Article

Encapsulated NOLA™ Fit 5500 Lactase—An Economically Beneficial Way to Obtain Lactose-Free Milk at Low Temperature

Katarzyna Czyzewska * and Anna Trusek *

Department of Micro, Nano and Bioprocess Engineering, Faculty of Chemistry,
Wroclaw University of Science and Technology, Norwida 4/6, 50-373 Wrocław, Poland
* Correspondence: katarzyna.czyzewska@pwr.edu.pl (K.C.); anna.trusek@pwr.edu.pl (A.T.);
Tel.: +48-71-3203314 (K.C.); +48-71-3202653 (A.T.)

Abstract: The current requirements of industrial biocatalysis are related to economically beneficial and environmentally friendly processes. Such a strategy engages low-temperature reactions. The presented approach is essential, especially in food processes, where temperature affects the quality and nutritional value foodstuffs. The subject of the study is the hydrolysis of lactose with the commercial lactase NOLA™ Fit 5500 (NOLA). The complete decomposition of lactose into two monosaccharides gives a sweeter product, recommended for lactose intolerant people and those controlling a product's caloric content. The hydrolysis reaction was performed at 15 °C, which is related to milk transportation and storage temperature. The enzyme showed activity over the entire range of substrate concentrations (up to 55 g/L lactose). For reusability and easy isolation, the enzyme was encapsulated in a sodium alginate network. Its stability allows carrying out six cycles of the complete hydrolysis of lactose to monosaccharides, lasting from two to four hours. During the study, the kinetic description of native and encapsulated NOLA was conducted. As a result, the model of competitive galactose inhibition and glucose mixed influence (competitive inhibition and activation) was proposed. The capsule size does not influence the reaction rate; thus, the substrate diffusion into capsules can be omitted from the process description. The prepared 4 mm capsules are easy to separate between cycles, e.g., using sieves.

Keywords: NOLA™ Fit 5500; sodium alginate; encapsulation; competitive inhibition; glucose activation; lactose-free milk; sweet-milk; low-temperature catalysis; heterogeneous enzymatic process

1. Introduction

Dairy products are present in our daily life. The benefits of milk and milk-related food consumption are obvious and well-known. Unfortunately, lactose intolerance and the high calorific value of dairy desserts are recognised as significant challenges to create healthy, balanced, and wholesome food products [1–3]. The lactose-free dairy market is a rapidly growing area of the food industry and was valued at EUR 9 billion in 2022 [4].

Various methods of lactose decomposition or separation are applied to overcome the hindrance of lactose intolerance. Enzymatic lactose hydrolysis is considered an economically beneficial and environmentally friendly strategy [5]. Moreover, the variety of commercially available lactases (β-galactosidases, E.C. 3.2.1.23) allows creating more sophisticated technological solutions. Nola ™ Fit 5500 (NOLA) from *Bifidobacterium bifidum*, produced by a selected strain of *Bacillus licheniformis*, is an interesting lactase representative applied in milk and cheese manufacture [6–8]. It is characterised by great activity in operational conditions related to dairy processes, e.g., related to pH, temperature, and the ionic components present in milk.

Enzymatic lactose hydrolysis is well characterised in the literature. Most of the described results concern lactose conversion at 35–50 °C [5,9]. The commercial preparation of NOLA also uses this range of temperatures [8,10]. Unfortunately, experimental data

obtained in the laboratory scale do not fully respond to the industrial operational conditions, especially regarding temperature, which should be kept below 20 °C. The literature reports provide little information about enzymatic lactose hydrolysis at low temperatures, which can be performed during milk storage and transportation. Low-temperature biocatalysis is characterised by commercial, economic, and environmental benefits. The prevention from spoilage, microbial contamination, energy-saving, retention of labile and volatile flavour compounds, and high taste quality are considered the most important benefits [11].

To meet the expectations of modern industrial biocatalysis, the enzymes are immobilised with high efficiency, which guarantees long application [12–15]. Enzyme encapsulation inside a natural polymer matrix is common in food technologies due to creating a favourable internal environment and naturally derived carriers [16]. Moreover, the available molecular models on the analysis of carrier and enzyme structure allow selecting the appropriate hydrogel matrix [17].

