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Magnetic CLEAs of β-Galactosidase from *Aspergillus oryzae* as a Potential Biocatalyst to Produce Tagatose from Lactose

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Abstract: β-galactosidase is an enzyme capable of hydrolysing lactose, used in various branches of industry, mainly the food industry. As the efficient industrial use of enzymes depends on their reuse, it is necessary to find an effective method for immobilisation, maintaining high activity and stability. The present work proposes cross-linked magnetic cross-linked enzyme aggregates (mCLEAs) to prepare heterogeneous biocatalysts of β-galactosidase. Different concentrations of glutaraldehyde (0.6%, 1.0%, 1.5%), used as a cross-linking agent, were studied. The use of dextran-aldehyde as an alternative cross-linking agent was also evaluated. The mCLEAs presented increased recovered activity directly related to the concentration of glutaraldehyde. Modifications to the protocol to prepare mCLEAs with glutaraldehyde, adding a competitive inhibitor or polymer coating, have not been effective in increasing the recovered activity of the heterogeneous biocatalysts or its thermal stability. The biocatalyst prepared using dextran-aldehyde presented 73.6% recovered activity, aside from substrate affinity equivalent to the free enzyme. The thermal stability at 60 °C was higher for the biocatalyst prepared with glutaraldehyde (mCLEA-GLU-1.5) than the one produced with dextran-aldehyde (mCLEA-DEX), and the opposite happened at 50 °C. Results obtained for lactose hydrolysis, the use of its product to produce a rare sugar (D-tagatose) and operational and storage stability indicate that heterogeneous biocatalysts have adequate characteristics for industrial use.

Keywords: β-galactosidase; immobilization; mCLEA; D-galactose; D-tagatose

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1. Introduction

In several industrial sectors, the use of enzymes has become common for process modification and optimisation, as well as the introduction of new methods to solve previously neglected issues [1,2]. Currently, in industrial processes related to the food industry, β-galactosidase (E.C. 3.2.1.23) is one of the most used enzymes due to its ability to hydrolyse lactose, a disaccharide consisting of glucose and galactose, whose presence in certain products and by-products may represent a hindrance.

β-galactosidase is a member of the glycoside hydrolases superfamily, possessing an (α-β)8-barrel catalytic domain among its six overall domains. The *Aspergillus oryzae* β-galactosidase has glutamic acid residues in the active site that function as an acid/base catalyst (Glu200) and another that works as a nucleophile (Glu298). During catalysis, in the active site, the β-galactose molecules make hydrogen bonds with other residues (Tyr96, Asn140, Glu142, and Tyr364) around the domain [3,4].

Some people have a deficiency in the production of β-galactosidase in the body, and the accumulation of lactose can cause discomfort and some intestinal problems, thus requiring the consumption of products in which the lactose has been hydrolysed or removed industrially. In addition, the presence of lactose, a hygroscopic sugar, promotes

unwanted crystallisation, which may alter the texture of certain dairy foods, i.e., frozen desserts. The presence of lactose in the by-products of the dairy industries discharged into the environment, such as whey, is also undesirable because it considerably increases the biochemical demand for oxygen (BOD) in the environment, one of the most critical parameters for monitoring pollution. The monomers that compound lactose can also be used later for various purposes, such as the production of ethanol and culture media for laboratory use [5,6]. One particular use of the galactose produced by lactose hydrolysis is its subsequent isomerisation to D-tagatose, a rare sugar beneficial to the intestinal microflora and with great potential to substitute sugar in low-caloric and hypoglycaemic diets [7,8]. The isomerisation can be carried out by another enzyme, L-arabinose isomerase (L-AI), in a cascade reaction coupled with β-galactosidase. The reaction starts with lactose as a substrate to produce D-tagatose [9–11]. Working with enzymes in an industrial environment requires certain precautions, such as controlling temperature, pH, and possible contamination, but especially regarding maximising efficiency and reducing losses. Thus, enzymatic immobilisation is necessary to supply such conditions, as it allows the reuse of molecules and increases enzyme stability, ensuring maximum use of cross-linked [12]. Among the various existing enzyme immobilisation methods, the formation of cross-linked enzyme aggregates (CLEA) is advantageous because it does not require specific support, reducing the costs associated with the strategy. CLEA consists of an aggregate of enzymes covalently linked to each other, which prevents its solubilisation and, consequently, allows its separation from the medium by simple techniques such as filtration and centrifugation. The method to prepare these aggregates unifies different stages of insolubility, such as precipitation and immobilization, in a single process, not requiring an extremely purified sample due to the specificity of the cross-linking reactions that involve only specific amino acid residues [13,14].

However, since the most commonly used separation methods for heterogeneous biocatalyst recovery, such as filtration and centrifugation, are also costly and difficult to be carried out on a large scale, there is an alternative that involves binding enzyme aggregates to superparamagnetic nanoparticles (MNP) so that the heterogeneous biocatalyst can be separated by a magnetic field [15,16]. These biocatalysts are known as magnetic cross-linked enzyme aggregates (mCLEA), a concept introduced in the early 2010s, in which the enzymes are covalently linked to the nanoparticles functionalised with amino groups in the same step that promotes the cross-linking of the enzyme molecules, giving all the heterogeneous biocatalyst magnetic properties [17–19]. Since then, the mCLEAs of different enzymes have been used in broad applications such as biodiesel production, biomass conversion, biodegradation of pollutants and cancer diagnosis [20–23]. The use of CLEAS and mCLEAs have also been described in the literature for the production of the rare sugar D-tagatose [11,24]. In a previous work by our group [24], mCLEAs of the recombinant L-arabinose isomerase (L-AI) from *Enterococcus faecium* were evaluated for the synthesis of D-tagatose from galactose. Stable and active insoluble L-AI biocatalysts were prepared. An amount of 110 mM of D-tagatose was produced, corresponding to 22% of galactose conversion, when m-CLEAs of L-AI were used as biocatalysts. It is also important to mention the work of Rai et al. [11], that studied the synthesis of D-tagatose from galactose, catalysed by mCLEAs of L-AI, and from lactose, using Combi-mCLEAs of L-Arabinose Isomerase and β-galactosidase, in a two-step cascade process. The authors achieved ∼50% yield of D-tagatose (at 24 h) from galactose isomerisation catalysed by the mCLEA, and ∼25% yield was found when D-tagatose was produced from lactose using Combi-mCLEA (β-galactosidase + L-AI). The work of Rai et al. [11] described, for the first time, a magnetic CLEA that combines the enzymes L-AI and β-Gal and that can be applied directly in the transformation of lactose into D-tagatose. The authors, however, used glutaraldehyde as a crosslinking agent for the preparation of Combi-mCLEA, which may be a disadvantage since glutaraldehyde is reported as a toxic/hazardous substance [25].

Along with the formation of cross-linked enzyme aggregates (CLEA), chemical modifications of the enzymes are an essential tool for immobilisation and enhancing enzyme

properties, such as stability and are used to modify catalytic features [26]. Bifunctional cross-linking agents are the key to enabling carrier-free immobilisation or self-immobilisation [27]. Glutaraldehyde, a small dialdehyde molecule, is the most commonly used bifunctional compound for the preparation of CLEA, due to its high reactivity with amine groups and conversion yield, low steric effects, stability, as well as high market availability and economic efficiency [27,28]. Glutaraldehyde has widely been used as a protein cross-linker, as an activator of supports, and as a cross-linker of enzymes and support [17,29]. Despite its vast application, glutaraldehyde is a toxic compound, and its application in the environmental, food and pharmaceutical industry is relatively limited [30].

