Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2018

www.elsevier.com/locate/jbiosc

Effectiveness of cross-linked enzyme aggregates of cellulolytic enzymes in hydrolyzing wheat straw

Shuddhodana, Munishwar N. Gupta, and Virendra S. Bisaria*

Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India

Received 25 December 2017; accepted 15 April 2018
Available online xxx

Development of industrially potent cellulolytic enzymes is one of the greatest challenges faced in lignocellulosic feedstock based bio-refining. In the current work cross-linked enzyme aggregates (CLEAs) of commercial cellulase mix were successfully prepared and their performance to be used as potential industrial enzymes in terms of stability and wheat straw hydrolysis was evaluated. The CLEAs were more stable compared to native enzymes with half-lives being 2.30-, 1.56-, 3.07- and 1.67-fold higher at 70°C for filter paper activity (FPA), endoglucanase, β-glucosidase and xylanase, respectively. CLEAs retained 77.4% of endoglucanase and 85.9% of xylanase activity after five cycles of hydrolysis of soluble substrates such as carboxymethyl cellulose and xylan, respectively. A maximum saccharification yield of 31.8% by soluble enzymes and 32.9% by CLEAs were obtained when alkali-pretreated wheat straw was subjected to hydrolysis. On repeated batch hydrolysis for five consecutive cycles of 24 h each, the CLEAs showed an overall higher saccharification yield of 43.3% compared to 31.8% with soluble enzymes.

[**Key words:** Bio-refining; Cellulase; Xylanase; Cross-linked enzyme aggregates; Lignocellulose; Wheat straw]

Bio-refineries based on renewable resources for the production of biofuels and other bio-based commodity chemicals using natural or engineered microbial processes have gained importance in the recent decades (1–3). In this context, lignocellulosic feedstocks constitute an important and sustainable source for producing alternate fuels giving rise to second generation of biofuel development. The pretreatment of lignocellulosic biomass and their enzymatic hydrolysis are the two major bottlenecks for commercial utilization of lignocellulose in bio-refineries (4–6). Hence, development of stable cellulolytic enzymes is of great importance in lignocellulose based bio-refineries (7,8).

An emerging technique of enzyme immobilization is to cross-link the enzymes after precipitating them from the solution called as cross-linked enzyme aggregates (CLEAs) (9–11). CLEAs are simple, cost effective and several studies have revealed that CLEAs generally tend to be thermally and operationally more stable (12–15). This technique can be applied to immobilize more than one enzyme at a time as a single preparation called combi- or multipurpose-CLEA (16). This feature of CLEA is well suited for stabilization of cellulolytic enzymes which generally contain a set of enzymes acting synergistically. For these reasons CLEAs seems to be one of the most promising techniques for stabilization of cellulolytic enzymes and this area is less explored with respect to enzymatic degradation of lignocellulosic biomass.

The aim of this work was to prepare stable CLEAs of cellulolytic enzymes and study their effectiveness by determination of kinetic and stability parameters. Furthermore, the prepared CLEAs were evaluated in comparison to their soluble counterparts by applying them to hydrolyze lignocellulosic biomass (wheat straw) pretreated differently for production of fermentable sugars.

MATERIALS AND METHODS

Chemicals and materials Enzyme SaccharISEB C6L containing cellulase (measured as filter paper activity of 280 ± 20 IU/ml), endoglucanase (2800 ± 100 IU/ml), β-glucosidase (570 ± 25 IU/ml) and xylanase (4250 ± 150 IU/ml) was kind gift of Advanced Enzymes, India. Carboxymethyl cellulose (0.7 DS) was obtained from Acros Organics, Belgium. *p*-Nitrophenyl-β-D-glucopyranoside (*p*-NPG), *p*-nitrophenol and xylan from oat spelts were obtained from Himedia, India. Glutaraldehyde was obtained from Sigma–Aldrich, USA. Ammonium sulphate and all other chemicals and reagents used in the study were obtained from Merck, Germany.

Enzyme assays Cellulase activity by filter paper assay and endoglucanase (EC 3.2.1.4) activity were measured according to the method recommended by International Union of Pure and Applied Chemistry (IUPAC) (17) using Whatman No. 1 filter paper and carboxymethyl cellulose (CMC) as substrates, respectively. β-Glucosidase (EC 3.2.1.21) activity was measured using substrate *p*-nitrophenyl-β-D-glucopyranoside (18). Xylanase (EC 3.2.1.8) activity was determined using xylan from oat spelts as substrate (19).