The available literature reports suggest the various models of kinetic description during lactose hydrolysis catalysed by β-galactosidase. Depending on the β-galactosidase isolation source and reaction conditions, the kinetics based on the Michaelis–Menten model or product inhibition can be found. In the second case, glucose, galactose, as well as mixed inhibition, is possible [18–22]. The most common strategy is related to competitive galactose inhibition (Equation (1)).

$$r = \frac{k_3 \cdot C_{enzyme} \cdot C_{lactose}}{K_m\left(1 + \left(C_{galactose}/K_i\right)\right) + C_{lactose}} = \frac{r_{max} \cdot C_{lactose}}{K_m\left(1 + \left(C_{galactose}/K_i\right)\right) + C_{lactose}} \qquad (1)$$

where:

C—concentration (g/L)
K_i—inhibition constant (g/L)
K_m—Michaelis–Menten constant (g/L)
k_3—kinetic constant (1/min)
r—reaction rate (g/(L·min))
r_{max}—maximum reaction rate (g/(L·min))

The main goal of the presented work is to characterise the preparation of NOLA, which can be utilised during lactose hydrolysis on an industrial scale. The key assumption leads to lactose bioconversion at 15 °C by native and encapsulated NOLA. The selected temperature value directly corresponds to the temperature of milk transportation and storage. A slightly acidic environment (pH 6.6) results in the pH of raw milk. According to literature reports, the lactose concentration in raw cow milk is noted as 37–55 g/L [23] and, thus, 55 g/L was the highest tested concentration.

The model of product inhibition or activation for both native and encapsulated NOLA was verified for the kinetic description. For efficient enzyme immobilisation, the encapsulation inside the sodium alginate matrix was selected. In the following section, the usefulness of encapsulated NOLA was determined. In this case, NOLA reuse, the half-time value, and diffusion resistance influence are discussed.

2. Results

2.1. Kinetic Characteristic of NOLA in the Native Form

The selection of NOLA concentration was performed in the range of 0.26–7.15 g/L. The linear dependence of the relation v = f(C_{enzyme}) was observed until 1.5 g/L. At 15 °C, this enzyme concentration allowed obtaining the almost complete lactose decomposition in 30 min independent of the initial substrate concentration (Figure 1).
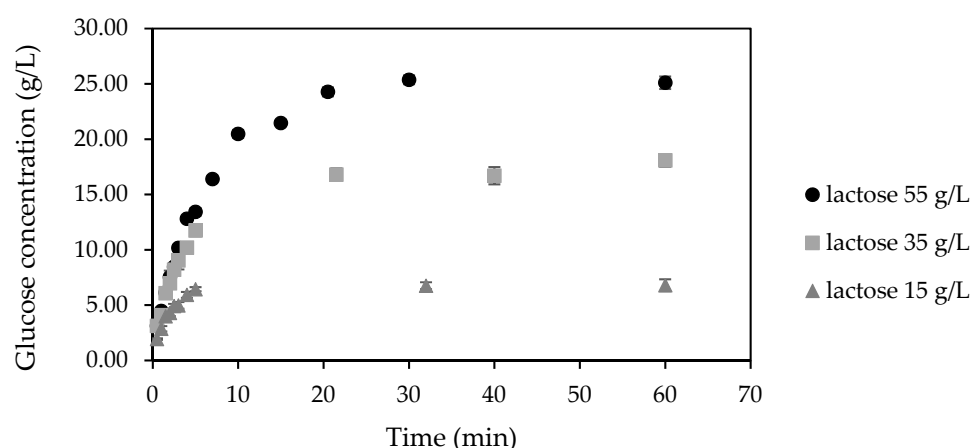
Figure 1. The lactose decomposition expressed as product (glucose) concentration changes by 1.5 g/L native NOLA, 15 °C, pH 6.6.

