CAFEi2016-142

Immobilization of cyclodextringlucanotransferase from *Bacillus maceranson* bleached kenafbast micro-fibre

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Abstract

Enzyme immobilization is the process of enzyme attachment on solid support that enables repetitive use of enzyme. In this study, kenafbast fibre was bleached with acidified sodium chloriteuntil7 to 13µm in diameter. Then it was functionalized with hexamethylenediamineandglutaraldehyde to form spacer arm and ligand, respectively. Cyclodextringlucanotransferase (CGTase) from *Bacillus macerans*was immobilized *via*couplinginteraction with ligand onmicrofibre surface. The results show that protein loading and enzyme activity recovery were 27.60% and 21.19%, respectively when 54.196 U/ml of CGTase was used. Besides, storage stability of the immobilized CGTase at 4°C, 25°C and 60°C resulted in 60%, 40% and 15% from its initial activity after 15 days storage. The optimum operating temperature shifted from 60°C to 70°C compared to free CGTase. For reusability study, the immobilized CGTase was able to retain 33% from its initial activity after 7 cycles of batch reaction. Therefore, bleached kenafbast microfibre has high potential to be applied in enzyme immobilization. This can contribute in building sustainable environment as less energy is needed for enzymatic processes in industries. Problems such as effluent disposal and wastage of enzymes can also be eliminated by using the immobilized enzymes developed in this study.

Keywords: immobilization, cyclodextringlucanotransferase, kenafmicrofibre, glutaraldehyde, hexamethylenediamine

INTRODUCTION

Cyclodextringlucanotransferase (CGTase) is a type of amylolytic enzyme, which belongs to α-amylase family. It is able to catalyze up to 4 reactions, which are disproportionation, hydrolysis, coupling and cyclization (Martin et al. 2003). CGTase is found to be produced by many bacteria especially from those of bacillus genus such as *Bacillus circulans, Bacillus macerans, Bacillus agaradhaerans, Bacillus firmus*and others (Cao et al., 2005). In cyclization, the enzyme reacts with starch to produce cyclic compounds known as cyclodextrins (CDs) that has wide applications in the field of cosmetics, analytical chemistry, pharmaceutical and food. CDs consist of hydrophobic internal cavity that allows them to form inclusion complex with other molecules (Astray et al., 2009). The most typical examples of CDs are α-CD, β-CD and γ-CD, which are made up of 6, 7 and 8 D-glucapyranose units, respectively (Goh et al. 2009).

Kenaf, Hibiscus cannabinus is a type of plant from the Malvaceae family. It can produce bast fibresand core fibres, which comprises 35% and 65% of the plant, respectively (Khalil et al., 2010). Kenaf has been drawing much attention because of its application such as in paper making and in production of required parts and components for automobile (Saraswati and Mahanum, 2008).

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It has been identified that the strength properties of kenafbast fibre are high (Ajiet al. 2009). Besides, kenafbast fibre is also hydrophilic and non-toxic. All these characteristics have proven kenafbast fibre as potential support matrix in enzyme immobilization.

In industrial biotechnology, enzyme stabilityis usually achieved by immobilization because it is simple, reliable and not very costly (Lyeret al., 2003).When the enzyme isimmobilized, itsincreased rigidity can help in preserving itsproperties, which may in turn retains their stability under changed conditions (Rodrigues et al., 2013).One of the most popular methods in immobilization is covalent immobilization as it minimizes enzyme to release from the support.Applying covalent enzyme immobilization on bleached kenafbast fibre is believed to have better efficiency because the microsized bleached fibre can provide a larger surface area per volume for the immobilization and thus it increases the loading rate of enzyme per unit mass (Jiaet al., 2003). Due to this, the enzyme immobilized on bleached kenafbastfibre is expected to show good performance and characteristics. The main objective of this research is to study the characteristics and the performance of cyclodextringlucanotransferase immobilized on bleached kenafbast microfibre.

