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IMMOBILIZATION OF MAXILACT BY CROSS-LINKED ENZYME AGGREGATES

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ABSTRACT

Maxilact L2000 (β-galactosidase from *Kluyveromyces lactis)* was aggregated using ammonium sulphate and the resultant aggregates, on cross-linking with glutaraldehyde, produced an insoluble and catalytically active enzyme. These cross-linked enzyme aggregates (CLEAs) Maxilact were obtained in the optimal conditions. The activity of a biocatalyst, immobilized with the obtained preparation, was 5.89 U/mg. The operating stability of the immobilized Maxilact was determined for 10 successive batches. Residual activity was 8.24 U/mg after 10 cycles of batch operation. The above studies show that moderately stable preparation of catalase can be economically prepared by the cross-linked enzyme aggregates. The preparation and characterization of CLEAs Maxilact is reported for the first time.

Keywords: Maxilact, immobilization, cross-linked enzyme aggregates

INTRODUCTION

Every year, the world produces about $6 \cdot 10^6$ tons of lactose. But over the past 50 years, almost half of the global production has been converted into secondary products. Lactose is discharged to water courses, which is a direct threat to the environment. Lactose is a hygroscopic sugar with low solubility, which makes its direct use impossible. The hydrolysis of lactose by means of acids is disadvantageous due to the contamination of the ion exchange of resins. Enzy-

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matic hydrolysis is a better alternative [1]. For this purpose, it is possible to use the enzyme lactase (β-galactosidase - EC 3.2.1.23) which hydrolyzes the β-(1-4)-glycosidic bond between glucose and galactose (Fig. 1).

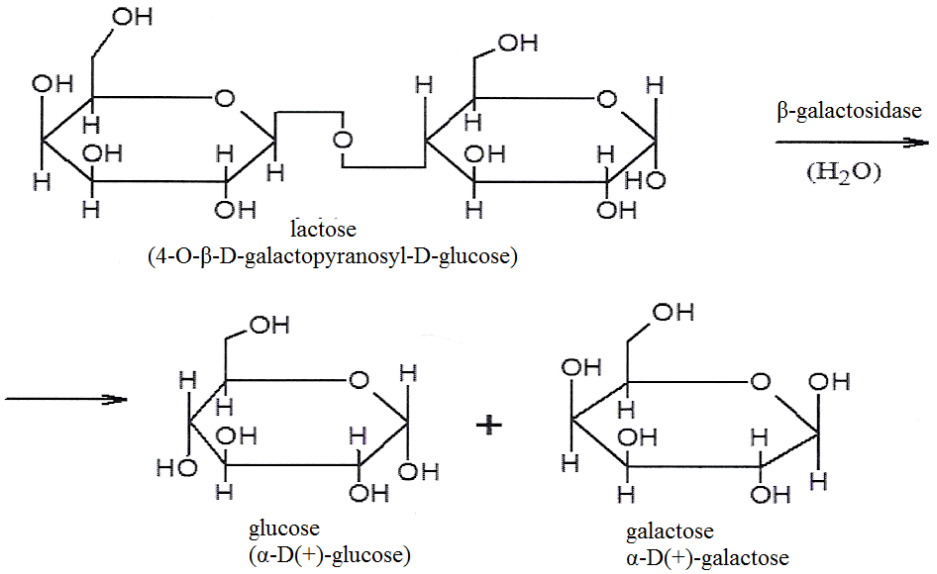

Fig. 1. Hydrolysis of lactose

β-galactosidase is produced by various bacteria, fungi and yeast. Special and growing interest in β-galactosidase from yeast *Kluyveromyces lactis* is now observed in science and the dairy industry [2, 3]. The crystal structures of tertmameric β-galactosidase from *Kluyveromyces lactis* is presented in Figure 2.