The search for adequate cross-linking agents for human health and environmental applications has been studied [31,32]. Among these agents, dextran-aldehyde, prepared by the periodate oxidative cleavage of 1,2-diol moiety inside dextran, has been pointed out as a promising compound for preparing CLEAs, since no toxicity issues are reported [33–35]. Dextran-aldehyde is a random multifunctional coil polymer with the properties of a coating material and a macromolecular cross-linker. It can be used to stabilize complex protein and lipid-based nanoparticles due to the chemical reaction between oxidized dextran and proteins, implying the formation of the Schiff base complex and creating covalent binding through imine bonds [35]. This compound has been used to enhance enzymatic biocatalysts systems: modify free or immobilised enzymes, modify supports involved in enzyme manipulation with different objectives, or even as a spacer arm in enzyme immobilisation [36–38]. Although dextran-aldehyde has been used to prepare CLEAs [38], the crosslinking step can be a bottleneck since it involves the reaction of reactive primary amino groups on the protein surface, which may not be available in large amounts. Thus, in such cases, the preparation of CLEAs is not simple, and depends on the enzyme in question. To the best of our knowledge, the use of dextran-aldehyde as a non-toxic alternative for glutaraldehyde for the preparation of mCLEAs of β-galactosidase has not been reported.

In this work, different strategies to prepare heterogeneous magnetic biocatalysts of beta-galactosidase were compared, aiming at efficient lactose hydrolysis, to later use its products for various purposes, such as the synthesis of the rare sugar D-tagatose, a cascade reaction. The magnetic biocatalysts (mCLEAs) were obtained using two cross-linking agents, glutaraldehyde and dextran-aldehyde, in order to allow the enzyme to bond through different areas. The use of dextran-aldehyde in this work aims to avoid the possibility of product contamination by weakly bonded glutaraldehyde molecules. Thus, it is expected to optimise their catalytic efficiency, thermal and operational stabilities, as well as the recovery of those heterogeneous biocatalysts after use in industrial processes.

2. Results and Discussion

2.1. Preparation of β-Galactosidase Magnetic CLEAs Using Different Glutaraldehyde Concentrations

Three different glutaraldehyde concentrations (0.6, 1.0 and 1.5%) were investigated for the preparation of β-galactosidase magnetic CLEAs (mCLEAs) to evidence the most efficient concentration for the cross-linking of the aggregates. It should be noted that the protein concentration was the same for all immobilisations (10 mg·mL^{-1}).

In this work, the theoretical activity was 10.93 U, corresponding to the free enzyme's activity in solution. High immobilisation yields (100%) were obtained at any investigated conditions since no activity was detected on the supernatant after protein precipitation.

Table 1 presents the results of the effect of the different cross-linker concentrations on the heterogeneous biocatalysts activity and recovered activity of mCLEAs. Figure S1 (in the Supplementary Materials) shows the thermal stability of mCLEAs at 60 °C, and Table 2 summarises the half-lives and deactivation parameters.

The biocatalyst prepared with the higher glutaraldehyde concentration (mCLEA-GLU-1.5) was the one with the highest recovered activity (32.33%), higher than mCLEA-GLU-1 (21.11%) and mCLEA-GLU-0.6 (11.96%), as shown in Table 1. Similar results were also

reported by Podpreošk et al. [39], who describe that 1.5% glutaraldehyde is the ideal concentration to achieve a high recovered activity in both CLEAs and mCLEAs.

Table 1. Effect of different glutaraldehyde concentrations on magnetic cross-linked enzyme aggregates (mCLEAs) parameters of β-galactosidase: Immobilized enzyme activity (At_d) and Recovered activity (At_r).

Sample	At_d (U)	At_r (%)
mCLEA-GLU-0.6	1.31 ± 0.47	11.96 ± 4.30
mCLEA-GLU-1	2.31 ± 0.40	21.11 ± 3.66
mCLEA-GLU-1.5	3.53 ± 0.36	32.33 ± 3.29

The results are presented as an average value with standard deviations. The recovered activity is the ratio between the activity of the heterogeneous biocatalyst and the theoretical activity.

Table 2. Sadana and Henley model parameters for thermal inactivation at 60 °C of the free enzyme and mCLEA of β-galactosidase produced with different concentrations of glutaraldehyde.

Sample	R^2	$t_{1/2}$ (min)	SF	α	K_d (min^{-1})
mCLEA-GLU-0.6	0.99	16.46	0.74	0.32	0.08
mCLEA-GLU-1	0.97	7.45	0.33	0.12	0.15
mCLEA-GLU-1.5	0.96	26.57	1.19	0.03	0.03
Free enzyme	0.97	22.24	-	0.06	0.03

The conditions employed for inactivation were 50 mM potassium phosphate buffer (pH 6.6). R^2 is the correlation factor; $t_{1/2}$ is the half-life of the enzyme. SF is the stability factor, a ratio between $t_{1/2}$ of the heterogeneous biocatalyst and $t_{1/2}$ of the free enzyme; α represents the fraction of the enzyme or heterogeneous biocatalyst that loses activity; K_d is the thermal inactivation constant.

The thermal stability at 60 °C and pH 6.6 for the three investigated mCLEAs biocatalysts are shown in Figure S1 (in the Supplementary Materials). The biocatalysts presented a progressive thermal decay profiles within the analysis time, presenting half-lives between 7.45 and 26.57 min. The biocatalyst that showed the highest thermal stability at 60 °C was produced with the highest concentration of glutaraldehyde, mCLEA-GLU-1.5 ($t_{1/2}$ = 26.57 min), followed by the biocatalysts mCLEA-GLU-0.6 ($t_{1/2}$= 16.46 min) and mCLEA-GLU-1 ($t_{1/2}$ = 7.45 min), respectively. The half-life of the free enzyme was 22.24 min, lower than the mCLEA-GLU-1.5, which indicates that immobilisation increased the thermal stability of the enzyme.

The mCLEA of β-galactosidase prepared with the highest concentration of glutaraldehyde (1.5%) as cross-linker yielded the most active biocatalyst (At_d = 3.53 U and At_r = ~32%) and the most stable biocatalyst ($t_{1/2}$ = 26 min). Therefore, mCLEA-GLU-1.5 was selected for the following experiments.

2.2. Effects of the Presence of an Inhibitor or Enzyme Coating in the Preparation of β-Galactosidase Magnetic CLEAs

The effect of adding galactose (competitive inhibitor) during cross-linking (immobilisation) and the heterogeneous biocatalyst coating with PEI were evaluated regarding the activity and thermal stability of the heterogeneous biocatalysts. These techniques were tested to obtain a better biocatalyst performance, reduce the loss of activity during the immobilisation process and increase the half-life of the heterogeneous biocatalyst at 60 °C.

Since galactose is a reversible competitive inhibitor of β-galactosidase [40], this carbohydrate was added to the immobilization buffer at a concentration above its inhibition constant (K_i = 15 mM [41]), to occupy the active sites of the enzymes during the cross-linking step, preventing amino acid residues from these regions from participating in the cross-linking reactions. The heterogeneous biocatalysts produced in the presence of the

inhibitor were also cross-linked with a concentration of 1.5% glutaraldehyde, being called mCLEA-GAL.

PEI, in turn, was added after the formation of mCLEAs (mCLEA-GLU-1.5), without galactose, to cover the molecules with their branched chains, promoting a possible increase on the thermal stability of the heterogeneous biocatalyst. These heterogeneous biocatalysts were called mCLEA-PEI. Some reports have previously found success in applying PEI coating on immobilised enzymes to enhance their stability, including β-galactosidase [42], which is why this approach was tested in the present work.

