

Rapid Screening of Additive Formulations for Enhancing Xylanase Stability in Pulp Bleaching and Storage Conditions

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Enzyme deactivation is a significant parameter to restrain the industrial commercialization of enzymes. In this study, a high-throughput screening technique using differential scanning fluorimetry (DSF) incorporated with enzyme activity analysis was thus developed in order to identify a suitable additive formulation for promoting enzyme stability in terms of conformational stability and functional activity. To demonstrate the concept feasibility, endo-xylanase obtained from metagenome of termite gut-inhabiting bacteria (XYN12) was used as a study model and several group of additives including salts, polymers, polyols and sugars at various concentrations and different combination patterns (single, double and triple pairing) were applied for investigating their effects on enzyme stability. As a result, a total of 80 reaction mixtures were retrieved including a native control (enzyme without any additives). DSF assay was performed using real-time PCR instrument to detect unfolding of protein in the presence of a fluorescence dye. Accurate melting temperature (T_m) values of each enzyme formulation were calculated using Boltzmann equation and compared to the T_m of the native control in order to find ΔT_m . After that, the residual activity of xylanase in all reaction mixtures were measured using pHBAH method in automated liquid handling system. For single additives, glycerol had a stabilizing effect to the target enzyme, as observed by a significant increase in the ΔT_m , while $(\text{NH}_4)_2\text{SO}_4$, CaCl_2 , NaCl destabilized it. In addition, cocktail additives included glycerol, PEG4000 and trehalose dramatically enhanced the enzyme stability by positive shifting of T_m from 54 to 56 °C and increasing of relative activity by more than 4 folds compared to single additive. Storage stability of the enzyme was improved when mixed with this cocktail additive as indicated by remaining of the enzyme activity (86 %) after kept at 30 °C, pH 7.4 for 262 days, whereas native control lost almost of its activity within 40 days when stored at the same condition.

1. Introduction

Enzymes have been used for a long time in several applications and recognized as an alternative greener technology for reducing or replacing the use of harsh chemicals in the industrial processes (Masutti et al., 2012). Although enzymes are remarkable catalysts in terms of high catalytic and specific activity with ability to function under mild conditions, however they are generally unstable and can be inactivated rapidly by heat, chemical agents, autolysis (proteases) or ionic strength, resulting in enzyme denaturation during manufacturing, storage and application in industry. Generally, increase of the enzyme stability could be achieved by the addition of the protective and stabilizing additives such as salts, polyols, polyelectrolytes, bovine serum albumine (BSA), organic osmolytes, sugars and others. However, the selection of the suitable additive formulation relies on the nature of the enzyme and requires automated- and integrated- screening platform to develop the reliable and appropriate formulation at a record speed under a variety of stress conditions. In this study, the stability of β -1,4-endoxylanase enzyme obtained from metagenome of termite gut inhabiting microbes (XYN12) was of interest in order to use as a bleach boosting agent in pulp bleaching process (Nimchua et al., 2012). In enzymatic bleaching process, xylanases, particularly β -1,4-endoxylanase

are the core enzymes responsible for partial hydrolysis of xylan in pulp structure through the random cleavage of β -(1,4) glycosidic linkages of heteroxylan backbone (Ashikin et al., 2017), which leads to the removal of xylan in lignin-carbohydrate complexes (LCC) produced during pulping process. By eliminating of the LCC, the enzyme-pretreated pulp is thus more permeable to chemical bleaching, leading to a significant reduction in the dosage of chemicals required for bleaching with improved pulp qualities included brightness and strength. However, an aggressive operating conditions of pulp bleaching process at alkaline pH and relatively high temperature range are considered to affect the xylanase stability. Although the functional properties of this enzyme is most effective in the temperature range of 40-60 °C and pH 8-9 (Nimchua et al., 2012), but almost half of its activity lost within 20 min in these conditions. Therefore, the aim of this work was to improve the stability of the XYN12 during pulp-bleaching simulated conditions and long-term storage by using the high-throughput approach for rapid screening of appropriate additive stabilizers.