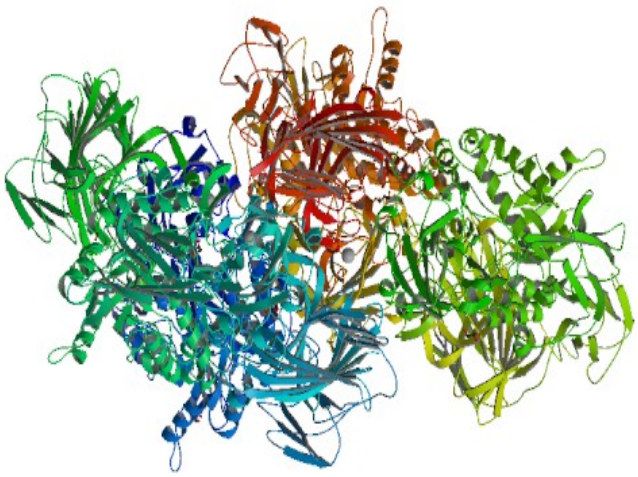

Fig. 2. β-galactosidase from *Kluyveromyces lactis* [4]

In this paper, Maxilact 2000 L (commercial preparation of β-galactosidase from *Kluyveromyces lactis* DSM Food Specialties) was used in the study. It was the aim of the study to produce Maxilact, immobilized by cross-linked enzyme aggregates CLEAs, produced in the optimum conditions (i.e., the immobilized lactase must be characterized by maximum activities). Information on cross-linked enzyme aggregates CLEAs β-galactosidase from *Kluyveromyces lactis* is scarce in the literature.

β-galactosidase is an important industrial enzyme, used in the hydrolysis of whey, milk and lactose. The enzymatic hydrolysis of lactose helps to avoid health-related and environmental problems caused by the disaccharide. Furthermore, the enzyme enables the formation of galacto-oligosaccharides, prebiotic additives which are so-called "health" food products.

β-galactosidase is one of the relatively few enzymes that are used in the process, in native or immobilized form [5]. There are various methods of immobilization of β-galactosidase, for example, β-galactosidase from *Aspergillus oryzae* was immobilized in calcium alginate, in starch, in cross-linked glutaraldehyde. The other types galactosidase are immobilized in the pores of such materials as cotton, aluminum, silanized glass, porous phenolic resin, corn grit and Sepharose 4B, silica gel, on low-pressure plasma, on magnetic nanoparticles [5-8].

The technology of immobilization by cross-linked enzyme aggregates Maxilact was studied. It is one of the three methods of cross-linking (Figure 3).

a) b) c)

Fig 3. The methods for immobilization by the cross-linking of (a) the dissolved enzyme – CLEs, (b) the aggregates of the enzyme – CLEAs, (c) the crystals of the enzyme – CLECs

The methods presented in Figure 3 are characterized:

a) Cross-Linked Enzymes – these products are mechanically unstable and the method is difficult to use – Fig. 3a [9,10].
b) Cross-Linked Enzyme Crystals – these products are highly stable in severe conditions such as extreme pH values and high temperatures. The produced aggregates are characterized by excellent mechanical properties. However, the main disadvantage is the high cost of the biocatalyst, which results from the need to obtain a high purity of the enzyme. The method is rarely used in practice – Fig. 3c [9,11,12].

The disadvantages of the CLEs and CLECs technologies had caused the introduction of the cross-linked enzyme aggregates CLEAs technology to the market. The biocatalyst production technology consists of two steps. The first step is the aggregation of soluble enzymes by the addition of salt, an organic solvent, or polymer. The process is carried out under mild conditions in order to ensure enzymatic activity. In the second step, the units are stabilized by cross-linking, usually by glutaraldehyde [9] or dextran, siloxane, such as $(MeO)_4Si$. The scheme of preparation CLEAs is shown in Figure 4 [13].

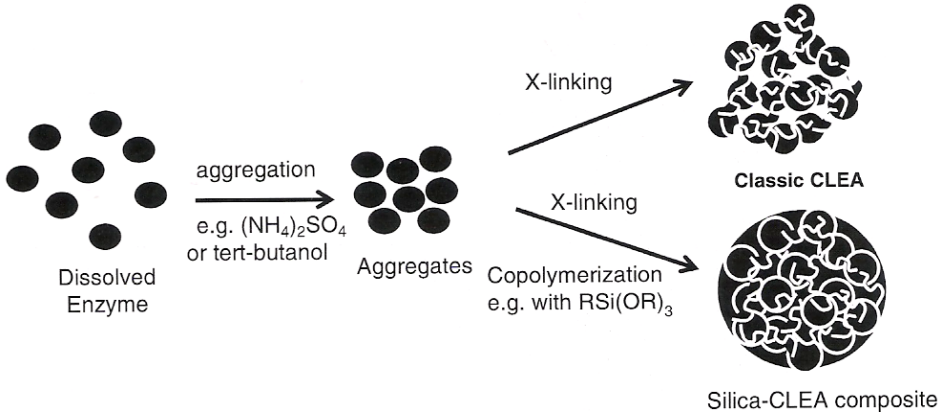

Fig. 4. Scheme of preparation of CLEAs

The cross-linking ingredient – glutaraldehyde – is a product, characterized by good efficiency and low cost. It is also stable and is certified GRAS (called General Recognized as Safe).