MATERIALS AND METHODS

Materials

Kenafbast fibre was kindly contributed by Lembaga Kenaf & Tembakau Negara (LKTN) Malaysia .Hexamethylenediamine and sodium methoxide were bought from Sigma-Aldrich (M) Sdn. Bhd., and glutaraldehyde was bought from Ajax Finechem Pty. Ltd. (Australia). CGTase from *Bacillus macerans*was purchased from Amano Enzyme Inc. (Japan). Water soluble starch powder (potato starch) was purchased from Fluka. Sodium chlorite and α-CD were from Acros Organics (US), and methyl orange was from Systerm.

Bleaching of kenaf bast fibre

Kenafbast fibre was bleached by following the method mentioned by Tee et. al.(2013)untilits size, ranging from 7 to 13 μm was obtained which was visualized by Dino-Lite (Model: AM-4113T5X, ANMO Electronics Corporation, Taiwan). The bleached microfiber was then dried at 105°C for 1 day.

Support activation and enzyme immobilization

Bleached Kenafbast microfibre was firstly coupled with hexamethylenediamine that act as spacer arm according to the procedures described by Chang et. al. (1987). After that the fiber coupled with spacer was added into 0.5% (w/v) glutaraldehyde in phosphate buffer (0.1M, pH 8.0) and the mixture was stirred at 4°C for 12 hours. The activated fibre was then washed with distilled water until it is clear of unreacted glutaraldehyde. Then 2g activated microfibre was added to 100 ml phosphate buffer (0.05M, pH6.0), containing 5419.6 U CGTase. The mixture was stirred for 12 hours at 4°C.

Bound enzyme determination

After immobilization, the supernatant was collected. The support was washed with buffer until no protein was detectable in the washed solution. The amount of bound enzyme was calculated based on amount of enzyme added (mg) minus total amount of enzyme (mg) in collected supernatant and washed solutionafter being determined by Bradford analysis at 595 nm with bovine serum albumin as the standard protein to construct the calibration curve.

Enzyme activity assay

CGTasefrom *B. macerans*produces mainly α-CD. To analyze the presence of α-CD, methyl orange was used by following the procedures described by Lejeune et al. (1989). Free and immobilized CGTase were added to 4 ml of 5% starch solution in phosphate buffer (0,05M, pH 6.0) at temperature 60°C. The reaction was incubatedfor 10 minutes and then195 μl of 6.0M HCL was added to stop the reaction. After that, 5.73 ml of methyl orange solution (0.061mmol/L) was added to the mixtureand the tube wascooled down at 16°C for 10

minutes. The absorbance of the mixture was then measured at 505 nm using UV-VIS spectrophotometer. The definition of the enzyme activity; 1unit CGTase activity is equal to 1 micro mole of α-CD produced per minute.

Effect of Temperature

Free and immobilized CGTase were added to 4 ml of 5% starch solution in phosphate buffer (0.05M, pH 6.0)at temperatures ranging from 30°C to 90°C. Then the enzyme activity was assayed as described above.

Operational Stability

20mg kenafbast microfiber that contains immobilized CGTase was added to 6ml of 5% starch solution in phosphate buffer (0.05M, pH 6.0) at 60°C. The mixture was incubated for 30 minutes, and then it was centrifuged at 10000rpm for 10 minutes. Then the supernatant was collected for α-CD analysis while the immobilized CGTase (with microfibre) was washed with distilled water and re-incubated in newly prepared starch solution as a new cycle. The procedures were repeated until 7 cycles were achieved.

Storage Stability

The immobilized CGTase was stored at different temperatures (4°C, 25°C and 60°C). Enzymatic activity was then assayed at initial, day 1, day 2, day 6, day 8, day 11, day 14 and day 15.

RESULTS AND DISCUSSION

Analysis of KenafBast Micro Fibre Size

Figure 1 shows the size of Kenafbast microfibre under the magnification using Dino-Lite. The analysis shows that the diameter of the microfibre after bleaching is 0.008 mm which is equivalent to 8 µm. This falls in the range of 7 to 13µm which is the average size of the microfibre obtained in this work.

