



Co-immobilization of pullulanase, glucoamylase and glucose isomerase together with cross-linked enzyme aggregates: Fructose conversion from three enzymes and starch in one step

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Received: 03 May 2018, Revised: 07 June 2018; Accepted: 10 June 2018

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ABSTRACT

In this study, in order to obtain fructose syrup from starch, pullulanase (PUL), glucoamylase (GA) and glucose isomerase (GI) were immobilized together with cross-linked enzyme aggregates (CLEAs), and named as 'combi-CLEAs'. The values of V_{max} and K_m from the kinetic parameters of combi-CLEAs [(GA + PUL) + GI] were found as 12 U g⁻¹ and 0.1157 mg ml⁻¹ in optimum conditions, respectively. After 50 uses, it was observed that the remaining activity of combi-CLEAs [GA + PUL + GI] was preserved at 46%. The thermal stability of combi-CLEAs [GA + PUL] + GI] was observed to be 44% and 30%, respectively, at 60 and 80 °C after 24 hours. At the end of 30 days, the storage stabilities of combi-CLEAs [(GA + PUL) + GI] was determined as 56% and 13% at the temperatures of 4 and 25°C, respectively.

Keywords: Combi-CLEAs, fructose syrup, enzyme immobilization.

Pullulanaz, glukoamilaz ve glukozizomerazın çapraz bağlı enzim agregatları ile beraber immobilizasyonu: Tek basamakta üç enzim ile nişastadan fruktoz dönüşümü

ÖZ

Bu çalışmada nişastadan fruktoz şurubu elde etmek için pullulanaz (PUL), glukoamilaz (GA) ve glukozizomeraz (GI) çapraz bağlı enzim agregatları (CLEAs) ile beraber immobilize edildi ve 'combi-CLEAs' olarak isimlendirildi. Combi-CLEAs [GA + PUL] + GI'nin optimum şartlardaki V_{max} and K_m değerleri sırasıyla 12 U g⁻¹ ve 0,1157 mg ml⁻¹ olarak bulundu. Combi-CLEAs [GA + PUL] + GI'nin 50 kullanımdan sonra kalan bağlı aktivitesinin % 46' da koruduğu gözlemlendi. 24 saat sonunda, 60 ve 80 °C' de Combi-CLEAs [GA + PUL] + GI'nin termal kararlılığının sırasıyla % 44 ve % 30 olduğu gözlemlendi. 30 günün sonunda, Combi-CLEAs [GA + PUL] + GI'nin 4 ve 25 °C' deki depolama kararlılığı ise sırasıyla % 56 ve % 13 olarak belirlendi.

Anahtar Kelimeler: Combi-CLEAs, fruktoz şurubu, enzim immobilizasyonu.

1. INTRODUCTION

Pullulanase (pullulan 6-glukanohidrolaz, EC 3.2.1.41) is an enzyme which hydrolyzes the pullulan, starch, amylopectin, and β -limit dextrin, α -1,6 glycosidic bonds.¹ Whereas glucoamylase converts starch into glucose, glucose isomerase converts glucose into fructose.²

In the food industry, in order to obtain fructose syrup, first the glucose syrup is obtained from starch, and then fructose syrup is obtained from glucose syrup. In the industry, this process is done in three stages. In the first stage, the starch is gelatinized by using alpha amylase

(EC 3.2.1.1) or pullulanase (EC 3.2.1.41). In the second stage, glucose syrup is obtained by using glucoamylase (EC 3.2.1.3). In the third stage, fructose syrup is obtained from glucose by using glucose isomerase.³

Free enzymes used commercially are for single use and have limited usage areas. With using of enzymes multiple times, the enzyme immobilization technology provides great financial advantages for the industrialists in terms of economic sense.

Recently, several studies related to the immobilization of two or three enzymes by combi-CLEAs (combined cross-linked enzyme aggregates) have been conducted.⁴⁻⁸ In the combi-CLEAs, the product ge-

nerated by an enzyme is substrate of another enzyme.