2. Experimental

2.1 Substrate and additives

Beechwood xylan purchased from sigma (Karlsruhe, Germany) was used as a substrate in all experiments. The salts, sugars and glycerol used in these experiments were obtained from Carlo erba (Val de Reuil Cedex, France). Poly (ethylene glycol) (PEG) with molecular mass of 3350 and 4000 was obtained from sigma-aldrich (Darmstadt, Germany).

2.2 Enzyme preparation

The enzyme was produced from *E. coli* Rosetta™ strain transformed with pET28a(+) (Invitrogen, USA) harboring gene encoded endo-xylanase obtained from metagenome of termite gut-inhabiting bacteria (XYN12).

To achieve high productivity of XYN12, high cell density fermentation of the *E. coli* expressing XYN12 was carried out in 5 L bioreactor by maintaining dissolved O₂ concentration at 30 % of saturation. The total gas flow rate was restricted between 1 and 2 VVM and the pH was controlled at 7.0 ± 0.2 by adding 25 % (v/v) NH₄OH and 10 % (v/v) H₃PO₄. All experiments were conducted at 37 ± 0.5 °C (growth and induction phases) by controlling the temperature of the water re-circulating in bioreactor jacket. After culturing, the cells were harvested from 1 L of culture broth by centrifugation at 7,000 rpm at 4 °C for 20 min, then re-suspended in 200 mL of 50 mM sodium phosphate buffer (pH 7.4), and disrupted using high pressure homogenizer (Microfluidizer/M-110P, PCL Holding, USA). Cell-free extract was prepared by centrifugation in order to separate cell debris at 19,000 rpm at 4 °C for 30 min. The target enzyme was further purified from the crude extract using a HisTrap column (GE Healthcare, USA) following the manufacturer's protocol. After that, the purified enzyme was concentrated on an Amicon Ultrafiltration Unit (Millipore, Billerica, USA) and determined the protein concentration by Bradford assay (Bio-Rad, USA) using bovine serum albumin (BSA) as a standard.

2.3 Enzyme activity assay

Xylanase activity was determined based on the amount of released reducing sugars by the Para-hydroxybenzoic acid hydrazide (pHBAH) method modified from Mellitzer et al. (2012). Briefly, the assay solutions contained 20 μ l of proper dilutions of the enzyme and 30 μ l of 1 % (w/v) of beechwood xylan in 100 mM Tris-HCl buffer, pH 8.5 as the substrate were transferred to a 96-well plate. The plates were then incubated at 55 °C for 10 min. The quantity of reducing sugars was determined by adding of 150 μ l of 1 % (w/v) pHBAH solution (prepared freshly by diluting a stock solution of 5 % (w/v) pHBAH (Sigma, USA) in 0.5 % (v/v) HCl with 0.5 M NaOH at a ratio of 1:4 v/v) into the reaction plates. After that, 120 μ l of the reaction mixtures were transferred to a new 96-well PCR plate and incubated at 95 °C for 5 min prior to cooling at 4 °C in PCR thermal cycler (Applied Biosystems, USA). The absorption in the reaction samples was investigated at 410 nm in a spectra MAX M5 (Molecular Device, USA). All 96-well pipetting steps were performed by robotic liquid handler (Multiprobe II PLUS EX, PerkinElmer, USA). One unit of enzyme activity was defined as the amount of enzyme which releases 1 μ mol of reducing sugar per minute under the assay conditions and was calculated by use of the formula:

$$\text{Activity (U/ml)} = \frac{\Delta OD_{410} \times D \times \frac{V_1}{V_2}}{m \times t}$$

Where ΔOD_{410} is the absorbance at 410 nm of the sample subtracted from blank, D is dilution factor, V_1 is total reaction volume (μ L), V_2 is enzyme volume, m is the slope of the standard curve (μ mol/ml) and t is the incubation time (min).