Table 3 shows the immobilisation parameters for the heterogeneous biocatalysts produced with these changes compared to mCLEA-GLU-1.5.

Table 3. Effect of the presence of galactose or PEI coating on magnetic cross-linked enzyme aggregates (mCLEAs) parameters of β-galactosidase: Immobilized enzyme activity (At_d) and Recovered activity (At_r).

Sample	At_d (U)	At_r (%)
mCLEA-GLU-1.5	3.53 ± 0.36	32.33 ± 3.29
mCLEA-GAL	1.26 ± 0.29	11.58 ± 2.65
mCLEA-PEI	0.74 ± 0.02	6.78 ± 0.18

The recovered activity is a ratio between the activity of the heterogeneous biocatalyst and the theoretical activity.

It is noteworthy that high immobilisation yields (100%) were achieved under all conditions, indicating that the protein of interest was precipitated entirely. In addition, the biocatalysts showed similar behaviour regarding theoretical activity (10.9 U), as the ones prepared in the absence of galactose or PEI. However, mCLEAs prepared in the presence of galactose as an inhibitor (mCLEA-GAL) or coated with PEI (mCLEA-PEI) showed lower recovered activity than mCLEA-GLU-1.5, reaching 11.58 and 6.78%, respectively. The lower recovered activity may be due to the physical interference of the added components, preventing an adequate performance of the cross-linking reactions in the case of galactose or occupying the active sites and blocking the substrate access in the case of PEI.

Thermal stability tests were also carried out for these biocatalysts at 60 °C, and the results are shown in Figure S2 (Supplementary Materials). Table 4 summarises the half-lives and deactivation parameters. The half-life of mCLEA-GLU-1.5 was 2.3-fold higher than the biocatalyst produced in the presence of the inhibitor (mCLEA-GAL). Moreover, the half-life of mCLEA-GLU-1.5 was 4.5-fold higher than the PEI-coated heterogeneous biocatalyst (mCLEA-PEI). These results show that both mCLEA-GAL and mCLEA-PEI also performed worse than mCLEA-GLU-1.5 against thermal deactivation at 60 °C. Therefore, these immobilisation alternatives were not further investigated.

Table 4. Sadana and Henley model parameters for thermal inactivation at 60 °C of free enzyme and magnetic β-galactosidase CLEA biocatalysts produced with an inhibitor or subsequent coating with PEI.

Sample	R^2	$t_{1/2}$ (min)	SF	α	K_d (min^{-1})
mCLEA-GLU-1.5	0.96	26.57	1.19	0.03	0.03
mCLEA-GAL	0.99	11.36	0.51	0.29	0.11
mCLEA-PEI	0.98	5.95	0.27	0.07	0.13
Free enzyme	0.97	22.24	-	0.06	0.03

The conditions employed for inactivation were 50 mM of potassium phosphate buffer (pH 6.6). R^2 is the correlation factor; $t_{1/2}$ is the half-life of the enzyme. SF is the stability factor, a ratio between $t_{1/2}$ of the heterogeneous biocatalyst and $t_{1/2}$ of the free enzyme; α represents the fraction of the enzyme or heterogeneous biocatalyst that loses activity; K_d is the thermal inactivation constant.

2.3. Preparation of β-Galactosidase Magnetic CLEAs Using Dextran-Aldehyde

The use of dextran-aldehyde (of approximately 450,000–650,000 Da) to prepare mCLEAs of β-galactosidase (mCLEA-DEX) caused a significant increase in the heterogeneous biocatalyst activity (8.02 U) and recovered activity (73.66%), when compared to mCLEA-GLU-1.5, as can be seen in Table 5. The mCLEAs-DEX activity was higher (2.3-fold) than that obtained with glutaraldehyde as a cross-linking agent. Moreover, the use of glutaraldehyde may have partially caused enzyme inactivation, as seen by the 32% recovered activity.

Table 5. Effect of the cross-linking agent on the magnetic cross-linked enzyme aggregates (mCLEAs) parameters of β-galactosidase: Immobilized enzyme activity (At_d) and Recovered activity (At_r).

Sample	At_d (U)	At_r (%)
mCLEA-GLU-1.5	3.53 ± 0.36	32.33 ± 3.29
mCLEA-DEX	8.02 ± 3.79	73.66 ± 34.67

mCLEA-GLU-1.5 is prepared with 1.5% glutaraldehyde and mCLEA-DEX is prepared using dextran-aldehyde. The recovered activity is a ratio between the activity of the heterogeneous biocatalyst and the theoretical activity.

As shown by Talekar et al. [27], the conformational flexibility of the enzyme decreases with the formation of covalent bonds between glutaraldehyde and basic enzyme residues, which also causes changes around the active site. Thus, one reason for the difference in recovered activity observed in the present work is that the action of glutaraldehyde promoted the formation of covalent bonds in lysine residues [26], critical for the correct spatial conformation of the active site in β-galactosidase molecules, preventing the enzymatic action of most of them. Although there are no lysine residues directly involved in the catalysis mechanism of β-galactosidase, some residues close to the active site can influence the molecule's spatial conformation (Figure 1). Mateo et al. [43] also evaluated the effectiveness of cross-linkers according to their molecular proportions, attesting that dextran-aldehyde, due to its large dimensions, cannot react with residues from the active site of the enzymes, resulting in high immobilisation yields and immobilized enzyme activities.

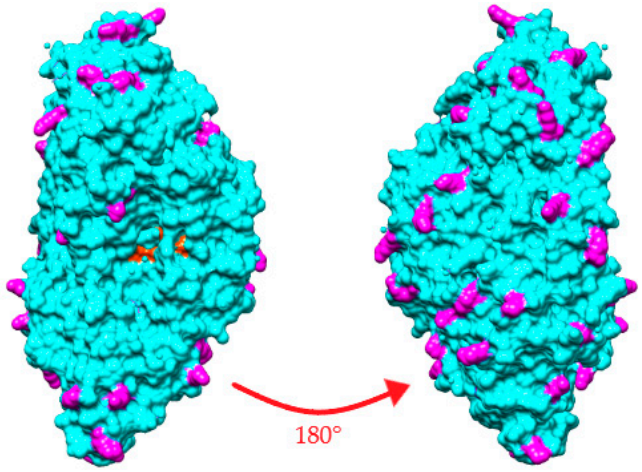


Figure 1. Tridimensional visualization of *Aspergillus oryzae* β-galactosidase structure. The active site is marked in orange, and the lysine residues are marked magenta. This model was obtained from Protein Data Bank, under the access code 4IUG (https://www.rcsb.org/structure/4i.ug, accessed on 1 December 2022) [4], and modified on the software Chimera 1.16.

The thermostability of the immobilised enzyme mCLEA-DEX at 60 °C (Figure S3, Supplementary Materials) was slightly lower than that of mCLEA-GLU-1.5, but very similar (considering the experimental error) to the free enzyme. These results are probably due to the higher flexibility of dextran when compared with glutaraldehyde [38]. The Sadana and Henley deactivation model [44] was fitted to the experimental data, and model parameters, as well as half-lives ($t_{1/2}$), are summarised in Table 6. The half-life for the

heterogeneous biocatalyst produced with dextran-aldehyde was lower than that of its analogue with glutaraldehyde.

Table 6. Sadana and Henley model parameters for thermal inactivation at 60 °C of free enzyme and magnetic β-galactosidase CLEA biocatalysts produced with glutaraldehyde and dextran-aldehyde.