EXPERIMENTAL

Materials

β-Galactosidase from *Kluyveromyces lactis* – (EC 3.2.1.23) Maxilact L 2000 was kindly provided by DSM Food Specialties (The Netherlands). For the purpose of the study Maxilact was diluted 1:1000 (v/v) in a phosphate buffer at pH=6.6. ONPG (ortho-nitrophenyl-β-D-galactoside), glucose oxidase *Aspergillus niger*, horseradish peroxidase, were purchased from Sigma-Aldrich. Lactose, 4-aminoantipyrine, phenol were purchased from POCH, Gliwice. All of the other chemicals used were of analytical quality.

Assay of the activity of Maxilact

The activity of the immobilized β-galactosidase was determined by the amount of the product (glucose) resulting from the hydrolysis of lactose. Maxilact in the amount of 0.5 cm^3 was added to 5 cm^3 of lactose of an initial concentration of 0.146 M (5% w/w). The hydrolysis was carried out at a room tem-

perature. Samples were collected in the amount of 0.05 cm^3 at 0 min, 15 min, 30 min, 60 min, 120 min and added to 2 cm^3 of the enzyme kit. The enzyme kit was composed by glucose oxidase (GOD)>250 μkat/dm^3; peroxidase (POD)>20 μkat/dm^3; phenol 5mM; 4-aminoantipyrine 5mM and phosphate buffer (pH = 6.6) 250 mM.

Absorbance was measured spectrophotometrically at a wavelength of 500 nm. The absorbance reading was taken after 30 seconds and 1 minute after addition of the samples in the amount of 0.05 cm^3 to the enzyme kit. During determination of glucose by the enzymatic method the colour of the solution was observed to change from colourless to pink.

Preparation of cross-linked enzyme aggregate

Maxilact aggregate

A 50% solution of $(NH_4)_2SO_4$ in the amounts: 1:2, 1:4, 1:6, 1:8, respectively, was added to Maxilact in order to produce the Maxilact enzyme aggregates. The solution was stirred for 0.5 hr, 1 hr, 2 hrs, 4 hrs and 24 hrs. The aggregates were centrifuged at 10 000 rpm for 15 min. Then the filtrate was separated from the aggregates. The activity of the Maxilact aggregates was determined during hydrolysis of lactose after the lapse of 15 min and 30 min.

Glutaraldehyde cross-linking aggregates of Maxilact

In the first step, glutaraldehyde at concentrations of 5%, 10%, 25%, 50% (w/w), respectively, was used for cross-linking and was stirred for 4 hrs of cross-linking time. Each sample was centrifuged at 6 000 rpm for 5 min. The obtained aggregates were washed twice using a phosphate buffer solution. The amount of enzyme in the filtrates and aggregates was used in the hydrolysis of lactose. The activity of the cross-linking aggregates was determined during the hydrolysis of lactose after the lapse of: 0 min, 15 min, 30 min, 60 min, 120 min. The next step was the preparation of aggregates using the 25% (w/w) glutaraldehyde. The final concentration of glutaraldehyde in the solution as a cross-linking ingredient was studied in the range from 0.5% to 3%. The next step was to study the effect of cross-linking time after 0.5h, 1h, 2hrs and 4hrs [11]. Each sample was centrifuged and washed twice using a phosphate buffer solution. The activity of cross-linking aggregates was determined during the hydrolysis of lactose after the lapse of: 0 min, 15 min, 30 min, 60 min, 120 min.

Study of operating efficiency of immobilized Maxilact (CLEAs)

The activity of the prepared Maxilact CLEAs was tested ten times during hydrolysis of lactose for 30 minutes.

Figure 5 shows a five-step optimization procedure to obtain the cross-linked enzyme aggregate which was presented by Schoevaart *et al*. 2004 [13]. The procedure was used for Maxilact but optimal conditions had to be found.