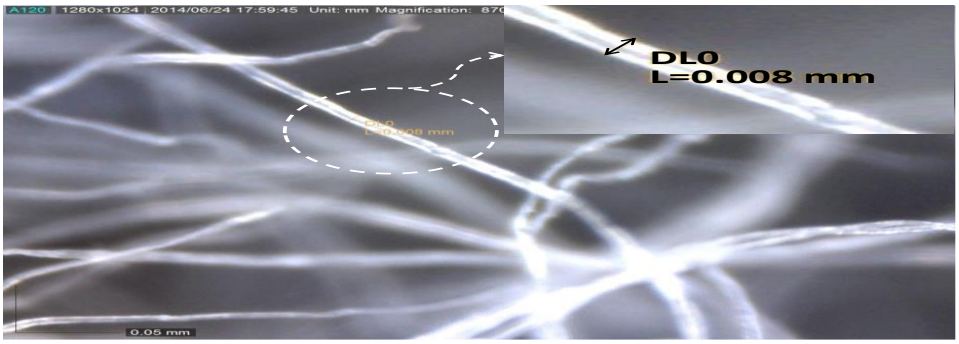

Figure 1. Size of bleached Kenafbast fibre under Dino-Lite (200 X).

Protein Loading and Activity Recovery

From Table 1, it clearly shows that the protein loading for immobilized CGTase is 27.60%. Meanwhile, the enzymatic activity that retained from free CGTase after immobilization was 21.19%. The reduction in enzymatic activity can be caused by the steric hindrance resulted during the immobilization process. As a result, diffusional restriction will occur and this will prevent the binding and reaction of the substrate molecules with the active site of the enzyme, and thus it decreases the activity of the immobilized CGTase (Tardioli et al., 2006 and Amud et al., 2008).

Table 1. Protein loading and activity recovery of immobilized CGTase.

Properties	Value
Protein added, mg (a)	80.00

Protein Loaded, mg (b)	22.08
Protein loading, % (b/a x100)	27.60
Enzyme Added, U (c)	5419.60
Unbound Enzyme, U (d)	3884.54
Immobilized Enzyme Activity, U (e)	325.23
Activity Recovery, % (e/(c-d) x 100)	21.19

Storage Stability

Storage stability of immobilized enzymes is a key feature showing relative importance for itsapplication in industry (Prousoontornet et al., 2007). Table 2 shows the result of storage stability for free CGTase and CGTase immobilized on Kenafbast microfibre with the use of hexamethylenediamine and glutaraldehyde as spacer arm and ligand, respectively. It clearly shows that at 4°Cand 25°C, the activity retention for free CGTase is better because the amount of α-CD produced by free CGTase at these 2 temperatures after 15 days storage were relatively higher than immobilized CGTase. However under 60°C storage, the activity retention for immobilized CGTase was better than free CGTase. The possible reason for this is that at low storage temperature, a small amount of lysis from immobilized CGTase occurs due to slight corrosiveness of glutaraldehyde at low concentration. However at high storage temperature, free CGTase denatured very significantly compared to immobilized CGTase that has higher thermal stability after immobilization.

Table 2. Storage stability of free and immobilized CGTase at different temperatures.

Storage Time (Day)	Amount of α-CD produced (μmol)					
	By Free CGTase			By Immobilized CGTase		
	4°C Storage	25°C Storage	60°C Storage	4°C Storage	25°C Storage	60°C Storage
0 (Initial)	8.80±0.00	8.80±0.00	8.80±0.00	8.68±0.10	8.68±0.10	8.68±0.10
6	8.61±0.14	7.68±0.07	1.08±0.09	7.01±0.11	6.32±0.13	2.99±0.10
11	8.17±0.21	7.04±0.03	0.00±0.00	6.04±0.10	4.79±0.29	1.88±0.29
15	8.56±0.19	6.85±0.05	0.00±0.00	5.28±0.21	3.33±0.22	1.25±0.00