Sample	R^2	$t_{1/2}$ (min)	SF	α	K_d (min^{-1})
mCLEA-GLU-1.5	0.96	26.57	1.19	0.03	0.03
mCLEA-DEX	0.99	15.87	0.71	0.10	0.05
Free enzyme	0.97	22.24	-	0.06	0.03

The conditions employed for inactivation were 50 mM of potassium phosphate buffer (pH 6.6). R^2 is the correlation factor; $t_{1/2}$ is the half-life of the enzyme. SF is the stability factor, a ratio between $t_{1/2}$ of the heterogeneous biocatalyst and $t_{1/2}$ of the free enzyme; α represents the fraction of the enzyme or heterogeneous biocatalyst that loses activity; K_d is the thermal inactivation constant.

Since mCLEA-DEX presented higher values of immobilized enzyme Activity (At_d) and recovered activity, and mCLEA-GLU-1.5 was better in terms of thermal stability at 60 °C, the two heterogeneous biocatalysts were chosen to further experiments.

2.4. Thermal Stabilities of mCLEAs at 50 °C

As tagatose production is conducted at 50 °C, the thermal stability of the two biocatalysts at this temperature was evaluated and a significant difference between the stabilities of mCLEA-GLU-1.5 and mCLEA-DEX heterogeneous biocatalysts (Figure S4) was observed. For comparison, the thermal stability of free enzyme was also investigated and presented a slow drop in activity during the analysis. The mCLEA-DEX showed a slow decay ($t_{1/2}$ = 9.13 h or > 500 min) when compared to mCLEA-GLU-1.5 ($t_{1/2}$ = 1.63 h or > 95 min), with a half-life 5.6-fold higher, as shown in Table 7.

Table 7. Sadana and Henley model parameters for thermal inactivation at 50 °C of free enzyme and magnetic β-galactosidase CLEA biocatalysts produced with glutaraldehyde and dextran-aldehyde.

Sample	R^2	$t_{1/2}$ (min)	SF	A	K_d (min^{-1})
mCLEA-GLU-1.5	0.99	98	0.05	0.35	0.014
mCLEA-DEX	0.95	548	0.26	0.05	0.0013
Free enzyme	0.99	2080	-	0.36	0.0007

The conditions employed for inactivation were 50 mM potassium phosphate buffer (pH 6.6). R^2 is the correlation factor; $t_{1/2}$ is the half-life of the enzyme. SF is the stability factor, a ratio between $t_{1/2}$ of the heterogeneous biocatalyst and $t_{1/2}$ of the free enzyme; α represents the fraction of the enzyme or heterogeneous biocatalyst that loses activity; K_d is the thermal inactivation constant.

These results showed a different behaviour than that observed at 60 °C, indicating that under milder conditions, the biocatalyst produced with dextran-aldehyde can guarantee higher thermal stability to the enzyme molecules. Similar behaviours were presented by Gaur et al. [45], comparing different methods of immobilisation of β-galactosidase, where mCLEAs were only more stable than the enzyme immobilised in chitosan at one of the three temperatures studied.

2.5. Kinetic Parameters of Lactose Hydrolysis Catalysed by the Different β-Galactosidase Biocatalysts

The kinetics of lactose hydrolysis using the selected mCLEAs was studied using different substrate concentrations to measure the initial rates of the enzymatic reaction. Moreover, the effect of immobilisation on the enzyme's affinity for the substrate was also evaluated. Figure 2 shows the fitting of the Michaelis–Menten model to the experimental

data. The kinetic parameters K_M and V_{max} were determined for the free enzyme and immobilised by mCLEAs and the constants are shown in Table 8.

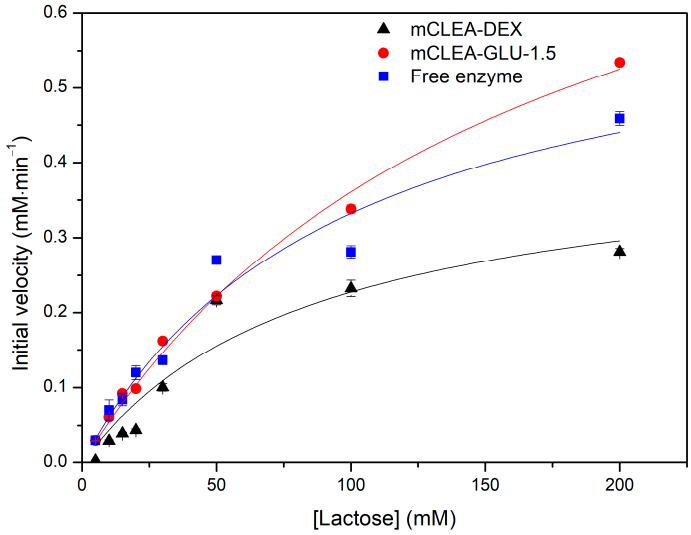


Figure 2. Influence of Lactose concentration in the initial rate of hydrolysis catalysed by free and immobilised β-galactosidase from *Aspergillus oryzae*. Reactions were carried out in potassium phosphate buffer (pH 6.6), with 0.1 mM $MnCl_2$ as a cofactor, at 37 °C. The lines represent the non-linear fit of the Michaelis–Menten model using Origin 8.5.

Table 8. Kinetic parameters for mCLEAs and free enzyme.

Sample	R^2	K_M (mM)	V_{max} (mM·min^{-1})
mCLEA-GLU-1.5	0.99	167.42 ± 18.09	0.96 ± 0.06
mCLEA-DEX	0.91	86.78 ± 22.32	0.42 ± 0.05
Free enzyme	0.95	96.39 ± 18.41	0.65 ± 0.06

The reactions were conducted in lactose solutions prepared in potassium phosphate buffer, pH 6.6. R^2 is the correlation factor; K_M is the Michaelis–Menten constant; V_{max} is the maximum speed.

No significant difference between the K_M of the mCLEA-DEX and the free enzyme was observed (Table 8); however, the V_{max} of the free enzyme was slightly higher. For mCLEA-GLU-1.5, the K_M value was considerably higher than that of the free enzyme, implying that immobilisation with glutaraldehyde caused a reduction in the enzyme's affinity for lactose. This increase in the K_M value between the free and immobilised enzymes, was also reported by Li et al. [46] and Gaur et al. [45], who used glutaraldehyde in the preparation of CLEAs of a β-galactosidase obtained from a metagenomic library and from *Aspergillus oryzae*, respectively.

2.6. Bioconversion of Lactose into Glucose and Galactose

How CLEAs are prepared can affect pore size, which is usually quite small, causing a reduction in substrate diffusion rate and an apparent decrease in enzyme activity [16]. Therefore, to evaluate if mass transfer limitations were impacting the reaction, lactose hydrolysis was conducted and the performance of mCLEAs with the free enzyme was compared. The more active (mCLEA-DEX) and the more stable biocatalysts at 60 °C (mCLEA-GLU-1.5) were assayed. The hydrolysis was carried out in a reactor with 5 mL of 164 mM lactose as substrate, and 0.3 g of mCLEA at 37 °C, pH 6.6 and the results of conversion and productivity are presented in Figure 3.