Preparation of CLEAs CLEAs were prepared as described in the literature (20). Briefly, ammonium sulphate was slowly added to chilled enzyme solution with stirring to cause precipitation and was kept for 30 min at 4°C. CLEAs were prepared by subjecting completely precipitated enzymes to chemical cross-linking by slow addition of glutaraldehyde and incubating the mixture at 4°C with constant shaking (300 rpm) for predetermined period of time. The suspension was centrifuged at 8000 × *g* for 10 min. Supernatant was decanted and the pellet was washed until there was no residual enzyme activity in the supernatant. Finally, CLEAs were stored in the form of a suspension in 0.05 M

* Corresponding author. Tel.: +91 11 26591002; fax: +91 11 26582282.
E-mail addresses: vbisaria@dbeb.iitd.ac.in, vsbisaria@hotmail.com (V.S. Bisaria).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.04.007

citrate buffer, pH 4.8, at 4°C for future use. Enzyme assays were performed to determine % activity recovery in CLEAs by using Eq. 1.

$$\%\ \text{Activity recovery in CLEA} = \frac{\text{Activity of enzyme in precipitate or CLEA (IU)}}{\text{Initial activity of the free enzyme (IU)}} \times 100 \quad (1)$$

Optimization of conditions for preparation of CLEAs Experiments for optimizing the process conditions for preparation of CLEA were performed with the help of response surface methodology (RSM) using three-level face centered central composite design (FCCCD). Various concentrations of BSA (25–125 mg/ml), glutaraldehyde (0.25–1.25% w/v) and incubation time (2–6 h) were selected as design parameters to prepare CLEA. A total of 20 runs were performed with six replicates at the center point. The statistical software package, Design Expert (version 10, Stat-Ease Inc., USA) was used to design the experiments and statistically analyze the results.

Effect of temperature and pH To determine the optimum temperature for different enzyme components of native soluble enzymes and CLEAs, enzyme assays were performed at different temperatures ranging from 40°C to 70°C as mentioned earlier. Similarly, optimum pH was determined by performing enzyme assays in the pH range of 4.0–7.0. The pH buffers were 0.05 M citrate buffer (pH 4.0–5.0) and 0.05 M sodium-phosphate buffer (pH 6.0–7.0).

Pretreatment of wheat straw Lignocellulosic biomass, wheat straw, was subjected to different pretreatments such as alkaline pretreatment (21), dilute acid pretreatment (22) and microwave mediated pretreatment (23,24).

Cellulose, hemicellulose and lignin contents of untreated and pretreated wheat straw on dry- and extractive free-basis were determined by NREL laboratory analytical procedures TP-510-42618, 42619, 42621, 42622, 48087 (25–28). Acid soluble lignin was obtained by measuring absorbance at 240 nm using an extinction co-efficient of 17.8 l/g•cm (25,29).

Enzymatic hydrolysis of wheat straw Enzymatic hydrolysis of wheat straw was conducted in 100 ml Erlenmeyer flasks containing 10% (w/v) of wheat straw on dry weight basis as substrate with an enzyme loading of 20 FPU/g of cellulosics (cellulose + hemicellulose), at a pH of 4.8 maintained by 0.05 M citrate buffer in a rotary shaker at 60°C and 150 rpm. The saccharification yield (or % hydrolysis) for each sample was calculated based on the percentage of cellulosic contents in each sample using Eq. 2.

$$\text{Saccharification yield (\%)} = \frac{W_{\text{sugar released}} \times \text{sugar conversion factor}}{W_{\text{biomass taken}} \times \text{total cellulosics fraction}} \times 100 \quad (2)$$

where W is amount of sugar released or wheat straw taken in mg, sugar conversion factor is 0.9 for conversion of cellulose and hemicellulose to sugars, and total cellulosics fraction is cellulosics (cellulose + hemicellulose) in each sample of wheat straw.

Hydrolysis of soluble substrates (CMC and xylan) Hydrolysis of CMC and xylan as substrates (10% w/v) was carried out separately with an enzyme loading of 20 FPU/g of cellulosics and the hydrolysis was carried out as described previously for several consecutive cycles. At the end of each cycle, CLEAs were separated by centrifugation at 8000 × *g*, washed and then resuspended in fresh substrate to perform next cycle of hydrolysis. Residual activity of cellulase components after each cycle was assessed considering the activity in the beginning of the first cycle as 100%.

