Bioresource Technology 167 (2014) 324–330

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Inhibitory effect of vanillin on cellulase activity in hydrolysis of cellulosic biomass

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## HIGHLIGHTS

- Vanillin reversibly inhibits the cellulase activity.
- Vanillin is a non-competitive inhibitor of cellulase.
- The inhibition kinetics of cellulase by vanillin follows the HCH-1 model.
- Aldehyde and phenolic hydroxyl groups of vanillin have inhibiting effect on cellulase.

## ARTICLE INFO

*Article history:*
Received 16 April 2014
Received in revised form 7 June 2014
Accepted 9 June 2014
Available online 17 June 2014

*Keywords:*
Vanillin
Cellulase
Inhibition kinetics
Inhibitory group

## ABSTRACT

Pretreatment of lignocellulosic material produces a wide variety of inhibitory compounds, which strongly inhibit the following enzymatic hydrolysis of cellulosic biomass. Vanillin is a kind of phenolics derived from degradation of lignin. The effect of vanillin on cellulase activity for the hydrolysis of cellulose was investigated in detail. The results clearly showed that vanillin can reversibly and non-competitively inhibit the cellulase activity at appropriate concentrations and the value of $IC_{50}$ was estimated to be 30 g/L. The inhibition kinetics of cellulase by vanillin was studied using HCH-1 model and inhibition constants were determined. Moreover, investigation of three compounds with similar structure of vanillin on cellulase activity demonstrated that aldehyde group and phenolic hydroxyl groups of vanillin had inhibitory effect on cellulase. These results provide valuable and detailed information for understanding the inhibition of lignin derived phenolics on cellulase.



## 1. Introduction

Researches on bioconversion of lignocellulosic materials for biofuel production have attracted great attention all over the world due to both the abundance of cheap and renewable cellulosic biomass and the consumption of nonrenewable fossil fuels (Galbe and Zacchi, 2012; Gomez et al., 2008; Kim and Kim, 2013). However, the current bioconversion process is still not commercially viable and cost-competitive in great part due to the recalcitrance of cellulosic biomass. It has been believed that the presence of lignin in lignocelluloses contributes greatly to the recalcitrance (Buranov and Mazza, 2008; Hansen et al., 2011). With tight association with cellulose and hemicellulose, lignin acts as a barrier to prevent the access of cellulolytic enzymes to the substrate (Nakagame et al., 2011, 2010). Moreover, lignin unproductively absorbs cellulase, decreasing the amount of available cellulase for hydrolyzing (Ju et al., 2013; Palonen et al., 2004; Sewalt et al., 1997).

In order to reduce the recalcitrance of lignocellulosic biomass, biomass-disrupting pretreatment is required prior to enzymatic hydrolysis (Chiaramonti et al., 2012; Haghighi Mood et al., 2013). Most of the prevalent pretreatments, however, are performed under severe conditions, resulting in formation of phenolic compounds from degradation of lignin (Jönsson etal., 2013; Mosier et al., 2005). These phenolic compounds identified in lignocellulosic hydrolyzates include simple phenolics (vanillin, ferulic acid, syringaldehyde and conifer alcohol, etc.) and oligomeric phenolics (ellagic acid, epicatechin and tannic acid, etc.). The amount and type of phenolic compounds depend on the biomass source, pretreatment methods and conditions.

The inhibition of lignin-derived phenols on fermentative strains have been widely investigated (Delgenes et al., 1996; Helle et al., 2003; Kim and Kim, 2013; Kim et al., 2013; Klinke et al., 2004; Mussatto and Roberto, 2004; Paul et al., 2003), whereas few reports are available on their inhibition on cellulose hydrolysis.

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http://dx.doi.org/10.1016/j.biortech.2014.06.035
0960-8524/

**Nomenclature**

| | |
|---|---|
| $a$ | Relative of activity (%) |
| $A$ | Initial enzyme activity without inhibitor in the hydrolysate |
| $B$ | Enzyme activity with inhibitor added into the hydrolysate. |
| $C$ | Product glucose concentration in the hydrolysate (g/L) |
| $E$ | Enzyme concentration (g/L) |
| $E^a$ | Adsorbed enzyme concentration (g/L) |
| $E^f$ | Free enzyme concentration (g/L) |
| $EG_x$ | Complexed enzyme concentration (g/L) |
| $G_s$ | Product concentration (g/L) |
| $G_x$ | Total cellulose concentration (g/L) |
| $G_X^f$ | Free cellulose concentration (g/L) |
| $i$ | Inhibition parameter (dimensionless) |
| $I$ | Inhibitor concentration (g/L) |
| $k$ | Reaction rate constant ($h^{-1}$) |
| $m$ | Slope (L/g) |
| $t$ | Time (h) |
| $V$ | Instantaneous reaction velocity |
| $\bar{V}$ | Average velocity |
| $V_r$ | Reaction volume (mL) |
| $v$ | Initial velocity of enzyme reaction (μmol/min) |
| *Greek* | |
| $\alpha$ | Lumped affinity constant (g/L) |
| $\beta$ | Inhibitor binding constant (L/g) |
| $\delta$ | Association constant (g/L) |
| $\varepsilon$ | Number of cellulose sites covered by adsorbed or complexed enzyme (dimensionless) |
| $\eta$ | Association constant (dimensionless) |
| $\kappa$ | Lumped rate constant ($h^{-1}$) |
| $\Phi$ | Fraction of total cellulose sites which are free (dimensionless) |