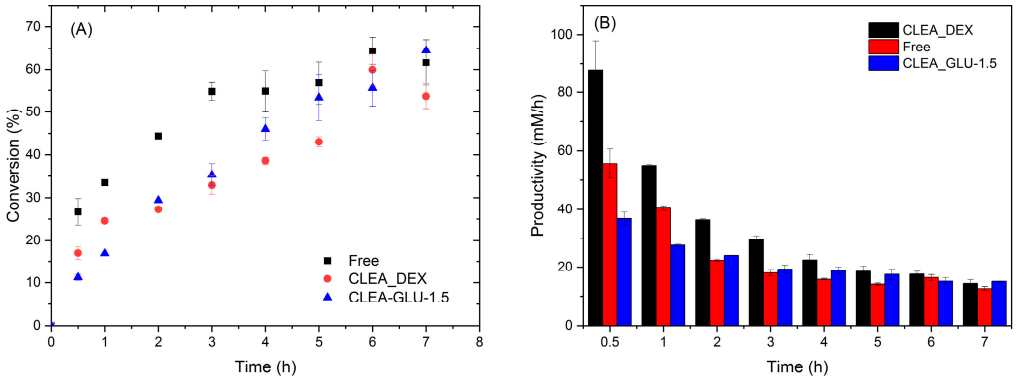


Figure 3. Enzymatic production of D-galactose and D-glucose catalysed by free and immobilised β-galactosidase from *Aspergillus oryzae* (mCLEA-GLU-1.5, mCLEA-DEX) in potassium phosphate buffer (pH 6.6), with 0.1 mM $MnCl_2$ as a cofactor, at 37 °C. Substrate conversion (**A**) and Productivity (**B**) are represented as a function of the reaction time. The initial concentration of lactose was 164 mM. The reaction was carried out in a final volume of 5 mL using 0.3 g of mCLEA.

The free enzyme enables ~55% conversion of lactose into galactose in 3 h (productivity of ~30 mMgalactose/h) and reaches ~60% conversion after 7 h (productivity of ~14 mMgalactose/h). Lactose conversion similarly took place using both mCLEAs, being a delay in conversion observed when compared to the free enzyme. During the first 1 h of analysis, mCLEA-DEX allowed a higher conversion rate (~25% and productivity of ~40 mMgalactose/h) compared to mCLEA-GLU-1.5 (~17% and productivity of ~28 mMgalactose/h). However, a higher conversion (~64%) within the analysis time (7 h) was achieved using mCLEA-GLU-1.5. The existence of some minor diffusion limitations using the mCLEAs may explain the delay in conversion observed. Still, it does not compromise the use of these biocatalysts since similar conversions (and productivities) were achieved at 7 h of the essay. Indeed, the rates of reaction using the heterogeneous biocatalysts may be increased by using higher enzyme concentrations. The higher amount of the immobilised enzyme will compensate the delay in conversion and enhance productivity without impacting the final cost of the process since the enzyme can be reused.

2.7. Immobilised Biocatalysts' Operational Stability

In order to investigate the operational stability (reusability) of β-galactosidase mCLEAs, repeated batches of lactose hydrolysis were carried out in a reactor using 0.5 g of biocatalyst in 5 mL of 164 mM lactose substrate (Figure 4).

At the end of 10 cycles of operation, there was a difference between the enzymatic activity presented by the mCLEA-GLU-1.5 and mCLEA-DEX biocatalysts. After 10 cycles, mCLEA-DEX and mCLEA-GLU-1.5 retained, respectively, around 60% and 80% of their initial activity, indicating the suitability of these heterogeneous biocatalyst for reuse. Similar results with CLEAs of β-galactosidase produced with glutaraldehyde were obtained by Li et al. [46] and Rai et al. [11], who also reached a residual activity close to 80% and >50%, respectively, at the end of 10 cycles.

The operational stability of both immobilised β-galactosidase systems suggests that they have a high potential for economic production in hydrolysis of lactose into glucose and galactose.

2.8. Storage Stability at 4 °C

The storage stability of mCLEAs was investigated by storing samples in a refrigerator (4 °C) for 10 weeks, and the results are shown in Figure 5. After 10 weeks, mCLEA-GLU-1.5 retained 100% of its activity. mCLEA-DEX, despite maintaining the relative activity in the first weeks, showed a reduction in activity at the end of the storage period (week 10), retaining 63% of its activity. For comparison, the dialysed free enzyme was stored at the same conditions and maintained 100% of its initial activity. Other methods authors

reported high storage stability of immobilized β-galactosidase, as 79% when entrapped in agar-agar after 30 days at 4 °C [47] and 85% when immobilized in synthesised silver nanosupport after 60 days at 4 °C [48]. These results indicate the suitability of maintaining the immobilised biocatalysts stored in cold for considerable periods without having an expressive loss of activity.

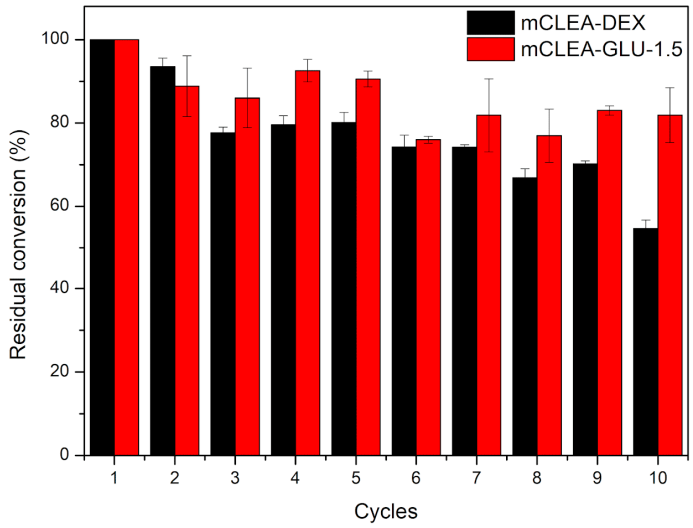


Figure 4. Operational stability of mCLEA-GLU-1.5 and mCLEA-DEX of β-galactosidase from *Aspergillus oryzae* during 10 cycles of 60 min operation. Subsequent batches of lactose hydrolysis were carried out in a potassium phosphate buffer (pH 6.6), with 0.1 mM Ma nCl_2 as a cofactor, at 37 °C, using lactose 164 mM (substrate).

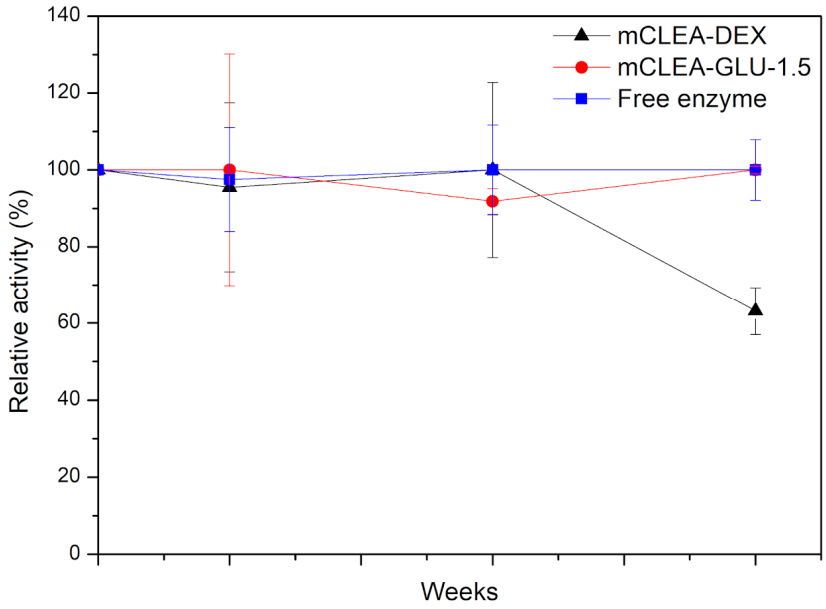


Figure 5. Storage stability of β-galactosidase from *Aspergillus oryzae* biocatalysts: mCLEA-GLU-1.5, mCLEA-DEX and Free enzyme. Analysis conditions: 0.1 g of heterogeneous biocatalyst in 1 mL of potassium phosphate buffer (pH 6.6), with 0.1 mM $MnCl_2$ as a cofactor, at 37 °C. The activity was obtained by reading the absorbance at 420 nm, for 2 min, by reacting 50 µL of the suspension in 2 mL of 1.25 mM ONPG.