Temperature Profile

Figure 2 shows the effect of temperature on free CGTase and immobilized CGTase activity. From the results, there is a shift in optimum temperature from 60°C of free CGTase to 70°C of immobilized CGTase. Besides, the relative activity of the immobilized CGTase at temperatures after the optimum point is slightly higher than free CGTase. This is because at high temperature, most of the free CGTase denatured while the rate of denaturation for immobilized CGTase is lower due to the increased rigidity after immobilization (Kim et al., 2006).

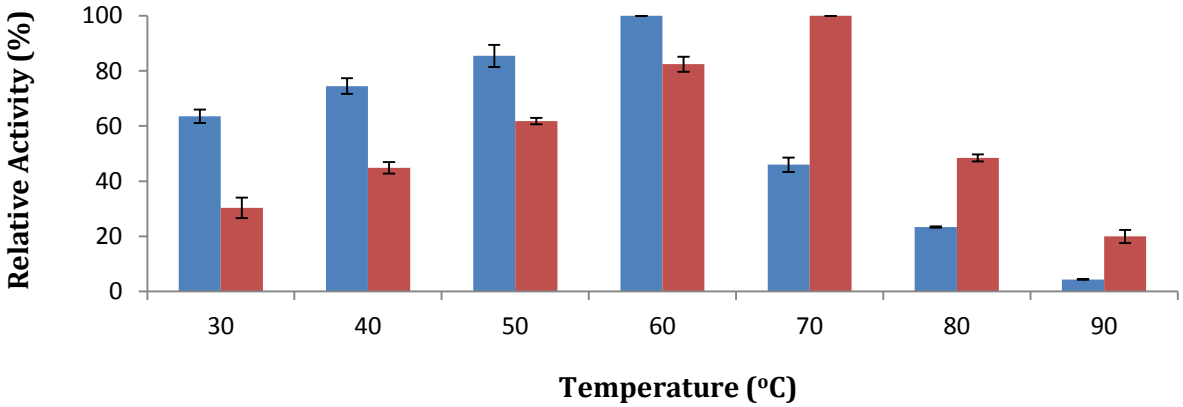

Figure 2. Effect of temperature on free and immobilized CGTase activity.

Reusability

Reusability or operational stability of enzymes is another important feature as it can help the industries to save production cost. From Table 3, it can be observed that the initial amount of α-CD produced by the immobilized CGTase was 9.93 μmol. The immobilized CGTase was then used in 6 other repeated batches and it was found out that at the end of 7th cycle, the enzyme was still capable of producing 3.27 μmol of α-CD, which is equivalent to 33% of the initial production. This retention of activity has shown the advantages of using immobilized enzyme as free enzyme can only be used for 1 time. Besides, using immobilized enzyme does not require tedious separation process to obtain higher product purity in industries unlike the use of free enzymes.

Table 3. Reusability of the immobilized CGTase.

Cycle Number	Amount of α-CD produced (μ mol)
1	9.93±0.34
2	8.18±0.09
3	6.72±0.09
4	5.45±0.17
5	4.66±0.26
6	3.82±0.26
7	3.27±0.00

CONCLUSIONS

The following conclusions can be drawn from the study:

- The immobilization of CGTase on bleached Kenafbast microfibre with the use of hexamethylenediamine and glutaraldehyde as spacer arm and ligand, respectively, was achieved successfully.
- Storage stability study has shown that free CGTase has better storage stability than immobilized CGTase at 4°C and 25°C but when it comes to high storage temperature such as 60°C, the storage stability of immobilized CGTase is better compared to free CGTase.
- Besides, the immobilized CGTase can sustain in higher operating temperature, comparing to free CGTase.
- In addition, good performance in enzyme reusability in this work has provided potential for the use of Kenafbast microfibre as enzyme immobilization support.

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