Generally in combi-CLEAs, by using inorganic salts and organic solvents, two or more enzymes are precipitated together without denaturation. Then, using dialdehyde or glutaraldehyde, the aggregates are connected.⁹

In the literature, there are some studies in which the enzyme has been immobilized by using different methods to obtain fructose syrup.^{4,11-13} For example, in the previous studies focusing on immobilization of enzyme to obtain fructose syrup, there has been no data related to storage stability, thermal stability and residual relative activity from the number of reuse, so far.^{4,12,13} In this study, it was aimed to obtain fructose syrup from starch by obtaining co-immobilization of pullulanase (PUL), glucoamylase (GA) and glucose isomerase (GI) together with CLEAs. Moreover, the storage stability and thermal stability of combi-CLEAs [(GA + PUL) + GI] and residual relative activity from the number of reuse were investigated. Because, it is very important that the immobilized enzymes to be used in industry are economical, reusable, able to be stored for a long time, and resistant at high temperatures.

2. MATERIALS AND METHODS

2.1. Materials

The mixture of glucoamylase and pullulanase enzymes and *Streptomyces Rubiginosus* Glucose Isomerase were taken from Sonar Company as a gift. All reagents used in the research were analytical purity and were taken from Merck or Sigma (St. Louis, MO) companies.

2.2. Methods

2.2.1. Preparation of combi-CLEAs [(GA + PUL) + GI]

The different amounts of (GA + PUL) and GI enzymes were mixed and dissolved in phosphate buffer, and then precipitated by adding ammonium sulphate. After the precipitation, glutaraldehyde was added, and it was kept in the water bath. Then, combi-CLEA [(GA + PUL) + GI] was washed and stored at 4°C for later use.

2.2.2. Determination of the relative activity of CLEAs [(GA + PUL) + GI]

The relative activity of combi-CLEAs [(GA + PUL) + GI] was calculated by measuring the amount of fructose in the relative activity of glucose isomerase. This was determined by measuring the amounts of fructose occurring in the reaction according to Resorcinol-HCl method used by Tolsma.⁷ One unit (U)

was calculated as the amount of enzyme that converts 1 μ mol glucose into fructose in 1 min.

2.2.3. Determination of the optimum temperature of combi-CLEAs [(GA + PUL) + GI]

The activities of combi-CLEAs [(GA + PUL) + GI] were measured by preparing at five different temperatures of 40, 50, 60, 70 and 80°C, and the temperature showing the maximum relative activity was determined.

2.2.4. Determination of thermal stability of combi-CLEAs [(GA + PUL) + GI]

Combi-CLEAs [(GA + PUL) + GI] was incubated for different time intervals (1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 24) at 60 and 80°C and remaining activities were determined.

2.2.5. Determination of storage stability of combi-CLEAs [(GA + PUL) + GI]

After the initial activities were determined, the samples of combi-CLEAs [(GA + PUL) + GI] were kept at 5 and 25°C for 1 month and their remaining activities were measured at regular intervals.

2.2.6. Determination of reuse stability of combi-CLEAs [(GA + PUL) + GI]

2 ml of starch solution of 1.5% was added to the column having different amounts of combi-CLEA [(GA + PUL) + GI] and after 30 minutes, the column was drained by opening the underlying tap. The relative activity of the enzyme was calculated by measuring the amount of the emerging fructose. After 20 second following the draining of the column, 2 ml of substrate solution was added to the column, and the relative activity was determined in the same manner. The remaining relative activity was calculated as the percentage of the relative activity in the first usage.

3. RESULTS AND DISCUSSION

In this study, pH, temperature, thermal stability and reuse parameters of combi-CLEA [(GA + PUL) + GI] were investigated.

3.1. Optimization of immobilization conditions of combi-CLEAs [(GA + PUL) + GI]

Optimization of immobilization conditions were performed so that combi-CLEAs [(GA + PUL) + GI] could show maximum relative activity. The immobilization environment, in which the final concentration of ammonium sulphate used as a precipitation to determine the optimum concentration of

ammonium sulphate was prepared as 20%, 30%, 40%, 50%, 60%, and 70% (w/v). The ammonium sulphate concentration showing the highest relative activity was determined as 40% (w/v). The results obtained are shown as relative activity (%) in Figure 1.

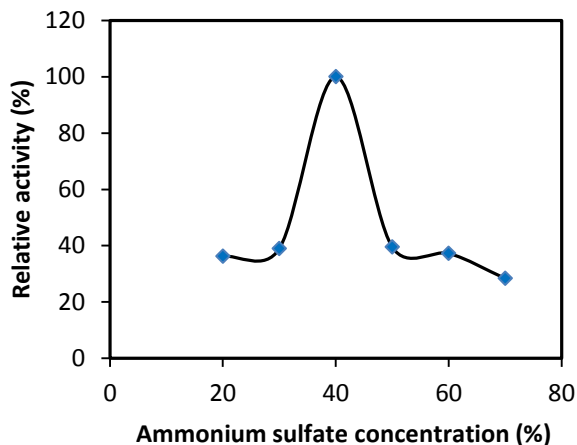


Figure 1. Relationship between the ammonium sulphate concentration in the immobilization medium and the activities of combi-CLEAs [(GA + PUL) + GI].