2.9. Structural Characterisation of mCLEA

The size and morphology of mCLEA-GLU-1.5 and mCLEA-DEX were evaluated by SEM (scanning electron microscope), see Figure 6. Although these aggregates initially

present a disorganised structure, this does not imply that there is no level of structural organisation, since CLEAs are formed by proteins with a constant and well-defined three-dimensional structure, whose conformation is maintained by the bifunctional (glutaraldehyde) or polyfunctional (dextran-aldehyde) agents [49].

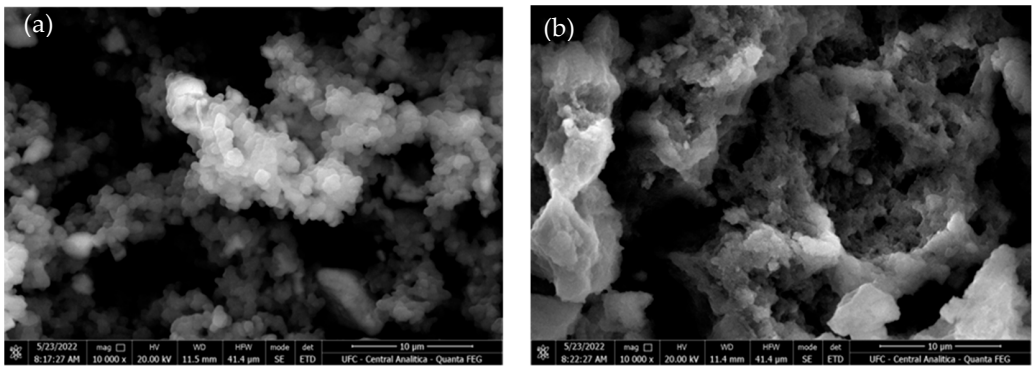


Figure 6. Electron microscopy of mCLEA-GLU-1.5 (**a**) and mCLEA-DEX (**b**), using 10,000× magnification.

According to Figure 6, mCLEAs have an amorphous structure, rough surface, heterogeneous morphology and mean aggregate diameter of approximately 10 µm. Similarly to what was described by other authors [11], a porous mesh-like architecture is observed for both heterogeneous biocatalysts. In addition, the microscopic structure of mCLEA-DEX has fewer void spaces between particles, while mCLEA-GLU-1.5 has higher spaces. Magnetic nanoparticles, when seen in SEM images, appear as small bright parts [49]. Still, they were not identified in Figure 6, possibly due to the cross-linking process that kept them together by covalent bonding.

The FTIR spectra are shown in Figure 7, and the band might identify the presence of magnetite (Fe_3O_4) cross-linking at 531 cm^{-1} attributed to the Fe-O deformation [50]. The characteristic bands of magnetite (Fe_3O_4) remained present in the FTIR spectra of the different CLEAs, which were obtained with different cross-linkers. Still, a sharper peak was observed when the dextran-aldehyde cross-linker was used.

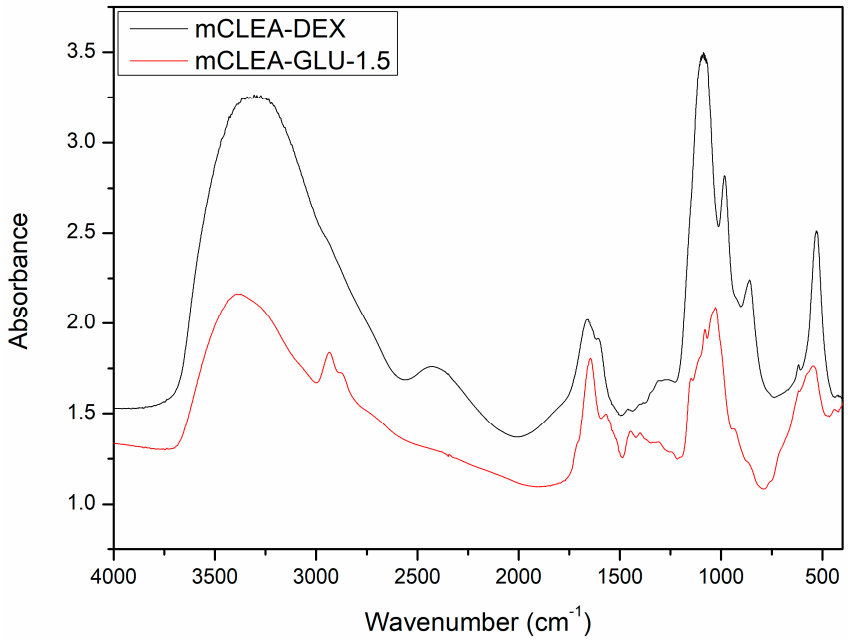


Figure 7. Fourier transform infrared (FTIR) spectra for mCLEA-DEX and mCLEA-GLU-1.5.

Comparing the FTIR spectra of mCLEA-GLU and mCLEA-DEX, it is observed that the band 2934 cm^{-1} represents the CH alkane stretch and the band of 1650 cm^{-1} refers to the characteristic a C=O, which is related to the insertion of aldehyde groups by oxidation with periodate and cross-linking with glutaraldehyde [49–54]. In the mCLEA-DEX spectra, the band 1088 cm^{-1} corresponds to the asymmetric stretching of the C-O-C bond of the dextran glycoside ring.

The peaks obtained between 3000 and 3500 cm^{-1} are associated with the vibration mode of O-H and N-H bonds, which are groups present in β-galactosidase. Also on the enzyme structure, the protein segments show a β-sheet conformation and might be correlated with the 1650 cm^{-1} band and those with helical structures with the 1658 cm^{-1} band [54].

2.10. Lactose Bioconversion into Tagatose by Immobilised β-Galactosidase and L-Arabinose Isomerase

The performance of mCLEAs of β-galactosidase from *Aspergillus oryzae* and L-arabinose isomerase from *Enterococcus faecium* [24] in converting lactose into D-tagatose, by a two-step reaction, was studied. The strategy to use sequential reactors (and not combined biocatalysts) was based on the results obtained when the mixed free enzymes were assayed for lactose hydrolysis (164 or 450 mM) plus galactose isomerisation. When 450 mM of lactose was assayed, the mixed free enzymes showed ∼70% lactose conversion and ∼2% yield of D-tagatose at 6 h. At 24 h, lactose was almost completely hydrolysed (∼93%) achieving only ∼6% yield of D-tagatose. No tagatose was produced when the initial lactose concentration was 164 mM. The production of tagatose from galactose is limited by the unfavourable thermodynamic equilibrium between products and substrates, which can be shifted by adding high substrate (galactose) concentrations at the beginning of the isomerisation reaction. Therefore, in this work, two bioreactors were used for the hydrolysis of lactose into glucose and galactose (reactor 1), and the subsequent conversion of galactose to tagatose (reactor 2).