The hydrolysis reactions were carried out in a wide range of lactose concentrations, 5.0–55.0 g/L, and for three different NOLA concentrations: 0.8, 1.2, and 1.5 g/L. An exemplary reaction course used for reaction rate (r) calculation (r = d$C_{glucose}$/dt) is presented in Figure 1. The initial reaction rate was calculated until the maximum 6% of lactose conversion, during which the influence of the products on the reaction run can be omitted. On the basis of 21 reactions, using the OriginPro 2021 programme, the obtained with the relative error 3.91% values of Michaelis–Menten equation are followed: k_3 = 3.28 L/min, K_m = 30.00 g/L.

Referring to the literature reports, the inhibition by products was checked. Galactose significantly inhibits the enzyme what is known from the previous reports [24,25]. This effect was visible for all tested lactose concentrations (5.0–55.0 g/L). The selected reactions are presented in Figure 2.
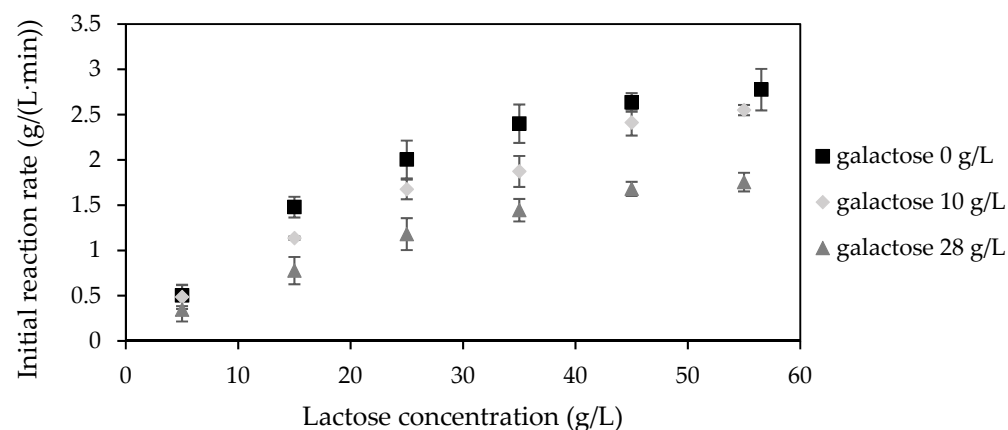
Figure 2. The influence of galactose on lactose hydrolysis rate (1.5 g/L native NOLA, 15 °C, pH 6.6).

The mathematical description of the process was well suited to competitive galactose inhibition; thus, Equation (1) was applied. The average value of inhibition constant (K_i) for galactose based on 24 reactions was determined as 26.28 g/L (Table 1), while the values of the constants k_3 and K_m were within the error range of the values determined from the initial reaction rates.

Table 1. The galactose effect—the values of the inhibition constant Equation (1); 1.5 g/L native NOLA, 15 °C, pH 6.6; k_3 = 3.28 ± 0.26 L/min, K_m = 30.00 ± 7.03 g/L.

Galactose (g/L)	K_i (g/L)
5	24.49 ± 2.93
10	29.78 ± 4.68
20	29.46 ± 3.64
28	21.39 ± 0.72

The effect of glucose on reaction rate was recognised on the basis of 28 reactions performed at different glucose and lactose concentrations. The initial rates of these reactions are presented in Figure 3.
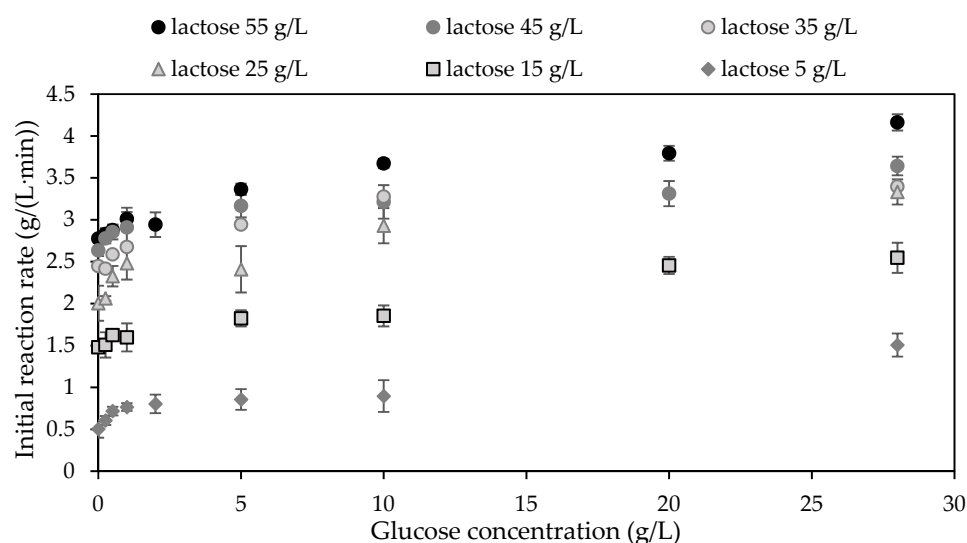
Figure 3. The glucose effect on reaction rate at various lactose concentrations (1.5 g/L native NOLA, 15 °C, pH 6.6).

