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Kinetic modeling of cellobiose by a β-glucosidase from *Aspergillus fumigatus*

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ABSTRACT

The final step in lignocellulose enzymatic saccharification is the cellobiose conversion to glucose by β-glucosidases (BG). In this work, a valid kinetic model to describe cellobiose degradation for an industrial mixture of BG enzymes present in *Aspergillus fumigatus* is selected. Firstly, the enzyme mixture was characterised in terms of protein content and enzymatic activity on p-NPG (1326 U $mL_{preparation}^{-1}$), determining the molecular weight of the only BG activity band observed in zymograms by SDS-PAGE and MALDI-TOF: 94 kDa. Subsequently, to select the correct kinetic model for the enzymatic hydrolysis of cellobiose, a combined strategy was performed: Firstly, non-linear regressions were applied to initial hydrolysis rate data for different enzyme concentrations and initial substrate and product concentrations, observing inhibition by cellobiose and glucose. Secondly, the optimal kinetic model was discriminated by a coupled non-linear regression-DOE numerical integration approach, by fitting several possible kinetic models involving different product inhibition mechanisms to progress curve data from runs at various initial substrate concentrations and temperatures. The best kinetic model involves non-competitive substrate inhibition and product competitive inhibition with two binding sites for glucose.

1. Introduction

In the years to come, biorefinery processes and products will be of utmost importance, being a more environmentally-friendly and renewable alternative or complement to the usual counterparts derived from petroleum. One of the best-known processes in biorefineries is the production of ethanol, widely used as a fuel additive (Choi et al., 2015). In 2015, 86 million tons of bioethanol from starch or sugar sources were produced (first generation bioethanol or 1GBE). To increase bioethanol sustainability and avoid interaction with food markets, lignocellulosic biomass feedstocks are envisaged as a more abundant and convenient raw material for bioethanol processing (second generation bioethanol or 2GBE). To this end, lignocellulosic biomass is pretreated, hydrolyzed with acids and/or enzymes and fermented with yeasts (Aditiya et al., 2016). The enzymatic saccharification step is key to achieve large amounts of economically feasible 2GBE (Gupta and Verma, 2015; Hasunuma et al., 2013). Hydrolysis releases glucose and other soluble sugars from celluloseby the action of endoglucanases (EDG), exoglucanases (EXG), β-glucosidases (BG) and other auxiliary enzymes (Hasunuma et al., 2013). Cellulose is depolymerised by EDG and EXG into cellobiose and other cello-oligosaccharides, which are substrates for BG, that catalyses their subsequent transformation into glucose (Singh et al., 2015).

BG acts on the hydrolysis of glycosidic bonds, which produces the release of nonreducing terminal glucosyl residues from glycoside or oligosaccharide molecules: thus, BG is active on many different substrates where this type of bond is present, including cellobiose, glucosyl ceramide, salicin or laminaribiose (Ketudat Cairns and Esen, 2010; Singh et al., 2015). Therefore, these enzymes arepresent in all

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Nomenclature

2GBE	Second generation bio-ethanol
AA	Auxiliary activities
AIC	Akaike's information criterion
ASI	Acompetitive substrate inhibition
BG	β-glucosidase enzyme
BPI	Competitive by-product inhibition
BSA	Bovine serum albumina
C_E	Enzyme concentration ($g\,L^{-1}$)
C_P	Product concentration (glucose) ($mol\,L^{-1}$)
C_S	Substrate concentration (cellobiose) ($mol\,L^{-1}$)
CAZy	Carbohydrate-active enzymes
CE	Carbohydrate esterase enzymes
CLD	Chain length distribution
CPI	Competitive product inhibition
E_{kcat}	Activation energy of the catalytic constant ($J\,mol^{-1}$)
EDG	Endoglucanase enzyme
EXG	Exoglucanase enzyme
F	Fischer's statistical parameter
GH	Glycoside hydrolase enzyme
GT	Glycoside transferase enzyme
HPLC	High performance liquid chromatography
k_{cat}	Catalytic constant ($mol\,min^{-1}\,g_E{}^{-1}$)
k_{cat0}	Preexponential factor of the catalytic constant ($mol\,min^{-1}\,g_E{}^{-1}$)
K_i	A competitive substrate inhibition constant ($mol\,L^{-1}$)
K_M	Substrate affinity constant ($mol\,L^{-1}$)
K_{NC}	Non-competitive product inhibition ($mol\,L^{-1}$)
K_P	Competitive product inhibition constant ($mol\,L^{-1}$)
K_X	Xylose competitive product inhibition constant ($mol\,L^{-1}$)
M-M	Michaelis–Menten kinetic model
N	Number of experimental data
NPI	Non competitive product inhibition
P	Number of parameter evaluated on kinetic model
P_1	Empirical parameters of experimental data adjusting (numerator)
P_2	Empirical parameters of experimental data adjusting (denominator)
PAGE	Polyacrylamide gel electrophoresis
PL	Polysaccharide lyase enzyme
pNPG	4-Nitrophenyl β-D-glucopyranoside
r	Reaction rate ($mol\,L^{-1}\,min^{-1}$)
r_0	Initial reaction rate ($mol\,L^{-1}{\cdot}min^{-1}$)
R	Ideal gas constant ($8.314\,J\,K^{-1}\,mol^{-1}$)
R_P	Glucose production rate ($mol\,L^{-1}\,min^{-1}$)
R_S	Cellobiose hydrolysis rate ($mol\,L^{-1}\,min^{-1}$)
RID	Refraction index detector
RMSE	Residual mean square error
SDS	Sodium dodecyl sulphate
SQR	Sum of quadratic residues
SSQ	Sum of squares
T	Temperature (K)
VE	Percentage of variation explained
v_{max}	Maximum reaction rate ($mol\,min^{-1}\,L^{-1}$)
X_C	Conversion of cellobiose into glucose
γ_l	Heteroscedasticity parameter for VE determination