In order to identify the optimum immobilization time of combi-CLEAs [(GA + PUL) + GI], at the end of 1-24-hours immobilization process, combi-CLEAs were prepared. The immobilization time showing the highest combi-CLEA [(GA + PUL) + GI] relative activity was obtained was determined as 5 hours. The results obtained are shown as relative activity (%) in Figure 2.

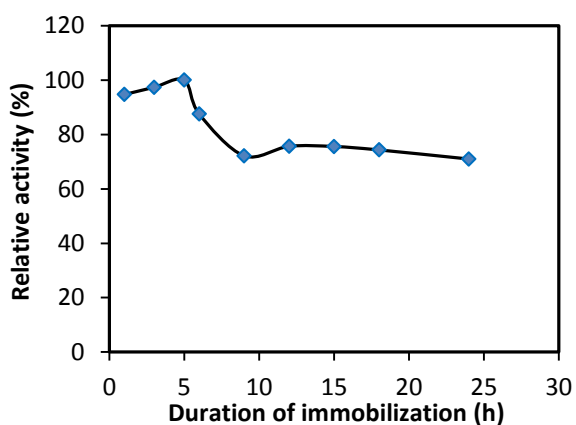


Figure 2. The effect of immobilization time on combi-CLEAs [(GA + PUL) + GI].

In order to identify the optimum glutaraldehyde concentration, the samples of combi-CLEAs [(GA + PUL) + GI] were prepared by using glutaraldehyde at the levels of 12.5%, 25%, 30%, 40% and 50%. The glutaraldehyde concentration indicating the highest combi-CLEAs [(GA + PUL) + GI] relative activity was

determined as 25%. The results obtained are shown as relative activity (%) in Figure 3.

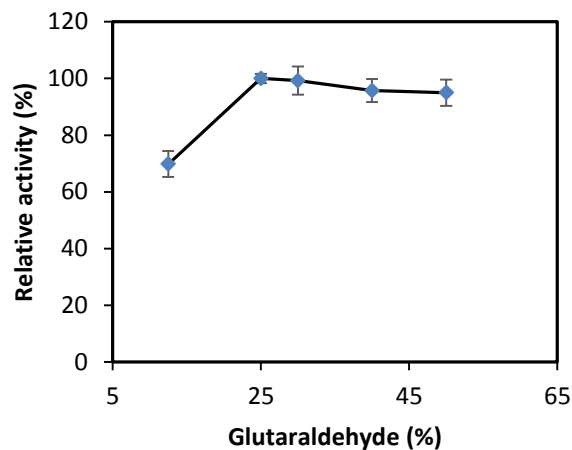


Figure 3. Relationship between glutaraldehyde concentration in the immobilization medium and the activities of combi-CLEAs [(GA + PUL) + GI].

The change of combi-CLEAs [(GA + PUL) + GI] relative activity depending on pH was measured by using buffers in different pH values at 60°C. The pH value showing the maximum relative activity of combi-CLEAs [(GA + PUL) + GI] was determined as 6. The results obtained are shown as relative activity (%) in Figure 4.

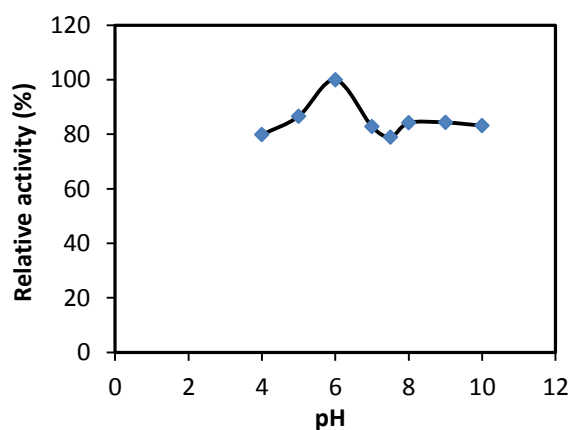


Figure 4. The pH stability of combi-CLEAs [(GA + PUL) + GI] at different pH values.