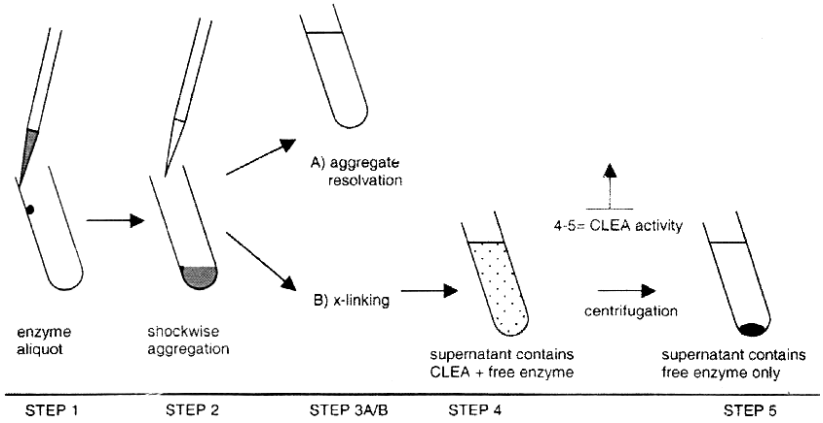

Fig. 5. The optimal method of production of CLEAs Maxilact

In the first step, 0.5 ml containing a few units of activity of Maxilact was added to a tube. Next, aggregation was induced using 2 ml of 50% (w/w) ammonium sulphate – the precipitant. At this stage, the presence of aggregates was not detected visually, although it was indicated, in this case, by an increased turbidity of the suspension. Aliquots were transferred to lactose to assess the activity of the aggregates. Comparison with initial activities indicated the effectiveness of the aggregation step. To determine the amount of the precipitant necessary to remove all activity from the supernatant, samples must be centrifuged with subsequent assaying of the supernatant.

The second stage of CLEAs Maxilact preparation was the aggregate cross-linking by adding the cross-linker (step 3B). After the lapse of the required cross-linking time, the then cross-linked aggregates were quenched with a buffer (step 4). A sample was drawn from the resulting suspension, which contains CLEAs Maxilact as well as residual free enzyme, and assayed for activity. Then, the CLEAs Maxilact were centrifuged and again a sample was drawn from the supernatant (step 5), which then contained only the free enzyme. The difference in activity between step 4 and step 5 was the CLEAs activity. In addition, the cross-linking time had an indirect influence since it set the exposure time of the enzyme to the precipitant. Although various cross-linkers are known and can be used, glutaraldehyde remains a cheap and very versatile agent.

RESULTS AND DISCUSSION

The implementation of the optimal method of production of CLEAs Maxilact was divided into several stages:

1) determination of the amount of the enzyme Maxilact used for 50% $(NH_4)_2SO_4$,
2) determination of the precipitation time after which activity of the aggregates of Maxilact was maximum, and in the centrifuged filtrate there was no activity of Maxilact,

3) determination of the concentration of glutaraldehyde (%) which was used in the cross-linking process when the Maxilact aggregates were formed,
4) determination of the cross-linking time when the Maxilact aggregates resulted from using glutaraldehyde.

The results of the precipitation tests indicate that Maxilact with precipitant concentrations of 1:4 (Fig.6) and the 24 hr time of precipitation (Fig.7) resulted in the highest enzyme activities.

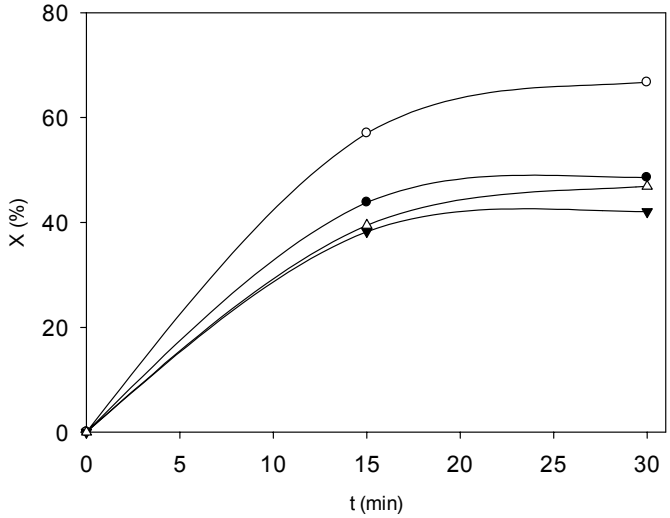

Fig. 6. The conversion of hydrolysis of lactose X vs time t for aggregates Maxilact obtained after 24 hours of precipitation with different amounts of enzyme and precipitant: 1:2 (●), 1:4 (○), 1:6 (▼), 1:8 (Δ)

