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Enumeration of monosugars' inhibition characteristics on the kinetics of enzymatic hydrolysis of cellulose

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ABSTRACT

A kinetic model based on Michaelis-Menten assumptions is developed here for enzymatic hydrolysis of cellulose using cellulase enzyme derived from *Trichoderma reesei*. Further, to assess the inhibition of enzyme and the nature of inhibition by different sugars (glucose, galactose, mannose, xylose and cellobiose) the primary kinetic model is modified with the inhibition kinetics. The enzymatic reaction was carried out at pH = 4.8, temperature = 50 °C, solid loading of 1:20 and at 100 rpm stirrer speed. It was observed among all the sugars, only xylose (five carbon sugars) showed non-competitive inhibition with K_i = 27.2 mM and V_{max} = 0.19 g $L^{-1}h^{-1}$. Glucose and cellobiose manifested competitive inhibition with almost comparable inhibition constants (K_i) of 24.7 and 26.3 mM respectively, while V_{max} = 0.26 g $L^{-1}h^{-1}$. On the contrary, with galactose and mannose, the inhibition constants decrease to 10.9 and 11.1 mM respectively. Statistical analysis shows that cellobiose attributes to a maximum inhibition with 72% reduction in reducing sugar production. On the contrary, the minimum inhibitory effect with 11% reduction in sugar production was observed with xylose. At the end, ANOVA analysis manifests that the effect of different sugars are much significant in inhibiting the enzyme compared to the substrate concentration in case with the cellulase enzyme from *Trichoderma reesei*.

1. Introduction

Cost effective production of fermentable sugars from cellulosic substrate attributes to a sustainable process towards biofuel production that might potentially compensate the limitations with fossil fuel resources [1–3]. However, the present technology like acid hydrolysis towards the production of fermentable sugars from cellulosic biomass has some limitations because of the production of fermentation inhibitors along with sugars. Therefore, enzymatic hydrolysis may be considered as an effective one with no co-formation of such inhibitors during reaction. Enzymatic hydrolysis with cellulase enzyme (EC: 3.2.1.4) is the most conventional one with high substrate loading along with high product yield [4,5]. Primarily, cellulase enzyme is characterized by the combinations of enzymes like endoglucanase, cellobiohydrolase and β-glucosidase, which breaks β-glycosidic chains of polymeric cellulose molecules towards the formation of glucose [6]. During enzymatic hydrolysis, the initial rate of production of glucose is higher and subsequently becomes steady after a substantial reaction hours manifesting an asymptotic nature of the reaction curve [7]. Such decrease in the rate of production attributes to the inhibition of enzyme ceasing the rate of reaction and the depletion of the substrate to glucose along with cellobiose. A substantial study had already been carried out on the mechanism of product inhibition during enzymatic hydrolysis of cellulose [8,9].

Enzymatic hydrolysis of cellulose requires a mechanistic and kinetic understanding of the process in order to implement the technology at large scale with optimum and economic design of the process towards enzymatic hydrolysis. However, one of the primary intricacies attributed to the kinetic study of the cellulase enzyme is the variations in the reaction rates because of enzyme consortium. Further, understanding of the kinetics becomes much difficult, when the reaction components either mechanically or chemically inhibit such a typical mixture of enzymes. Apparently, due to less solubility of cellulosic materials in water, the cellulosic solution becomes non-homogeneous and hence, a non-uniform distribution over the enzyme surface manifests less effective reaction. Such a heterogeneous composition also renders a limit on the mass transfer coefficient for the substrate to be diffused either towards the enzyme or vice versa. On the contrary, the chemical inhibition of the enzymes (endo, exo and β-glucosidase) by the product also becomes a critical factor in limiting the yield from the enzymatic reaction. Several studies are reported in enumerating the kinetics of such enzymatic hydrolysis of cellulose. It is postulated from those studies

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that the enzyme combination follows Michaelis-Menten kinetics during cellulose hydrolysis [10,11], which might also be re-coupled with the inhibition kinetics in order to infer on the feasibility of the enzymatic hydrolysis of cellulose [12,13].

Product inhibition is recognized as a cynosure restriction to the practical acceptability of cellulase enzyme for the conversion process of cellulosic material into sugars. Carvalho et al. [14] studied the kinetics of enzymatic hydrolysis of sugarcane bagasse using commercial complex of cellulase from *Trichoderma reesei*. Subsequently, Michaelis-Menten model was fitted to their experimental observations with an inference that glucose inhibits substantially in a competitive fashion. On the contrary, it was observed that glucose acts as a noncompetitive inhibitor for cellulase obtained from *Trichoderma reesei,* when corn stover was used as a feedstock [15]. Further, Miao et al. [13] carried out another kinetic study on enzymatic hydrolysis of pretreated rice straw and observed a competitive inhibition caused by both glucose and xylose with a dissociation constant (pK_a) of 1.24 and 0.33 respectively. This manifests almost four folds less glucose inhibition compared to xylose. Thus, the nature of the substrate molecule substantially influences the effect of the inhibition caused by the products from enzymatic hydrolysis. Gruno et al. [16] observed 100 fold higher competitive inhibition because of cellobiose, when exo-glucanase Cel7A acts on bacterial cellulose than that of low molecular weight substrate. In another investigation, Xiao et al. [17] studied the inhibitory effect of sugars derived from hemicelluloses, primarily mannose, galactose and xylose on cellulase enzyme using cellulose as the substrate. Around 32, 38 and 45% decrease respectively in the hydrolysis rate was observed, when 100 g/L of individual sugars were added externally into the reaction volume. Kinetic analysis of enzymatic hydrolysis with cellulase poses a major challenge in designing such a process with an accurate estimation of reducing sugars along with the feasibility analysis of the process. Inclusions of some other mechanistic parameters along with typical Michaelis-Menten model might be a good alternative to formulate a precise model on cellulose hydrolysis and thereafter, to understand the process feasibility. Michaelis-Menten model is more prone to conform to the kinetic pattern primarily arises from rapid initial hydrolysis of cellulose followed by an asymptotic decay in the rate. During prolonged reaction of enzymatic hydrolysis, the aforementioned model has been observed with bit limitations towards its practical adaptation in kinetic analysis as it hardly considers the continuous structural alteration of cellulose. Cellulase enzymes in general perpetuate more towards the accessible amorphous region and repel less accessible crystalline zone of cellulose. Hence, reduction in the crystallinity of the substrate continuously affects the kinetics of the hydrolysis reaction [18]. However, apart from the crystallinity, during an onset of enzymatic reaction, the extreme outer layer of the cellulose fibrils gets digested and the water-solid-enzyme reaction interface proceeds towards the core of the substrate molecule. Gan et al. [19] observed that several other parameters such as proper cellulase-cellulose interactions and enzyme inhibition-deactivation are the key parametric considerations that are required during the mechanistic model formulation of enzymatic cellulose hydrolysis in order to achieve more realistic predictions. However, inclusions of such variables will not only complicate the model, but also makes it difficult to solve.