2.4 Thermal shift determination

To investigate the influence of additives towards enzyme thermal stability, 20 μ l reactions of 50X SYPRO (2 μ l) Orange dye (Sigma, USA), test additives (Table 1; 5-15 μ l), 1 mg/ml protein (2 μ l) and distilled water were mixed in a 96-well PCR plate. DSF assay was performed according to the protocol of Seabrook and Newman (2013) by heating the plates in RT-PCR (Bio-RAD, USA) under FRET (Förster resonance energy transfer) scanning mode at raising rate of 0.5 $^{\circ}$ C/min starting from 25 to 95 $^{\circ}$ C. The controls were prepared as the same manner using distilled water instead of test compound. The wavelengths at 470 and 570 nm were used for excitation and emission, respectively. The changes of fluorescence intensity in the reactions were detected simultaneously by a fluorescence reporter molecule at a particular wavelength in every cycle and was plotted as a function of temperature, generating a sigmoidal curve of two-state transition of enzyme. The temperature midpoint for enzyme unfolding transition (T_m) was calculated from Boltzman equation as follows:

$$y = I_0 + \frac{I_1 - I_0}{1 + e^{-\frac{T_m - T_x}{a}}}$$

where I_0 and I_1 are the values of minimum and maximum intensities respectively, whereas a is the slope of the curve within T_m . The T_m value measured from enzyme-additive mixture was compared with the T_m value of the control, and the difference in unfolding temperature (ΔT_m) was calculated. After DSF assay, all reaction mixtures were determined for the remaining xylanase activity using pHBAH method as described above. All samples were carried out in triplicate.

Table 1: List of additives used in the thermal shift assay

| Additive pairing | Additive group | Additive compound | Concentration |
|------------------|-------------------------------|---|--|
| single | salts | (NH ₄) ₂ SO ₄ , CaCl ₂ , or NaCl | 50, 100, 150, 200 and 250 mM |
| | sugars | Fructose (Fru), Maltose (Mal), or Trehalose (Tre) | 10, 20, 30, 40 and 50 mM |
| | polyol | Glycerol (Gly) | 5, 10, 15, 20 and 25 % (v/v) |
| | polymers | PEG3350 (P3350) or PEG4000 (P4000) | 0.1, 0.2, 0.3, 0.4 and 0.5 % (w/v) |
| double | glycerol and sugars | Fructose, Maltose or Trehalose | glycerol:sugar = 5 or 25 % (v/v): 10 or 50 mM |
| | glycerol and polymers | PEG3350 and PEG4000 | glycerol:polymer = 5 or 25 % (v/v): 0.1 or 0.5 % (w/v) |
| triple | glycerol, polymers and sugars | PEG3350 or PEG4000 and Fructose, Maltose or Trehalose | (v/v):0.1 or 0.5 % (w/v):10 or 50 mM |

2.5 Effect of additives on enzyme stability

The cocktail additive formulation (0.5 % w/v PEG4000, 25 % v/v glycerol and 50 mM trehalose) showed the best stabilizing effect on enzyme by means of positive shift of T_m and remaining activity was added to 1 mg/ml xylanase, pH 8.5 (50 mM Tris-HCl) for determination its efficiency in the pulp bleaching simulated conditions (pH 8.5, 45 $^{\circ}$ C for 1 h). Control was prepared in parallel at the same enzyme concentration without addition of additives. The residual activity was measured after incubation at 0, 10, 20, 30, 40, 50 and 60 min.

2.6 Storage stability of native and xylanase incorporated with additives

Enzyme samples containing 10 mg/ml xylanase was mixed with the selected additive solution (0.5 % w/v PEG4000, 25 % v/v glycerol and 50 mM trehalose). The reaction mixture was incubated in 50 mM sodium phosphate buffer, pH 7.4 at 30 $^{\circ}$ C, for 262 days Samples were drawn from the reaction mixtures at proper time intervals for enzyme activity assay. Control was prepared in the same manner without any additives. All samples were conducted in triplicate and the results were reported as mean values.