In the first step, lactose (164 mM) was hydrolysed by immobilised β-galactosidase (m-CLEA-DEX) into galactose and glucose (step 1), resulting in 100% conversion within 24 h. Thereafter, galactose was isomerised by immobilised L-arabinose isomerase (m-CLEA-LAI) to produce D-tagatose (step 2), reaching ∼10% and 13% of conversion into D-tagatose (Figure 8) in 6 h and 24 h of reaction, respectively. Other authors [11] have studied the production of D-tagatose from lactose using a combined immobilised mCLEA (β-galactosidase and L-AI) and they observed that the yield was improved to ∼8% (at 6h) and ∼25% (at 24 h) when compared to the simultaneous use of the free enzymes (mixed β-Gal + L-AI), that showed ∼5% (at 6h) and ∼15% (at 24 h) yield. They attribute this increase in yield to a substrate channelling phenomenon in the cascade reaction when the Combi-mCLEA was used as biocatalyst.

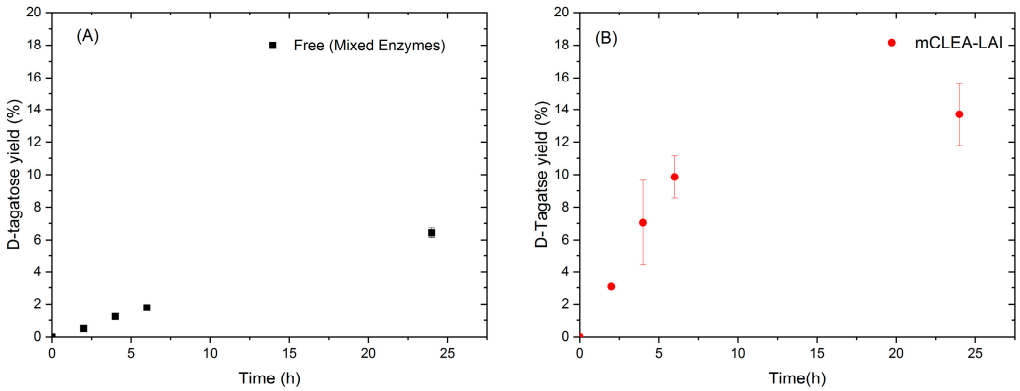


Figure 8. Bioconversion of lactose into D-tagatose, at pH 6.6: (**A**) simultaneous lactose hydrolysis and tagatose synthesis using free enzymes (mixed β-Gal + L-AI) at 50 °C and pH 6.6 and (**B**) Sequential lactose hydrolysis (first bioreactor) catalysed by mCLEA-DEX at 37 °C and pH 6.6 and galactose isomerisation 50 °C (second bioreactor) catalysed by mCLEA-LAI.

3. Materials and Methods

3.1. Synthesis and Cross-Linking of Magnetic Nanoparticles (MNP) with APTES

The magnetite synthesis was conducted as described by Bezerra et al. [55], with modifications. Initially, ferric chloride ($FeCl_3 \cdot 6H_2O$) and ferrous sulphate ($FeSO_4 \cdot 7H_2O$) were dissolved in ultrapure water (Milli-Q, Millipore, Burlington, MA, USA), previously acidified with 5% HCl (pH 3.0–4.0), under magnetic stirring during 30 min. The process also followed the steps described by Sousa et al. [24], by stirring the aqueous solution while adding ammonium hydroxide (NH_4OH). The functionalisation procedure followed the same methodology, adding 3-aminopropyl triethoxysilane (APTES) to the nanoparticles.

3.2. Production of Magnetic Cross-Linked Enzyme Aggregates (mCLEA) of β-Galactosidase

The β-galactosidase used in these experiments was produced by *Aspergillus oryzae*, purchased in lyophilised form from Sigma Aldrich (St. Louis, MO, USA). There were no additional treatments for the enzyme before immobilisation experiments.

As previously described by Gaur et al. [45], to produce heterogeneous biocatalysts using glutaraldehyde as a cross-linking agent, 0.020 g of β-galactosidase was dissolved in 1 mL of 0.1 M potassium phosphate buffer, pH 7.0 (immobilisation buffer), and the solution was kept in an ice bath under magnetic stirring. For the biocatalyst mCLEA-GAL, 164 mM galactose was added to the buffer. 0.550 g of ammonium sulphate was added to the solution, followed by 1 mL of ammonium sulphate solution at 55% w/v. Then, 0.020 g of magnetic nanoparticles was added to the solution, followed by glutaraldehyde (25%), kept under constant stirring for 16 h in an ice bath. The amount of glutaraldehyde added changed according to the biocatalyst: 50 µL, 80 µL or 120 µL was added to form mCLEA-GLU-0.6, mCLEA-GLU-1 and mCLEA-GLU-1.5, respectively. To prepare mCLEA-GAL and mCLEA-PEI, 120 µL of glutaraldehyde was added. Next, the solid was washed using the immobilisation buffer, followed by centrifugation, at 5000 rpm for 15 min, all at 4 °C. The heterogeneous biocatalyst mCLEA-PEI was yet put under rotation stirring in 10% polyethyleneimine (PEI) solution for 5 h.

To prepare dextran-aldehyde, 1.00 g of dextran (M_W 450,000–650,000) supplied by Sigma Adrich (St. Louis, MO, USA), was dissolved in 30.35 mL of ultrapure water, and oxidised with 2.3373 g of sodium periodate, for 90 min at 25 °C under magnetic stirring. The solution was dialysed 5 times using 5 L of water, each dialysis lasting 2 h, to obtain the final dextran-aldehyde solution. As previously performed by Mateo et al. [43], with some changes, 0.0200 g of β-galactosidase was dissolved in 1 mL of 500 mM potassium phosphate buffer pH 8.0, keeping the solution under stirring and in an ice bath. 0.5500 g of ammonium sulphate was added to the solution, followed by 1 mL of ammonium sulphate solution at 55% w/v. Then, 0.0200 g of magnetic nanoparticles and 2 mL of the dextran-aldehyde solution were added and kept under agitation in an ice bath for 16 h. Subsequently, washes were followed by centrifugations, at 5000 rpm for 15 min, and then for 10 min, all at 4 °C, resulting in a suspension of mCLEA-DEX heterogeneous biocatalysts.

3.3. Activity Assays and Determination of Immobilisation Parameters

The free enzyme solution was obtained by resuspending 0.020 g of β-galactosidase in 2 mL of 50 mM potassium phosphate buffer, pH 6.6, with 0.1 mM $MnCl_2$ (activity buffer). The activity was obtained by reacting 25 µL of the enzymatic solution in 2 mL of 1.25 mM o-nitrophenyl β-D-galactopyranoside (ONPG, calculated $\varepsilon = 4.53$ mL/(U×cm×min)) for 2 min, monitoring the absorbance at 420 nm during the process [56]. For the immobilised β-galactosidase, 0.1 g was resuspended in 1 mL of 50 mM potassium phosphate buffer supplemented with 0.1 mM $MnCl_2$, pH 6.6. The activity was obtained by reading the absorbance at 420 nm, for 2 min, by reacting 50 µL of the enzyme suspension in 2 mL of 1.25 mM ONPG. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce one µmol of product per minute under the reaction conditions.

For the immobilised L-arabinose isomerase, activity was determined according to Sousa et al. [24].

The immobilisation parameters were calculated according to Sousa et al. [24]. Immobilisation yield (IY) is the percentage of enzyme activity (free enzyme) that was immobilised and recovered activity (At_r) is the percentage of the immobilised enzyme that remains active in the biocatalyst.