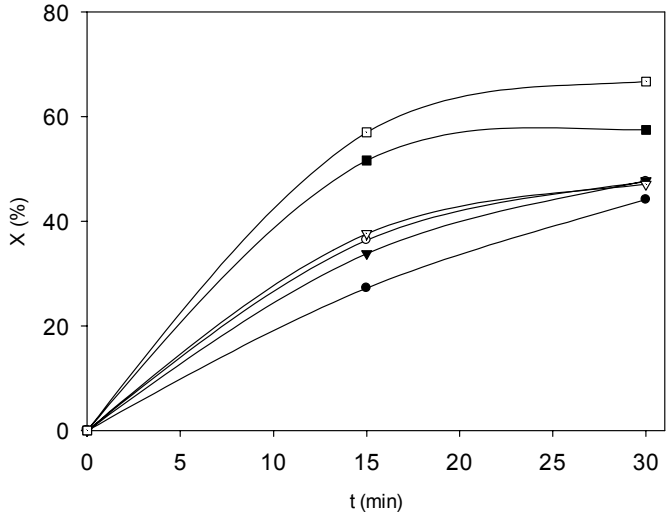

Fig. 7. The conversion of hydrolysis of lactose X vs time t for aggregates of Maxilact obtained with enzyme and precipitant (1:4) after different times of precipitation: 0.5hr (●); 1hr (○); 2hrs (▼), 3hrs (∇), 4hrs (■), 24 hrs (□)

The effect of using a 50% concentration of glutaraldehyde on the efficiency of the cross-linking process was studied. This concentration led to denaturation of all the enzyme, of which the activity reached zero. Next, the concentrations of glutaraldehyde: 5%, 10% and 25% were used in the study. An appropriate amount of the aldehyde was added so that its final concentration in the solution was 2%. The cross-linking was carried out for 4 hours. The highest results of the conversion of hydrolysis of lactose were obtained for the cross-linked aggregates of Maxilact by using a 25% concentration of glutaraldehyde (Fig. 8).

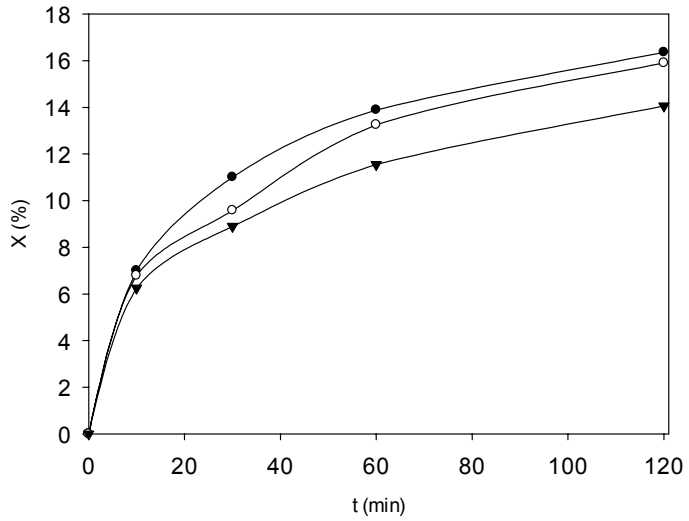

Fig. 8. The conversion of hydrolysis of CLEAs Maxilact vs time for CLEAs cross-linking by glutaraldehyde at concentrations of: 5%(▼), 10% (○), 25% (●)

Figure 9 shows the conversion of hydrolysis of lactose for CLEAs cross-linking 25% concentration of glutaraldehyde for different final concentrations of glutaraldehyde in the range from 0.5% to 3%. The maximum conversion was obtained for the hydrolysis of lactose by aggregates of Maxilact cross-linked using a glutaraldehyde concentration of 25% to achieve a 2% final concentration in the solution. Determination of the impact of the concentration of glutaraldehyde is necessary due to the potential inactivation of enzymes by this type of chemical. The use of 270 μM (25% concentration of glutaraldehyde to achieve a final 2% concentration in the solution) of glutaraldehyde as the cross-linking agent provides the highest activity of CLEAs Maxilact and recovery, compared with higher concentrations.