3. Results

3.1 Enzyme production

After high cell density fermentation, the *E. coli* expressed a high level of the alkalo-tolerant xylanase (activity of 10,179 u/ml and specific activity of 481 u/mg) on synthetic medium (José da Silva et al., 2013) incorporated with 80 g/L lactose as an inducer after 16 h of cultivation. The induced protein with His₆ tag was further

purified using His-trap column and the target protein fraction was eluted with 200 mM imidazole. The active fractions were pooled and concentrated by an Amicon Ultrafiltration Unit, resulted in a target enzyme yield of 8.63 % and purification fold of 8.13. The purified protein appeared as a single band of approximately 35 kDa against the protein marker on 10 % SDS-PAGE (Figure 1).

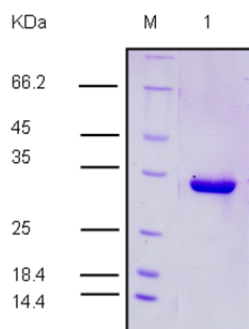


Figure 1: Analysis of the purified enzyme using SDS-PAGE (10 % polyacrylamide gel). Lane M protein marker, Lane 1 purified recombinant xylanase.

3.2 Additive screening

In this study, four additive groups at different concentrations and combination patterns (Table 1) which generated a total of 80 condition treatments have been investigated for the stabilizing effect towards the protein using DSF and pHBAH assays. Among the single additives tested, glycerol was the only compound that gave the greatest ΔT_m value (≥ 0.5 °C; Figure 2A), while other additives gave a positive shift of ΔT_m value less than 0.5. All tested salts including $(\text{NH}_4)_2\text{SO}_4$, CaCl_2 , NaCl induced a negative shift of ΔT_m value (data not shown). A positive ΔT_m can be linked to an increase of conformational compactness, while negative ΔT_m displays a sign of misfolding. As the result in enhancing of protein stabilization by single additives is rather limited, thus a new approach that use of cocktail additives by combining more than one compound to significantly increase the protein stability has been demonstrated. In cocktail approach, glycerol, the best stabilizer in single additives was combined with the other 2 groups of additives (polymers and sugars) in double and triple pairing, which allowed approximately 34 treatment conditions to be investigated. After screening, only 13 of 80 additive formulations were found to stabilize the enzyme by increasing of ΔT_m (≥ 0.5 °C; Figure 2A) and the relative activity of enzyme (≥ 100 %; Figure 2B). Of all combined formulations tested, almost of the triple formulations (no. 9-13; Figure 2A) showed the maximal ΔT_m of 2 °C, while double additive mixtures (no. 2-7; Figure 2A) showed relatively less ΔT_m values, indicated that triple combinations appear to be more favourable for protein stabilization. Among the triple mixtures, the formulation containing glycerol, PEG4000 and trehalose gave the highest relative activity (442.2 ± 6.9 %; Figure 2B) compared to the control, thus it was selected for study its efficiency in stabilization of the enzyme in pulp-bleaching simulated conditions and storage.

3.3 Effect of the selected additive mixture on xylanase stability

The stabilizing efficiency of the selected cocktail additive (0.5% w/v PEG4000, 25% v/v glycerol and 50 mM trehalose) on the activity of enzyme at pulp bleaching simulated condition was investigated with respect to the temperature of 45 °C and pH 8.5. The enzyme activity of native and additive-combined enzyme was measured by pHBAH assay after incubation at 10 min-intervals for 1 h. After adding the cocktail additive, the activity of enzyme revealed that the use of additives led to an increase of xylanase activity by approximately 30 % compared to the activity of control sample before starting the incubation (Figure 3). In addition, almost of the enzyme activity was rapidly loss (more than 90%) after incubation at 45 °C for 40 min in the enzyme without additives, whereas no sign of inactivation of xylanase activity was observed in additive incorporated enzyme (Figure 3). The obtained results showed consistent trend as reported in the study of Börjesson et al. (2006) that revealed the synergistic interactions of enzyme and its additive formulations by increasing the enzymatic hydrolysis of lignocellulose. In fact, addition of polyols, sugars and polymers to liquid solutions of enzymes has been demonstrated in enhancing strength of the hydrophobic interactions among non-polar amino acid residues, leading to protein stiffening and promoting the enzyme stability against operating environments. In this study, the result suggested that the presence of the synthesis additive mixture has a positive stabilizing effect on enzyme, making it more resistant to unfolding environments, particularly industrial pulp-bleaching process.