The effect of glucose on reaction rates is complex. It was described with the best accuracy as simultaneous competitive inhibition and activation—Equation (2).

$$r = \frac{k_3 \cdot C_{enzyme} \cdot \left(1 + \frac{C_{glucose}}{K_a}\right) \cdot C_{lactose}}{K_m \cdot \left(1 + \frac{C_{glucose}}{K_i}\right) + C_{lactose}} \tag{2}$$

where:

C—concentration (g/L)
K_a—activation constant (g/L)
K_i—inhibition constant (g/L)
K_m—Michaelis–Menten constant (g/L)
k_3—kinetic constant (1/min)
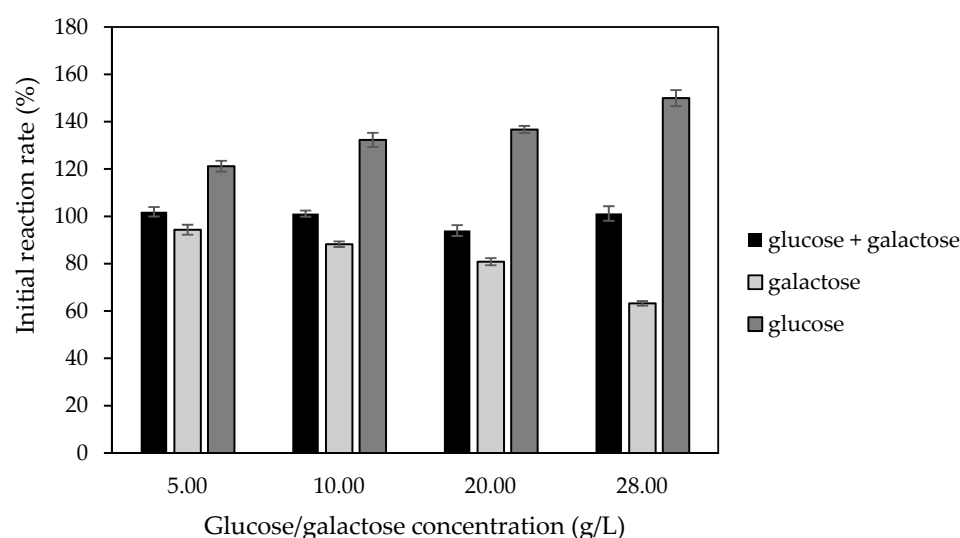r—reaction rate (g/(L·min))

The average value of inhibition constant (K_i) for glucose was determined as 15.79 g/L, activation constant (K_a) as 0.76 g/L, while the constant values k_3 and K_m were within the error range of the values determined from the initial reaction rates. Experimental values and those calculated with the above values of constants are presented in Table 2.

Table 2. The effect of glucose on the reaction rate (g/(L·min))—experimental data and values calculated using Equation (2); 1.5 g/L native NOLA, 15 °C, pH 6.6, k_3 = 3.28 $\pm$ 0.26 L/min, K_m = 30.00 $\pm$ 7.03 g/L.

