectic acid. Although PG enzymes play a key role in pectin hydrolysis, their actions have not been studied in details from kinetics point of view so far, while kinetical behaviour of many soluble and immobilized pectinase enzymes and enzyme-mixtures have been already characterised (Gillespie & Coughlan, 1989; Sariodlu, Demir, Acar, & Mutlu, 2001; Todisco, Calabro, & Iorio, 1994). Kulbe, Heinzler, and Knopki (1987) have assumed that the PG enzyme from *Aspergillus niger* was inhibited by its monomeric product, but no experiments were carried out to prove it and determine the mechanism.

Therefore the aim of this work was to study the kinetics of pectin hydrolysis by polygalacturonase enzyme from *A. niger* in details, to decide experimentally if inhibition occurs or not; and to describe the possible inhibition phenomena. Moreover we intended to design and operate a
reactor system, where product inhibition can be avoided and productivity of the process can be enhanced.

To avoid product inhibition in hydrolysis of macromolecules, like starch or cellulose, membrane bioreactors have been often applied, where the small inhibitory product molecules (e.g. glucose) can easily pass through the membrane and be removed continuously from the system, while the large molecules (substrate and enzyme) are retained by the membrane (Gergely, Bekassy-Molnar, & Vatai, 2003). Thermostated flat-sheet and hollow fiber membrane bioreactors have been successfully used for enzymatic hydrolysis of polysaccharides in our laboratories (Bélaﬁ-Bákó, Nemestóthy, Milisic, & Gubicza, 2002). Pectin hydrolysis in a membrane bioreactor has been studied by Kulbe et al. (1987) and Olano-Martin et al. (2001), however in the first case three types of pectinase enzymes were used simultaneously, thus action of polygalacturonase enzyme alone was not possible to study, while in the latter case the aim was to produce pectic oligosaccharides (not galacturonic acid).

2. Materials and methods

Pectin substrate (low esterification degree, LM-5CS) was received as a gift from Polding Ltd. (Budapest, Hungary). Polygalacturonase enzyme (PG) from A. niger was purchased from Sigma (USA), its activity was 1.7 U/mg. Activity definition: one unit is defined as the amount of enzyme which is able to produce 1 µmol galacturonic acid from polygalacturonic acid in 1 min in pH = 4.1 and 50 °C. The enzyme is able to hydrolyse pectin molecules, as well. All the other chemicals (analytical grade) were purchased from Fluka (Germany).

To study the kinetics of the reaction, shaking flask experiments were carried out in a New Brunswick Scientiﬁc (USA) shaking incubator. Citrate buffer was used (pH 4.1) to prepare the substrate solutions with various concentrations, and the operational conditions of the experiments were 50 °C and 150 rpm. To determine the product inhibition, galacturonic acid (product) was added initially to some of the substrate solutions.

Experiments were conducted in a membrane bioreactor, as well. The set-up of the system is presented in Fig. 1. Diluted substrate solution (citrate buffer, pH 4.1) containing the enzyme was circulated from a stirred thermostated vessel through a flat-sheet membrane module (thermostated). The material of the ultraﬁltration membrane was regenerated cellulose, cut-off 30 kDa. The membrane was able to retain the enzyme, while low molecule weight substance, like galacturonic acid product could pass through the membrane easily. The level of the substrate solution in the stirred vessel was controlled by a mechanical equaliser.

The hydrolytic reaction was followed by measuring the reducing sugar content using the dinitro-salicylic test DNS standard method—Miller (1959) which is based on the formation of a chromophore between DNS product and reducing groups of the (oligo)galacturonic acid molecules.

3. Results

3.1. Study on the kinetics

In the shaking flask experiments progress curves on the hydrolysis of pectin solutions were measured, where the reducing sugar contents (galacturonic acid) in the reaction mixtures as a function of time were determined with different initial substrate concentrations. Data on 2, 5 and 8 g/l initial substrate concentrations (using 0.01 g enzyme) are presented in Fig. 2. As it can be seen higher reaction rate and final conversion were achieved using higher initial

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**Fig. 1.** Set-up of the membrane bioreactor equipped with equaliser for continuous pectin hydrolysis.
substrate concentration, but it seems that the relations between the reaction rate data and the substrate concentrations were not proportional. These data implied that product inhibition might occur during the reaction. Therefore another series of experiments were carried out to study the effect of the product on the process. Various amounts of galacturonic acid were added initially to the reaction mixture using different substrate concentrations and the reducing sugar content was measured as a function of time. Fig. 3 presents experimental time course data using 2 g/l initial substrate concentration and 0, 2 and 4 g/l galacturonic acid concentrations. In Fig. 3 it is clearly shown that the galacturonic acid present had a significant inhibition effect on the reaction. The more product was added to the reaction mixture, the slower was the initial reaction rate. These experiments have proven that product inhibition occurred during the pectin hydrolysis by PG enzyme. From the experimental data (progress curves) initial reaction rates were calculated (Fig. 4), which were then transformed according to Lineweaver–Burk method (double reciprocal method). In Fig. 5 it can be seen that the $1/v$ intercepts of all the lines were in the same section, thus it can be concluded that the type of inhibition was competitive. The Michaelis–Menten model for the reaction rate completed with competitive product inhibition describes the process as follows:

$$v_i = \frac{v_{\max}(S)}{K_m + \frac{K_I + I}{K_I + S}}$$

where

- $v_i$ reaction rate
- $S$ substrate concentration
- $I$ inhibitor (product) concentration
- $v_{\max}$ maximal reaction rate
- $K_m$ Michaelis–Menten constant
- $K_I$ inhibition constant