Repeated batch hydrolysis of wheat straw Hydrolysis of wheat straw was carried out as described previously for five consecutive cycles. For each cycle of 24 h, the insoluble residues (non-hydrolyzed wheat straw and CLEA) from the previous hydrolysis cycle was mixed thoroughly with the fresh substrate to maintain the original initial total solids content (that is, 10% w/v). At the end of each hydrolysis cycle, the samples were centrifuged at 8000 × *g* to separate the solids and liquid fractions. The supernatant was used to estimate sugar concentration and saccharification yield was calculated for each cycle.

RESULTS

Preparation of CLEAs From the preliminary studies conducted for selection of precipitating agents (ammonium sulphate, acetone, ethanol and isopropanol) we found ammonium sulphate (90% saturation) to be the precipitant of choice with 95.1 ± 3.5% activity recovery in the precipitate and 33.4 ± 2.3% activity recovery in the form of CLEAs (data not shown). The preparation of CLEAs was carried out with the help of RSM using FCCCD as the design model. The critical parameters considered in FCCCD were glutaraldehyde concentration (0.25–1.25% w/v), BSA concentration (25–125 mg/ml) and reaction time (2–6 h). By applying multiple regression analysis on data generated by 20 experiments

TABLE 1. Kinetic parameters for different components of native soluble enzyme and CLEAs of commercial cellulase mix determined by performing assays for β-glucosidase, endoglucanase and xylanase at varying substrate concentrations.

Enzyme	Parameter	Soluble enzyme	CLEAs
β-Glucosidase	K_m (μM)	202.2	205.8
	V_{max} (μM/min)	1.1 x 10^3	1.2 x 10^3
	V_{max}/K_m (min^{-1})	54.95	59.17
	r^2	0.9978	0.9970
Endo-glucanase	K_m (mg/ml)	9.78	8.00
	V_{max} (mg/min.ml)	11.12	6.22
	V_{max}/K_m (min^{-1})	1.14	0.78
	r^2	0.9721	0.9877
Xylanase	K_m (mg/ml)	10.51	8.92
	V_{max} (mg/min.ml)	14.53	5.74
	V_{max}/K_m (min^{-1})	1.38	0.64
	r^2	0.9803	0.9954

The kinetic data were analyzed according to Michaelis–Menten kinetic model and the evaluation of the kinetic parameters was based on Hanes-Woolf plot. The experiments were conducted at least in triplicates and percent error in each set of readings was within 3%.

(Supplementary Table S1), following second order polynomial equations were obtained.

$$\%\ \text{activity recovery FPA} = 75.71 + 4.36\,A + 3.63\,B + 2.11\,C + 16.68\,AB - 1.61\,AC - 3.78\,BC - 15.87\,A^2 - 36.16\,B^2 + 6.97\,C^2 \quad (3)$$

$$\%\ \text{activity recovery } \beta\text{–glucosidase} = 87.93 + 5.53\,A + 16.43\,B + 2.35\,C + 11.09\,AB + 1.66\,AC - 5.12\,BC + 4.27\,A^2 - 44.11\,B^2 + 3.46\,C^2 \quad (4)$$

$$\%\ \text{activity recovery endoglucanase} = 55.24 + 2.98\,A + 8.81\,B + 2.70\,C + 13.30\,AB - 0.48\,AC + 1.49\,BC - 4.52\,A^2 - 25.58\,B^2 - 3.35\,C^2 \quad (5)$$

$$\%\ \text{activity recovery xylanase} = 38.81 + 3.19\,A + 7.39\,B + 0.45\,C + 6.39\,AB - 1.63\,AC + 0.21\,BC - 5.86\,A^2 - 18.50\,B^2 + 5.09\,C^2 \quad (6)$$

where A, B and C are BSA (mg/ml), glutaraldehyde (% w/v) and time (h), respectively.

The quadratic equation for each response were evaluated by *F*-test. Analysis of variance (ANOVA) performed on the equations revealed that the models were statistically significant with model *p* value (Prob > F) of <0.0001 for all the four models. Further multiple correlation coefficients (R-squared values) for each model were determined and it was found that predicted R^2 was in reasonable agreement with the adjusted R^2 (i.e., difference less than 0.2) (Supplementary Table S2). The models were further assessed by plotting three-dimensional response surface graphs which provide a means to visualize the interaction between these factors and estimate optimum levels of each of these factors for maximum response in terms of percent activity recovery. Representative RSM plots (Supplementary Fig. S1) show the interaction between the factors responsible for activity recovery of cellulase enzymes in terms of FPA in the form of CLEAs.

Based on the models developed using experimental results obtained by FCCCD, we found that 94.0 mg/ml of BSA, 0.87% (w/v) of