Lactose (g/L)	Glucose 5 g/L		Glucose 10 g/L		Glucose 20 g/L		Glucose 28 g/L	
	Exper.	**Calcul.**	**Exper.**	**Calcul.**	**Exper.**	**Calcul.**	**Exper.**	**Calcul.**
5	0.856	0.756	0.897	0.803	1.066	0.880	1.005	0.930
15	1.824	1.793	1.853	1.929	2.455	2.164	2.546	2.322
25	2.409	2.469	2.929	2.682	3.534	3.056	3.330	3.315
35	2.943	2.946	3.277	3.221	3.712	3.712	3.400	4.059
45	3.166	3.300	3.213	3.625	3.312	4.215	3.642	4.637
50	3.187	3.445	3.497	3.792	3.769	4.425	3.978	4.881
55	3.363	3.573	3.672	3.940	3.794	4.612	4.162	5.100

The hydrolysis reaction of lactose produces equal amounts of glucose and galactose. As studies have shown, although the effect of galactose and glucose on the reaction is different (inhibition vs. activation, different values of K_i constant), their equimolar concentration in the reaction system does not significantly change the reaction rate (Figure 4). Thus, the effect of inhibition in general description can be omitted.


Figure 4. Galactose and glucose impact on the reaction rate (55 g/L lactose, 1.5 g/L native NOLA, 15 °C, pH 6.6).

2.2. Encapsulated NOLA Characteristics

Before the encapsulation procedure, NOLA stability in the presence of calcium ions was studied. As a result, NOLA incubation in 15% $CaCl_2$ solution for 360 h does not change the enzyme activity (data not shown). This observation confirms the creation possibility of highly efficient preparation of encapsulated NOLA inside calcium alginate matrix.

In the next experiment, encapsulated NOLA reuse was examined. The number of cycles during which enzyme utilisation is possible in the subsequent batches is a crucial factor, especially in industrial biocatalysis. NOLA encapsulated into alginate capsules can be reused for six cycles, where the highest cycle in which the enzyme shows more than 20% initial activity is taken as the last cycle (Figure 5). Declining activity resulted in longer individual cycle times to achieve complete (>97%) lactose hydrolysis. This time was 110 min for the first cycle and 247 min for cycle six.
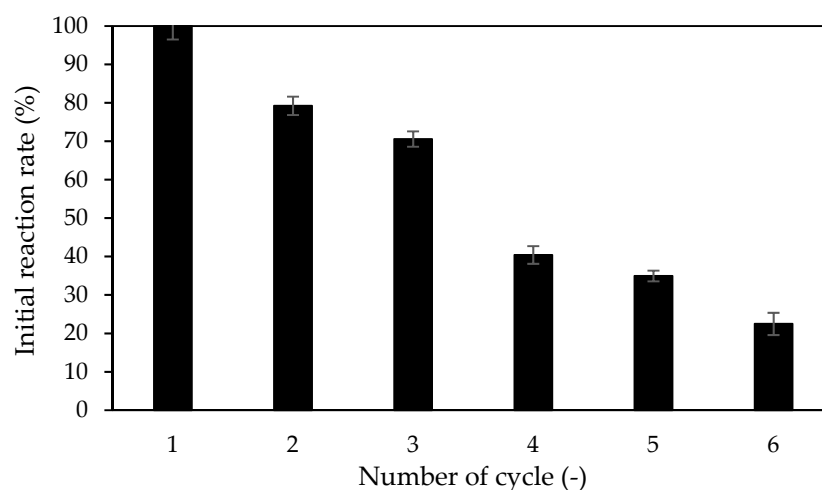
Figure 5. Reusability of encapsulated NOLA expressed by the percentage of the initial reaction rate. Enzyme activity was determined in the presence of 55 g/L lactose, 15 °C, pH 6.6.