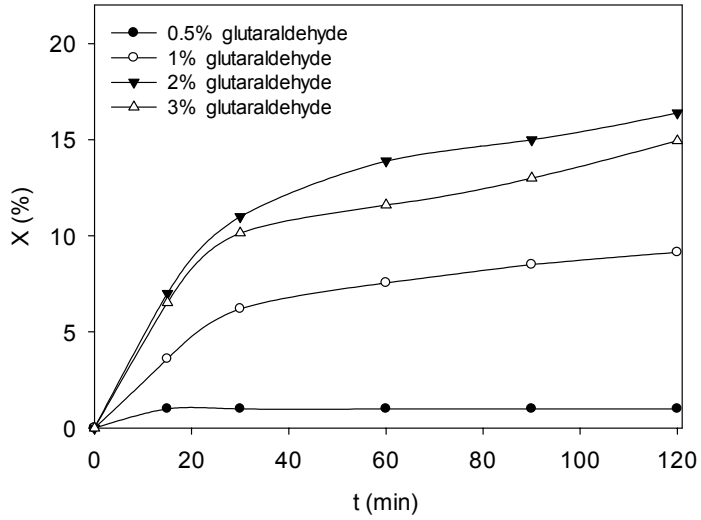

Fig. 9. The conversion of lactose hydrolysis by CLEAs Maxilact cross-linked with 25% glutaraldehyde dependent on different final concentrations of aldehyde in the solution

The reduction of the CLEA activity at glutaraldehyde concentrations above 270 μM could be explained by a rigidification of the enzyme's three-dimensional structure that hinders the conformational change needed for the oxidation of substrates at the active site.

The effect of the time of cross-linking by the glutaraldehyde solution on the cross-linking process was studied for the following times: 0.5 hr, 1hr, 2hrs and 4 hrs. The obtained value of the effect is presented in Figure 10.

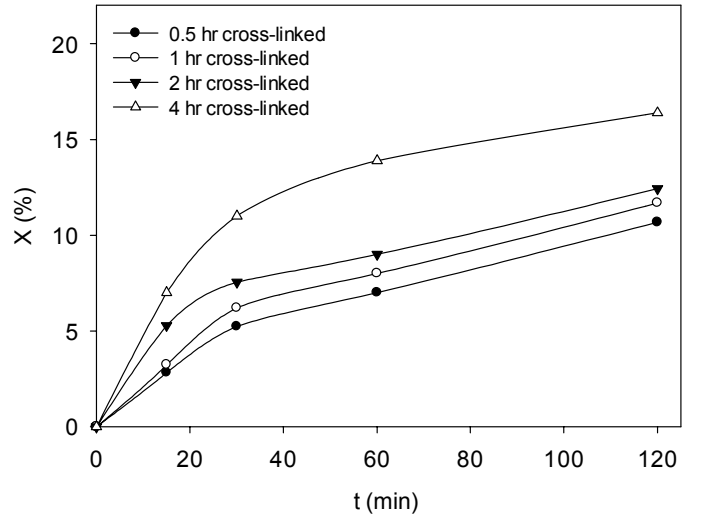

Fig. 10. The conversion of hydrolysis lactose by the produced CLEAs Maxilact cross-linked with 25% glutaraldehyde dependent on different cross-linked times

The value of conversion of hydrolysis of CLEAs Maxilact in the cross-linking time in the range from 0.5 h to 4 hrs was from 11.7% to 16.4%. The highest conversion of hydrolysis of CLEAs Maxilact was determined for the cross-linking time of 4hrs.

Comparison of the conversion of hydrolysis of lactose by the native Maxilact and CLEAs Maxilact is shown in Figure 11. The efficiency of the cross-linked enzymes aggregate (CLEAs) was 19%.