domains of living organisms, comprising Archaea, Eubacteria and Eukaryotes, in which they have proven an enormous variety of functions (Ketudat Cairns and Esen, 2010). According to the role developed by the enzymes involved in the carbohydrate polymer decomposition, a Carbohydrate-Active Enzymes database was elaborated in 1998, named CAZy (Cantarel et al., 2009), which classifies the different involved enzymes in five activities: glycoside hydrolases (GH), glycoside transferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE), and auxiliary activities (AA) (Cantarel et al., 2009). BGs, responsible for cellobiose hydrolysis, are classified as glycoside hydrolysis enzymes in the clan GHA, which contains families with similar conserved catalytic amino acids and catalytic domain structure. The most abundant family in BGs is GH1, yet they are also included in families GH5 and GH30 (Singh et al., 2015).

The importance of BG action in cellulose saccharification is notable both from the technical and economic viewpoints: these enzymes yield glucose from cellobiose and low-molecular weight cellooligosaccharides and, on the other side, their technical preparations at industrial level are 2–4 times more expensive than those with a high endo- and exo-glucanase activity (as these latter mixtures usually lack adequate BG activity and needs BG complementation). In this regard, strategies that are focused on the recycling of the enzymes, either by recycling the spend solid (Waeonukul et al., 2013) or by using immobilization techniques that permit a good contact enzyme-substrate and a good separation of the enzymatic support after operation (Verma et al., 2016, 2013b) are being developed to reduce BG cost in saccharification.

There is still much controversy and research effort regarding kinetic modelling of the depolymerisation step due to endoglucanases and cellobiohydrolases (Hosseini and Shah, 2011a, 2011b; Jeoh et al., 2017). In addition, kinetic information on the action of β-glucosidases still lacks precision, if present, as new BGs less prone to substrate and product inhibition are sought and proper kinetic modelling, looking for optimal kinetic models, is not always performed, even in the case of the most usual β-glucosidases, as those produced by molds of the *Trichoderma* genus. This lack of optima kinetic models is due to the mathematical tools and model discrimination approaches (linearization of non-linear models or model screening based only on reaction rate, for example). A robust approach to kinetic modelling for enzyme-driven processes is suggested by Al-Haque et al. (2012).

Several kinetic models have been proposed in the literature to explain the catalytic activity of BG enzymes, as compiled in Table 1. In the first model shown therein, the authors employed an intial rate approach and a Lineweaver–Burk linear regression. The model did not fit accurately to experimental data, specially after long experimental times, but it was proved that BG suffers from product inhibition as the enzyme has a much higher affinity for the product than for the substrate: K_M is 5.6 mM, K_i is 0.0244 mM (Hong et al., 1981).

Bravo et al. (2001) employed the previous model to fit to the experimental results achieved in the enzymatic hydrolysis of cellobiose by a commercial BG. Several initial pH values were tested and, whilst fitting one run at a time, very good fits were achieved. However, neither a relationship between pH and substrate and/or product inhibition nor a complete model able to reproduce the system kinetics for different pH values could be established (Bravo et al., 2001).

Corazza et al. (2005) used the enzyme employed by the previous authors and discriminated, by hybrid neural modelling, among the six possible combinations of models based on uncompetitive and noncompetitive substrate inhibition, on one hand, and competitive, uncompetitive and noncompetitive product inhibition on the other. They used the initial rate approach, concluding that the best-fitting models include competitive inhibition by the glucose and noncompetitive or acompetitive inhibition by the substrate (Corazza et al., 2005).

Resa and Buckin (2011) used ultrasonic technology to diminish experimental errorand establish a real-time on-line monitoring of enzyme reaction on cellobiose. Its enzymatic hydrolysis was carried out with a commercial BG at 50 °C and pH 4.9. In this work, a modification in the product inhibition pattern was included: a model that included two enzyme binding sites for glucose, as well as an uncompetitive inhibition on cellobiose, were fitted to experimental data. The best-fitting results have been achieved with the two-binding sites