The stability of the preparation was described according to first order kinetics (Equation (3)).
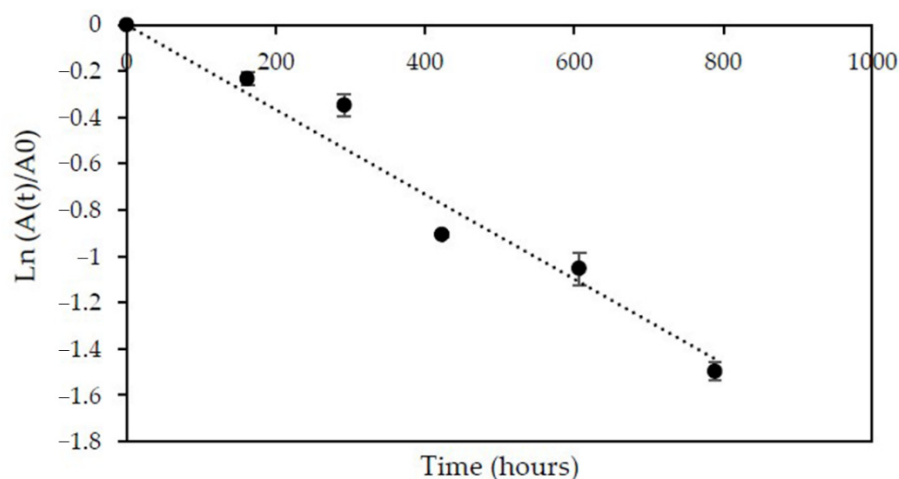
$$ln\frac{A(t)}{A(t=0)} = k_{inact.} \cdot t \tag{3}$$

where:

A—enzyme activity expressed by the initial reaction rate [g/L·h]
$k_{inact.}$—inactivation constant [1/h]
t—time [h]

The determined inactivation constant $k_{inact.}$ = 0.001828 L/h allowed calculation of the half-life of the activity, which is about sixteen days (Figure 6).

Figure 6. The half-life time of encapsulated NOLA (15 °C, pH 6.6).

Using the encapsulation method developed and described in [26], it can be assumed that the enzyme concentration inside the capsule is equal to the enzyme concentration in the alginate solution dropped into the crosslinking bath. Hence, the efficiency of the immobilisation itself is equal to 100%. Unfortunately, this is not directly reflected in the enzymatic activity. Although encapsulation is the mildest form of immobilisation and calcium chloride had no negative effect on NOLA activity, per mass of enzyme used in the whole volume (lactose solution and capsules volume), the activity of the immobilised

enzyme was lower. This resulted in the need to extend the reaction time to achieve complete substrate conversion—Figure 7.
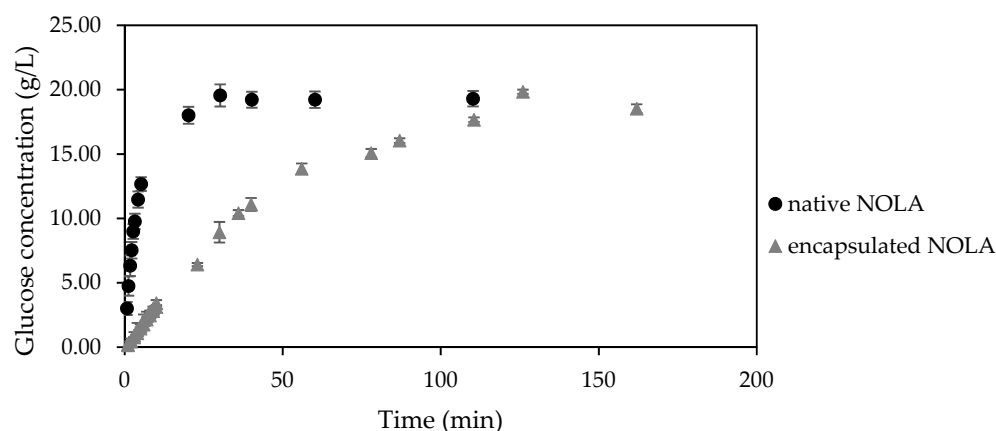
Figure 7. The course of the complete lactose conversion by native and encapsulated NOLA expressed by the product (glucose) concentration (39.29 g/L initial lactose concentration, 0.8 g/L enzyme concentration, 15 °C, pH 6.6).

The results shown in Figure 7 suggest the possibility of diffusion resistance accompanying heterogeneous catalysis, which can take place when an encapsulated enzyme is used [23]. Then, the rate of catalysis is significantly slower than that of substrate diffusion into the hydrogel interior, and the substrate concentration profile across the hydrogel capsule is flat. Its value is only a function of the substrate concentration in the solution surrounding the capsules and is the highest as possible. This situation corresponds to the kinetic regime of the process. In the diffusive regime, fast catalysis occurs at the surface and in the closest surrounding layers of the capsules, and the substrate molecules do not have the possibility to diffuse into the capsules. Thus, enzymes located near the centre of the capsule will not be involved in a reaction.