3.4. Thermal Stability of Immobilised and Free Biocatalysts

The thermal stability of β-galactosidase at 60 °C was determined as previously described by Freitas et al. [56]. Briefly, 2 mL of activity buffer was previously maintained at 60 °C, to which 200 mg of immobilised biocatalysts or free enzyme solution were subsequently added and incubated. Samples (0, 5, 15, 30 and 60 min) were withdrawn, and residual activity was determined. The Sadana and Henley [44] enzyme deactivation model was fitted to the experimental data of residual activity using the software OriginPro 8.5. The model parameters were used to determine the half-lives of heterogeneous biocatalysts and the free enzyme. The thermal stability at 50 °C of mCLEA-GLU-1.5 and mCLEA-DEX was performed in a similar way, using 2 mL of the activity buffer previously heated to 50 °C.

3.5. Enzymatic Kinetics

The kinetic parameters, maximum speed (V_{max}) and Michaelis–Menten constant (K_M), of the heterogeneous biocatalysts (mCLEA-GLU-1.5 and mCLEA-DEX) and the free enzyme were calculated for the hydrolysis of lactose. The reaction was conducted for 60 min, using increasing concentrations (5, 10, 15, 20, 30, 50, 100 and 200 mM) of the substrate, as previously described by Li et al. [46], with modifications. 0.025 g of immobilised enzyme (mCLEA) in 250 µL of lactose solution was used, while to free enzyme, 650 µL of the dialysed solution of β-galactosidase was added to 850 µL of lactose solution. The glucose concentration resulting from the hydrolysis was determined using the Bioclin® glucose kit (Quibasa, Belo Horizonte, Brazil).

3.6. Lactose Hydrolysis Assay

The lactose hydrolysis assay was performed as previously described [57], with modifications. Amounts of 5 mL of a 164 mM lactose solution and 0.2942 g of heterogeneous biocatalyst (mCLEA-GLU-1.5 or mCLEA-DEX) were added to a bench reactor, maintained at 37 °C and under constant mechanical stirring. Samples were withdrawn for up to 24 h of reaction to measure lactose hydrolysis into galactose and glucose. Glucose, a product of the reaction, was determined with the Bioclin® Glucose Kit (Quibasa, Minas Gerais, Brazil).

3.7. Operational Stability of Immobilised Biocatalysts

To determine the operational stability of the biocatalysts, mCLEA-GLU-1.5 and mCLEA-DEX were used to catalyse subsequent batches of lactose hydrolysis at 37 °C and pH 6.6. The lactose concentration in each cycle was 164 mM, and the volume was 5 mL. Each cycle lasted 60 min, and the initial mass of the heterogeneous biocatalyst was 0.53 g. At the end of each cycle, the biocatalyst was recovered with a magnet and washed with 5 mM sodium acetate buffer (pH 5.6) to remove the unreacted remaining substrate and products. Glucose concentration was measured using the Bioclin® glucose kit (Quibasa, Belo Horizonte, Brazil).

3.8. Storage Stability of Immobilised and Free Biocatalysts

Suspensions of 0.1 g of mCLEA-GLU-1.5 and mCLEA-DEX in 1 mL of activity buffer, as well as 1 mL of dialysed free enzyme, were kept at 4 °C. Samples were taken to measure the activity (ONPG hydrolysis) of these biocatalysts after 0, 2, 6 and 10 weeks of storage.

3.9. Structural Characterisation of mCLEA

Scanning electron microscopy (SEM) analysis of mCLEAs was performed on a Quanta FEG 450 microscope (FEI, Hillsboro, OR, USA) operated at 20 kV and 0.7 torr, according

to the methodology described by Ferreira et al. [58]. The samples were deposited on a carbon tape and metallised with gold by the Quorum QT150ES Metallizer (Quorum, Laughton, UK). Fourier transmission infrared (FTIR) spectroscopy analysis of mCLEAs was also performed using the 670-IR FTIR equipment (Varian; Mulgrave, Australia) [59]. The biocatalysts were ground and pelleted with KBr (1:100, *w*/*w*), followed by uniaxially pressure, being evaluated in a wavelength range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}.

3.10. Lactose Bioconversion to Tagatose by Immobilised β-Galactosidase and L-Arabinose Isomerase

β-galactosidase and L-arabinose isomerase were evaluated to produce D-tagatose from lactose, a cascade reaction. First, the two-step cascade reaction was carried out using a combined mixture of the free enzymes, β-Galactosidase (0.6 U/mL) and L-AI (0.6 U/mL), in 5 mL sodium phosphate buffer (50 mM, pH 6.6) containing 164 or 450 mM of lactose and in the presence of 1 mM Mn^{2+} at 50 °C. Samples were withdrawn at regular intervals, and the amount of galactose and D-tagatose produced were determined.

The sequential application was catalysed by the immobilized enzymes and consisted of the first step, lactolysis at 37 °C and pH 6.6 (bioreactor 1), using an immobilised β-galactosidase (mCLEA-DEX), followed by isomerisation of D-galactose in D-tagatose at 50 °C and pH 6.6 by the immobilised L-arabinose isomerase (mCLEA-LAI) (bioreactor 2). The batch process was initiated by adding 164 mM of lactose as substrate to the bioreactor 1 and the immobilised heterogeneous biocatalyst (0.3 g of mCLEA-DEX). After 24 h, the mCLEA-DEX was removed and the resulting reactional media, containing products was equilibrated to 50 °C. Next, 0.8 g of mCLEA-LAI was added to the bioreactor to produce D-tagatose.

Productivity was expressed in terms of carbohydrates (galactose or D-tagatose). For the first reaction, glucose using the Bioclin® glucose kit (Quibasa, Belo Horizonte, Brazil). For the isomerization, D-tagatose was determined via the cysteine-carbazole sulfuric acid method [24].

4. Conclusions

The immobilisation of β-galactosidase into magnetic CLEAs was successfully carried out using either glutaraldehyde or dextran-aldehyde as a cross-linking agent. The recovered activity was a crucial parameter and mCLEA-DEX showed a high value (>73.6%). Regarding thermal stability, each heterogeneous biocatalyst stood out at a different temperature, mCLEA-DEX performed better than mCLEA-GLU-1.5 at 50 °C and the opposite happened at 60 °C. Furthermore, both heterogeneous biocatalysts performed lactose hydrolysis similarly to the free enzyme but maintained high stability over storage and reusability. Moreover, the capability of obtaining the rare sugar D-tagatose from galactose produced by lactose hydrolysis catalysed by mCLEA-DEX was also attested. Therefore, mCLEA-GLU-1.5 and mCLEA-DEX were selected as promising biocatalysts in the dairy industry.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal13020306/s1, Figure S1: Thermal deactivation at 60 °C of heterogeneous biocatalysts produced in different glutaraldehyde concentrations and of the dialyzed free enzyme, in terms of activity, relative to the initial, by time, in minutes. The reactions took place in potassium phosphate buffer, pH 6.6; Figure S2: Thermal deactivation at 60 °C of the dialyzed free enzyme, and heterogeneous biocatalysts produced with a crosslinker concentration of 1.5%, including immobilization with an inhibitor and coating of heterogeneous biocatalyst, in terms of activity, relative to the initial for time in minutes. The reactions took place in a potassium phosphate buffer, pH 6.6; Figure S3: Thermal deactivation at 60 °C of heterogeneous biocatalysts produced with glutaraldehyde and dextran-aldehyde and of the dialyzed free enzyme, in terms of the activity relative to the initial by time in minutes. The reactions took place in a potassium phosphate buffer, pH 6.6; Figure S4: Thermal deactivation at 50 °C of immobilized β-galactosidase produced with glutaraldehyde and with dextran-aldehyde and the dialyzed free enzyme, in terms of relative activity by time in minutes. The reactions took place in a potassium phosphate buffer, pH 6.6.

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