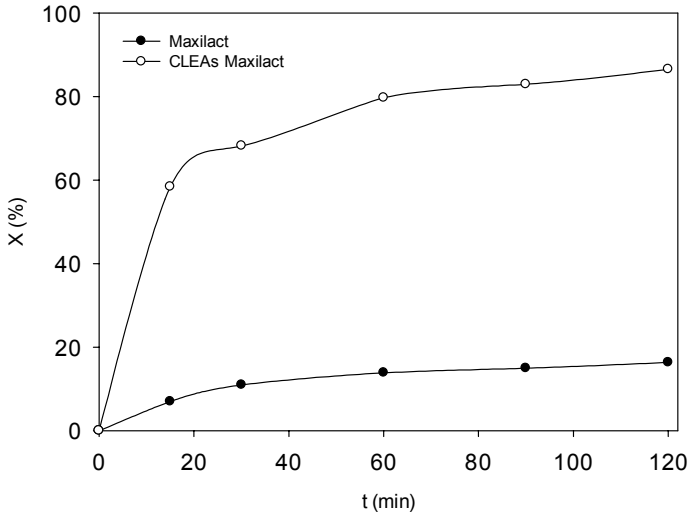

Fig. 11. Comparison of the degree of hydrolysis of lactose by the native and CLEAs Maxilact

The operational efficiency was studied by using immobilized CLEAs Maxilact in ten cycles in the hydrolysis of lactose (Fig. 12). Reusability of the immobilized enzyme is an important characteristic for its potential industrial applications.

During the test in subsequent measurements, the activity of CLEAs Maxilact was gradually increased by about 20% in measurement No. 3 and then increased by about 20% in measurement No. 5. The maximum activity was 9.12 U/mg for measurement No. 5. In subsequent measurements, there occurred a small decrease in the enzymatic activity. The final measurement of activity was 10% lower compared with the maximum value. The mean value of ten measurements was 7.89 U/mg. The results indicate that the enzyme aggregates, immobilized by cross-linking, improved the stability of Maxilact and similar results were reported for other immobilization methods [7, 8]. According to Güleç, *et al*. [7] the immobilized β-galactosidase on a plasma-modified membrane was successively reutilized for 10 cycles. The enzymatic derivative retained approximately all of its initial activity. The immobilization of β-galactosidase onto magnetic nanoparticles [8] maintained about 86% of its original activity after 10 cycles.

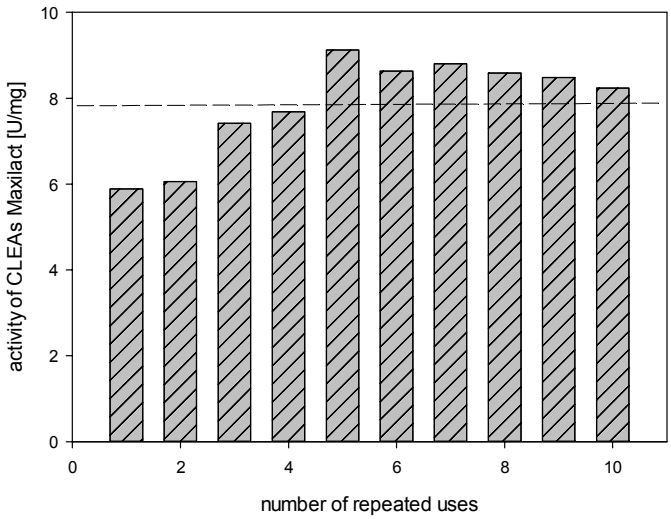

Fig.12. Maxilact CLEAs operating efficiency for ten cycles of lactose hydrolysis during 30 minutes.

CONCLUSIONS

The technology to obtain immobilized Maxilact by cross-linked aggregates (CLEAs) was developed. The maximum values of activity CLEAs Maxilact were obtained for aggregates cross-linked with glutaraldehyde:

1. The Maxilact amount relative to the amount of 50% $(NH_4)_2SO_4$ is 1:4. The time salt-out is 24 hours.
2. The cross-linking process of Maxilact aggregates was carried out with a 25% glutaraldehyde solution during 30 minutes.
3. Efficiency in the process of immobilization by the method of cross-linked aggregates of CLEAs Maxilact is about 19%.
4. The operating efficiency of the immobilized Maxilact by CLEAs was high. There was no decrease in the biocatalyst activity of the immobilized Maxilact, when used tenfold during the hydrolysis of lactose.

The resulting biocatalyst is characterized by high stability.

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