3.4 Storage stability of xylanase

The storage stability of native and enzyme incorporated with the additives (0.5% w/v PEG4000, 25% v/v glycerol and 50 mM trehalose in 50 mM Sodium phosphate buffer, pH 7.4) was investigated at 30 °C for 262 days. The activity profile of xylanase showed that negligible activity was detected from native enzyme after 40 days of storage, while additive mixture treated xylanase remained a relative activity of more than 86 % throughout the preserved time. The remarkable increase in storage time by the designed additive mixture might be explained by a preferential exclusion theory, proposes that the addition of additives such as PEG4000, glycerol and trehalose to the bulk water solution secludes water molecules away from the enzyme surface, leading to reduced hydration radius and increased compactness of the enzyme molecules, consequently stabilizing effect.

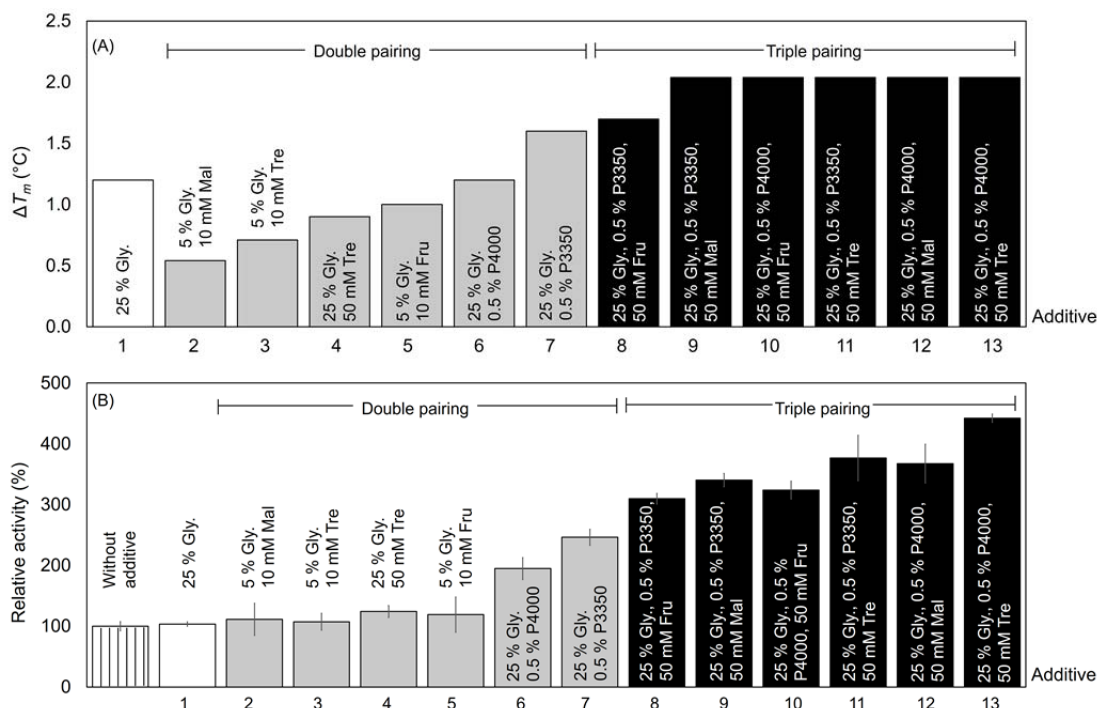


Figure 2: (A) Difference of the midpoint temperature (ΔT_m) and (B) relative activity of XYN12 in the selected additive formulations that showed $\Delta T_m \geq 0.5$ °C and relative activity $\geq 100\%$. For abbreviation, see Table 1.