The effect of temperature on the change of combi-CLEAs [(GA + PUL) + GI] relative activity was studied at five different temperatures of 40, 50, 60, 70, and 80°C, and the temperature showing the maximum relative activity was determined as 60°C. The results obtained are shown as relative activity (%) in Figure 5.

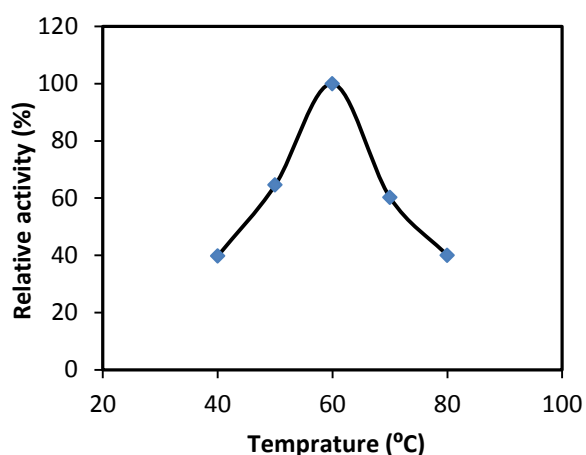


Figure 5. The effect of temperature on combi-CLEAs [(GA + PUL) + GI] relative activity.

The thermal stability of combi-CLEAs [(GA + PUL) + GI] was determined by measuring the remaining activities at two different temperature (i.e. at 60 and 80°C) as a function of time. The results obtained are shown as relative activity (%) in Figure 6. From Figure 6, it is observed that thermal stability is decreased as the temperature is increased. At the end of 24-hours incubation at 60°C, while the remaining relative activity of combi-CLEAs [(GA + PUL) + GI] was approximately 44% of the initial relative activity, its remaining relative activity at 80°C was found to be approximately 30% of the initial relative activity. These values showed that the thermal stability of combi-CLEAs [(GA + PUL) + GI] was very high.

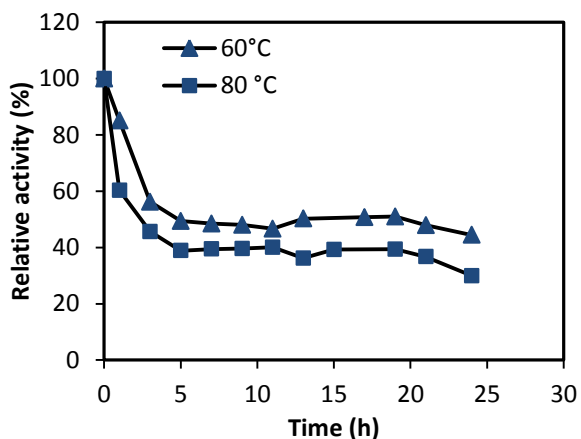


Figure 6. Change of combi-CLEAs [(GA + PUL) + GI] relative activities depending on thermal stability.

In order to determine storage stability of combi-CLEAs [(GA + PUL) + GI], the samples were held at 4 and 25°C, their activities were measured at certain days for 30 days, and their relative activities were calculated. The results obtained are shown in Figure 7. The remaining activities of combi-CLEAs [(GA + PUL) + GI]

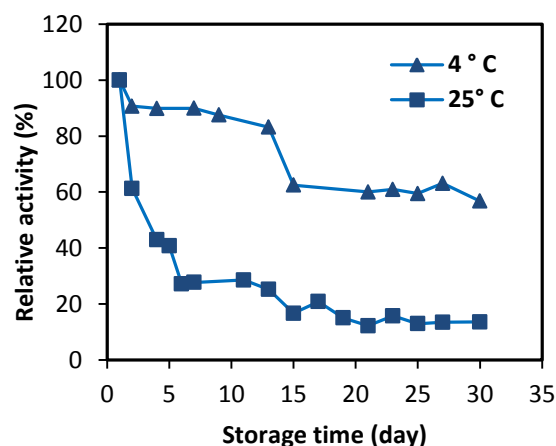


Figure 7. Relationship between combi-CLEAs [(GA + PUL) + GI] relative activity and storage time at different temperatures.

held at 4 and 25°C for one month were found as 56% and 13%, respectively. The high storage capability of combi-CLEAs [(GA + PUL) + GI] provides great economic advantages for the industrialists.