Applying the Lineweaver–Burk method, the parameters of the model were determined (which were checked by

![Fig. 2. Progress curves of pectin hydrolysis by polygalacturonase from A. niger (Reaction conditions: pH 4.1, 150 rpm, 50 °C, 0.01 g enzyme).](image)

![Fig. 3. Progress curves for studying the product inhibition (Reaction conditions: pH 4.1, 150 rpm, 50 °C, 0.01 g enzyme).](image)

![Fig. 4. Initial reaction rates in hydrolysis of pectin with various inhibitory galacturonic acid ($I$) concentrations by polygalacturonase enzyme from A. niger (Reaction conditions: pH 4.1, 150 rpm, 50 °C, 0.01 g enzyme).](image)

![Fig. 5. Lineweaver–Burk linearisation.](image)

Table 1
Parameters obtained for hydrolysis of pectin by PG from A. niger

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Michaelis–Menten constant</td>
<td>$K_m$ (g/l)</td>
</tr>
<tr>
<td>Maximal reaction rate</td>
<td>$v_{\max}$ (g/l/min)</td>
</tr>
<tr>
<td>Inhibition constant</td>
<td>$K_I$ (g/l)</td>
</tr>
</tbody>
</table>
numerical methods, as well). The parameters obtained are summarised in Table 1. We were going to check and compare these data to the ones in literature, but have not found any. The only data on inhibition kinetics of PG enzymes were found on PG from Penicillium capsulatum (Gillespie & Coughlan, 1989) in the Brenda data base, however it was about substrate inhibition. So no data was found in literature on product inhibition occurring in pectin hydrolysis by PG enzyme.

3.2. Experiments in the membrane bioreactor

Thermostated flat-sheet membrane bioreactor equipped with equaliser (shown in Fig. 1) was applied for the measurements on continuous pectin hydrolysis by polygalacturonase enzyme. Experiments similar to the shaking flask measurements were carried out in the membrane bioreactor (0.01 m² surface area) with 2 g/l initial substrate concentration. All the operation conditions were the same: 100 ml thermostated reaction vessel, citrate buffer pH 4.1, temperature 50 °C and 0.01 g enzyme (thus the substrate-enzyme S/E ratio was the same, 20). The reaction mixture containing the product was circulating through the thermostated flat-sheet membrane module and the galacturonic acid solution was collected from the secondary side of the membrane. A transmembrane pressure (pressure difference between the two sides of the membrane) was maintained at 0.1 bar. The accumulated amount of galacturonic acid as a function of time and the flux data calculated from the amount of permeate obtained are presented in Fig. 6(a) and (b). It can be seen that reliable operation was achieved in the course of 50 h reaction time, the enzyme has shown excellent working stability. The galacturonic acid product (as an aqueous solution) was gradually accumulated in the permeate side and more than 2.5 l permeate was collected during the process. In the course of the continuous hydrolysis the flux obtained were slightly decreasing, but no serious fouling could be observed in the long term operation.

The specific productivity (related to the enzyme concentration) of the systems were calculated based on the data of galacturonic acid produced during the batch and continuous process. It was found that the values of specific productivities were 6.9 and 9.7 g product/h g enzyme for the shaking flask and membrane bioreactor experiments, respectively. So the specific productivity value calculated in the continuous hydrolysis was 40.6% higher than in the batch system. The volumetric productivities calculated were similar, since both the volume of the reactors (100 ml) and the amount of enzyme (0.01 g) were the same in the shaking flask experiments and in the membrane bioreactor. These results have proven that it is worth to carry out pectin hydrolysis in a membrane bioreactor, since much higher productivity can be achieved due to the prevention of product inhibition. Moreover it is a continuous reaction possessing all the advantages of a continuous process over a batch operation.

4. Conclusions

Hydrolysis of pectin by polygalacturonase from A. niger was studied in details. It was found that strong product inhibition occurred during the process. The product, galacturonic acid is a comparative inhibitor of the enzyme, and the inhibition constant was determined experimentally \( K_I = 3.13 \text{ g/l} \) as a new finding. To avoid inhibition, membrane bioreactor was applied for continuous pectin hydrolysis and more than 40% higher productivity was achieved than in the shaking flask experiments.

The low esterification degree pectin used in this work was a suitable model substrate in the kinetical study. However, our aim is to extend the research work for other substrates, like sugar-beet pectin, since it can be recovered from a waste material (extracted sugar-beet pulp), thus an industrial process (sucrose production) can be completed with waste utilization step: manufacturing a valuable product, d-galacturonic acid. Thus the next step of our research is to involve sugar-beet pectin in the experiments, to study its hydrolysis in a membrane bioreactor and finally to scale-up the system.

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References