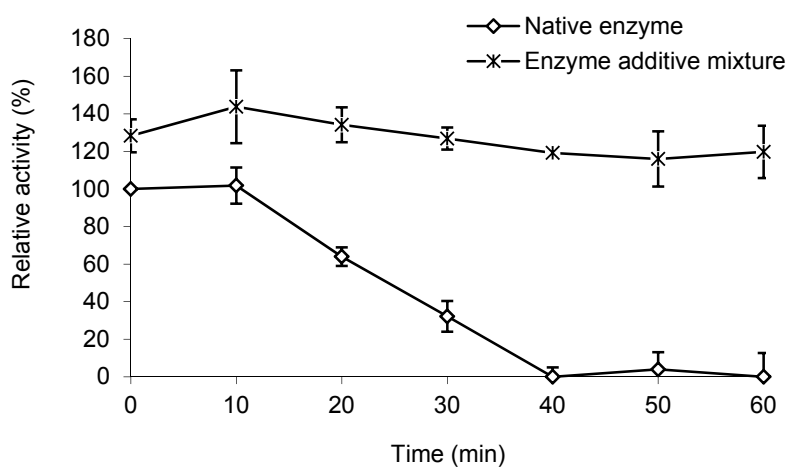


Figure 3: Activity profile of xylanase in presence and absence of additive mixture at pulp bleaching simulated conditions (pH 8.5 and 45 °C for 1 h)

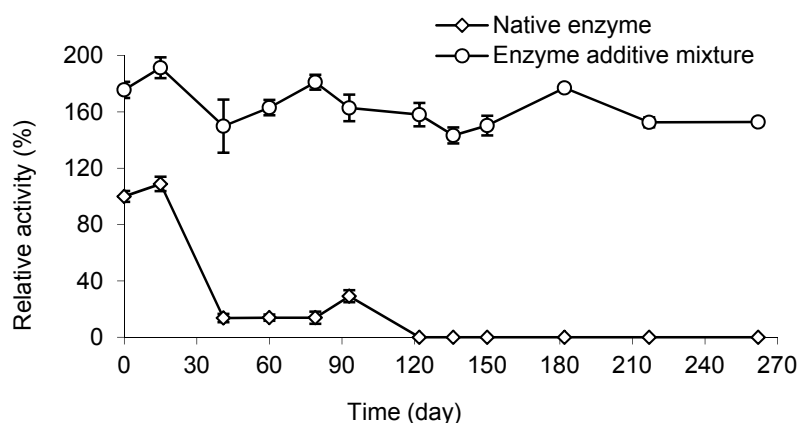


Figure 4: Activity profile of xylanase in presence and absence of additive mixture at storage conditions (50 mM sodium phosphate buffer pH 7.4 and 30 °C for 262 days)

4. Conclusions

This research assesses the stabilization efficiency of several additives in single and cocktail combinations on conformation and biological function of the alkalo-tolerant xylanase obtained from termite gut using DSF incorporated with activity analysis. The DSF assay recognized as a rapid and simple method, was successfully established to carry out a high-throughput screening technique for potential additives that helps to stabilize and enhance the enzyme activity. The data obtained from DSF assay can be used in complement with the data gathered from pHBAH method, where the same set of additives was applied to determine the residual activity. By co-considering of the DSF and pHBAH datasets, additive formulations showing positive result in both assays become the potential candidates for further study in stabilizing the enzyme at operating and storage conditions. In this study, compared to single additive approach, the DFS and pHBAH results revealed that the triple pairing of glycerol with polymers and sugars had a dramatic stabilizing effect, increasing the ΔT_m by up to 2 °C and the activity of at least 300 %. The best stabilizing performance in pulp-bleaching simulated conditions was observed from the additive mixture comprising glycerol, PEG4000 and trehalose. Correspondingly, it appeared to be appropriate for long-time storage stabilization of the target enzyme at 30 °C. Therefore, this result clearly establishes the proof of principle that the “cocktail approach” in which more than one additive are used, is a potential method to efficiency enhance the thermal stability and prevent the activity loss of protein in both pulp-bleaching simulated and storage conditions.

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