The number of reuse of combi-CLEAs [(GA + PUL) + GI] was determined by using the buffer of pH 6 at 60°C. Depending on number of reuse, the change in relative activity of combi-CLEAs [(GA + PUL) + GI] is shown in Figure 8. After 50 usage, it was seen that combi-CLEAs [(GA + PUL) + GI] was able to maintain the 46% of the initial relative activity. This result is a very big advantage for the industry.

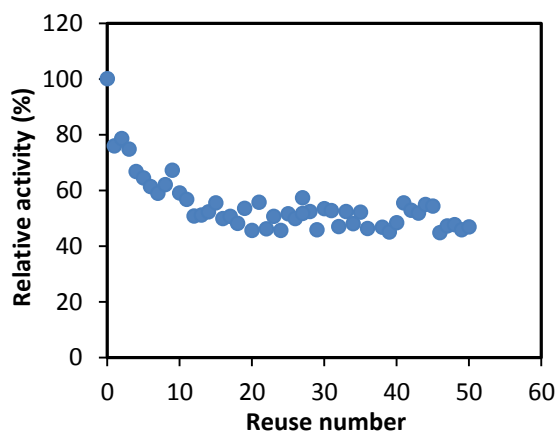


Figure 8. Relationship between the relative activity of combi-CLEAs [(GA + PUL) + GI] and reuse number.

3.2. Kinetic parameters

From the kinetic parameters of combi-CLEAs [(GA + PUL) + GI], V_{max} and K_m were calculated by means of Eq. (1). This equation is known as Michaelis-Menten equation¹⁴ as shown in the following.

$$V = V_{\max} [S] / K_m + [S] \quad (1)$$

where, $[S]$ is the concentration of substrate S, V_{\max} is the maximum velocity achieved by the system, at maximum (saturating) substrate concentrations. K_M is the Michaelis constant indicating the substrate concentration at which the reaction velocity is 50% of the V_{\max} .

Herein W_{\max} and K_m were found as 12 U g^{-1} and $0.1157 \text{ mg ml}^{-1}$, respectively. This parameter changes are shown in Figure 9. If an enzyme has a low K_m , it has high affinity for the substrate. The K_m of the enzyme indicates that the enzyme and substrate relationship is very good.

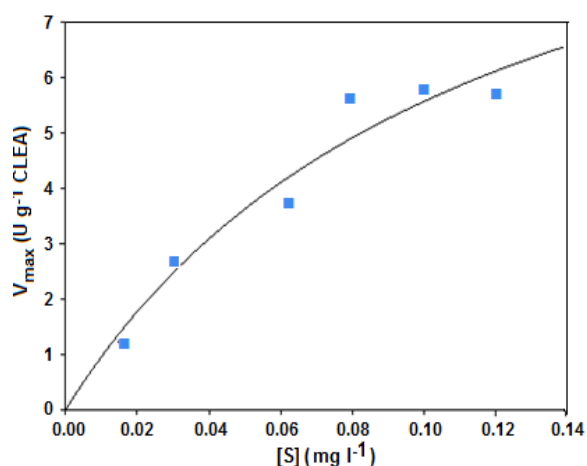


Figure 9. Kinetic graph of combi-CLEAs [(GA + PUL) + GI].

4. CONCLUSION

In this study, in order to obtain fructose syrup from starch, glucoamylase, pullulanase and glucose isomerase were immobilized in the form of combi-CLEAs [(GA + PUL) + GI]. In this immobilization study, the optimum conditions of ammonium sulfate, glutaraldehyde, pH, temperature and immunization time were found as 40%, 6, 60 °C and 5 hours, respectively. The remaining relative activity of combi-CLEAs [(GA + PUL) + GI] after 50 uses was identified as 46%. The remaining activities of combi-CLEAs [(GA + PUL) + GI] after 24-hour incubation at 60 and 80 °C were found as 44% and 30%, respectively. In terms of the storage stability of combi-CLEAs [(GA + PUL) + GI], at the end of 30 days at the temperature level of 4 and 25 °C, the remaining activities were calculated as 56% and 13%, respectively. V_{\max} and K_m values of combi-CLEAs [(GA + PUL) + GI] were calculated as 12 U g^{-1} and $0.1157 \text{ mg ml}^{-1}$, respectively.

Conflict of interest

Authors declare that there is no a conflict of interest with any person, institute, company, etc.

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