Kim et al. (2013) investigated the effect of soluble inhibitors on cellulase and found that phenolic compounds and xylo-oligosaccharides were the most important causes leading to the decrease in cellulase activity. Ximenes et al. (2011, 2010) confirmed that vanillin, syringaldehyde, trans-cinnamic acid and hydroxybenzoic acid inhibited cellulose hydrolysis in wet cake. What is more, their work demonstrated that vanillin was the strongest inhibitor for cellulase among those four kinds of phenols. Although previous work has identified vanillin's inhibiting effect on cellulase, to the best of our knowledge, detailed information on the inhibition type, kinetics, mechanism and inhibitory group in vanillin is still unclear.

The objective of the present study was to investigate the inhibiting action of lignin derived vanillin on cellulase. The inhibition kinetics and model of cellulase by vanillin were figured out. Furthermore, the effect of three phenolics similar to vanillin in structure on cellulase activity were compared with that of vanillin so as to identify which group on phenyl ring of vanillin exerted the inhibiting effect on cellulase.

## 2. Methods

### 2.1. Materials

Microcrystalline cellulose powder was purchased from Sinopharm Chemical Reagent Co., Ltd, China. The commercial cellulase derived from *Trichoderma reesei* with a filter paper activity of 30.43 FPU/g was supplied by Hersbit Imperial Jade Bio-Technology Corp, Ningxia, China. Vanillin was purchased from Longxi Chemical Co., Ltd, Guangdong, China. Guaiacol, 3,4-dimethoxybenzaldehde and p-hydroxybenzaldehyde were purchased from J&K Scientific Ltd, Beijing, China.

### 2.2. Inhibitory kinetics of cellulase by vanillin

#### 2.2.1. Effects of vanillin on the enzymatic hydrolysis

Experiments were first conducted to investigate the possible inhibitory effect of vanillin on the enzymatic hydrolysis. Enzymatic hydrolysis was carried out in citrate buffer (pH 4.8) in 50 mL flasks (working volume 30 mL) at 10% (w/v) substrate loading (microcrystalline cellulose). The commercial cellulase was used for enzymatic hydrolysis experiments. The enzyme loading was 20 FPU/g cellulose. The vanillin concentration was 0, 1, 5 and 10 g/L. To prevent microbial contamination, 30 μL penicillium sodium solution (100 g/L) was added to each flask. All the reaction flasks were incubated in a shaker (THZ-C, Taicang Experimental Equipment Factory, Jiangsu, China) at 150 rpm and 50 °C for 72 h. Samples were collected at 1, 2, 4, 6, 12, 24, 48, 72 h and then stored at −20 °C for further analysis of glucose concentration.

#### 2.2.2. Effects of vanillin on the activity of cellulase

The enzymatic hydrolysis was carried out in citrate buffer (pH 4.8) in 25 mL tubes (working volume was 10 mL). The concentration of microcrystalline cellulose and cellulase loading were kept at 25 g/L and 20 FPU/g cellulose, respectively. The vanillin concentration was 0, 5, 10, 20, 40 and 60 g/L. In addition, to prevent microbial contamination, 10 μL penicillium sodium solution (100 g/L) was added to each tube. All the reaction tubes were incubated in a shaker (THZ-C, Taicang Experimental Equipment Factory, Jiangsu, China) at 150 rpm and 50 °C for 1 h. These tubes were then heated for 5 min in a boiling water bath to stop the enzymatic hydrolysis. Samples were taken after cooling to analyze sugars and then determine the activity of cellulase.

#### 2.2.3. The type of inhibition (irreversible or reversible inhibition) of cellulase by vanillin

The experiments were carried out in citrate buffer (pH 4.8) in 25 mL tubes (working volume was 10 mL). The concentration of microcrystalline cellulose was 25 g/L and the vanillin concentrations were 0, 5, 10, 20, 40 and 60 g/L. The enzyme loadings were 5, 10, 20, 40 and 60 FPU/g cellulose. Otherwise mentioned, the operating conditions were the same as that mentioned in Section 2.2.2.

#### 2.2.4. Non-competitive inhibition

The experiment was carried out in citrate buffer (pH 4.8) in 25 mL tubes (working volume was 10 mL). The vanillin concentrations were 0, 10, 20 and 40 g/L. In this experiment, the concentration of cellulose ranged from 25 to 80 g/L. The enzyme loading was 20 FPU/g cellulose. Except otherwise mentioned, the operating conditions were the same as that mentioned in Section 2.2.2.

### 2.3. Inhibition model

The Michaelis–Menten model is the classic model to interpret enzymatic reaction, which predicts the reaction rate as linear in enzyme dosage. However, it was found that the relationship between the reaction rate and enzyme dosage was not linear in