Cellulase enzyme commercialized from *T. reesei* is well known to contain substantial amount of CBHI and CBHII, comprises of 60% and 15% respectively of the total secreted protein by *T. reesei.* The synergistic action of both the proteins facilitates the enzymatic hydrolysis of cellulose into cellobiose. Further, the intrinsic substantial β-glucosidase activity of cellulase enzyme manifests the production of glucose without an instantaneous accumulation of cellobiose in the reaction medium, which alleviates the chances of product inhibition on a high extent. Although, a number of investigations as discussed above had already been carried out on cellulase activity, enzyme specifically obtained from *T. reesei* still demands a detailed kinetic understanding of the process along with its inhibition during cellulose hydrolysis. Therefore, the present work focuses on the development of the kinetic model for enzymatic hydrolysis of cellulose using cellulase enzyme derived from *Trichoderma reesei* based on such insight mechanisms. The kinetic parameters were evaluated through regression from experimental observations followed by an in-depth understanding of the inhibition characteristics of the model for different sugars (glucose, cellobiose, galactose, mannose and xylose). A rigorous statistical analysis using ANOVA test manifests that the reaction kinetics is sensitive to the sugars', while it is insignificant to the substrate concentration.

2. Materials & methods

2.1. Materials

Cellulose (CAS 9004-34-6), glucose (CAS 50-99-7), xylose (CAS 58-86-6), galactose (CAS 59-23-4), mannose (CAS 3458-28-4) and cellobiose (CAS 528-50-7) were procured from Sisco Research Laboratories Private Limited. Cellulase enzyme (EC 3.2.1.4) isolated from *Trichoderma reesei* was purchased from Sigma Aldrich, India. Buffer preparation chemicals sodium hydroxide (CAS 1310-73-2) and citric acid (CAS 5949-29-1) were procured from Merck, India. De-ionized water was collected from double distilled de-ionized water system (make: Merck millipore water system).

2.2. Enzymatic hydrolysis of cellulose and inhibition study

Enzymatic hydrolysis was conducted with cellulose as substrate at 50 °C temperature in 0.05 (M) sodium citrate buffer (pH = 4.8). Enzyme of 20 FPU of cellulase/g cellulose was given into a reaction mixture of 40 mL and the reaction was carried out for 24 h. For kinetic study, solid substrate to liquid (enzyme in buffer solution) ratio was varied at different proportions of 1:25, 1:20, 1:15 and 1:10. The samples were withdrawn at different time intervals till 24 h, where rate of reaction became steady. Subsequently, to study product inhibition characteristics, glucose, xylose, galactose, mannose and cellobiose were added externally at a concentration of 20 g/L, 10 g/L, 10 g/L, 10 g/L and 20 g/L respectively into a 10 mL of enzymatic reaction system and the samples were collected at 24 h for further analysis.

Withdrawn sample was heated in boiling water bath for 5 min to cease the enzymatic reaction. Sample was centrifuged at 10,000 rpm for 10 min and the supernatant was collected for further analysis. The amount of total reducing sugars (TRS) present in the hydrolysate was analyzed by DNS method [20] and the total sugars were analyzed using a protocol described by Haldar et al. [21].

2.3. Cellulose hydrolysis and inhibition of cellulase: mechanistic elaboration

Substantial amount of pretreated biomass as feed to enzymatic reactor facilitates hydrolysis of cellulose with increased yield of reaction products, which also poses an issue with the product inhibition. However, the propensity towards inhibition reaction depends on the enzyme's molecular structure and the chemical nature of its active "substrate binding site", characteristic of which depends on the source of the enzyme. Cellulase enzyme from *Trichoderma reesei* primarily consists of two domains – one, the cellulose binding domain (CBD) is responsible for the adhesion of the enzyme to the surface of the substrate molecules and the other is known as the catalytic domain (CD) containing an active site that involves in catalysis during enzymatic hydrolysis of cellulose. These two domains are ordered by a long flexible peptide linkage, which efficiently accommodates both the domain [6]. TrCel7A is known as the most active exo-glucanase and comprises 60% of the total cellulase obtained from *T. reesei.* According to Silveira et al. [6] the flexible tunnel shaped TrCel7A binds to the substrate at the reducing terminal of the cellulose chain and holds the substrate in proper orientation through an accessory loops of the enzyme manifesting enzymatic hydrolysis. However, it is also reported that during