The values of kinetic constant, substrate diffusion coefficient, and capsule diameter included in the Thiele modulus (φ) determine the process regime [27]—Equation (4).

$$\varphi^2 = R^2 \cdot \frac{k}{D_S} \tag{4}$$

where:

Ds—substrate diffusion coefficient (m^2/h)
k—kinetic constant (1/h)
R—capsules diameter (m)
φ—Thiele modulus

Hence, this parameter should be controlled and optimised when the capsule size directly influences the profile of substrate concentration inside the capsules and directly impacts the reaction process rate [27]. To test the effect of diffusion on the reaction, NOLA capsules with four different diameters in the range 2.32–4.02 mm were used. As no differences in process rate were observed (data not shown), the effect of diffusion on hydrolysis can be excluded.

2.3. Kinetic of Encapsulated NOLA

The rate of reaction for encapsulated enzyme was calculated as follows.

$$r = \frac{\Delta m_{glucose}}{\Delta t \cdot \left(V_{buffer} + V_{capsules} \right)} \tag{5}$$

where:

$m_{glucose}$—glucose (product) mass (g)

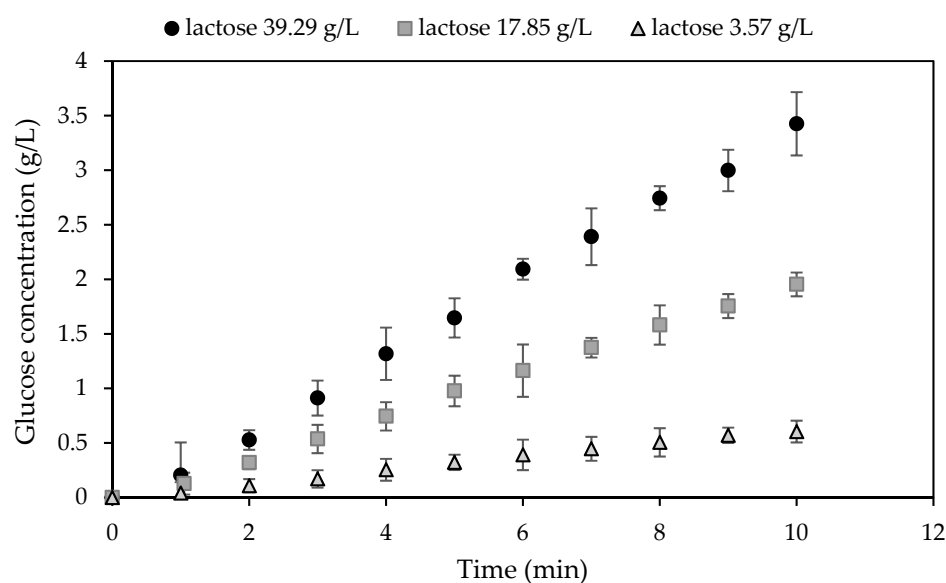r—reaction rate (g/(L·min))

t—time (min)

V_{buffer}—volume of buffer with substrate (L)

$V_{capsules}$—volume of capsules (L)

Examples of the hydrolysis process expressed by glucose concentration changes are shown in Figure 8.

Figure 8. Lactose hydrolysis by encapsulated NOLA expressed by the product (glucose) concentration (C_{enzyme} inside capsules is 0.8 g/L, capsule diameter is 4.02 mm, $V_{capsules}/V_{solution}$ = 0.4, 15 °C, pH 6.6).

The initial reaction rate corresponding to hydrolysis of lactose mass in 5% at different lactose concentrations is presented in Figure 9. On the basis of these values, using OriginPro 2021, the constants of Michaelis–Menten equation were calculated. The obtained values of Michaelis–Menten constants with 4.85% relative error are as follows: k_3 = 0.60 L/min, K_m = 21.19 g/L.
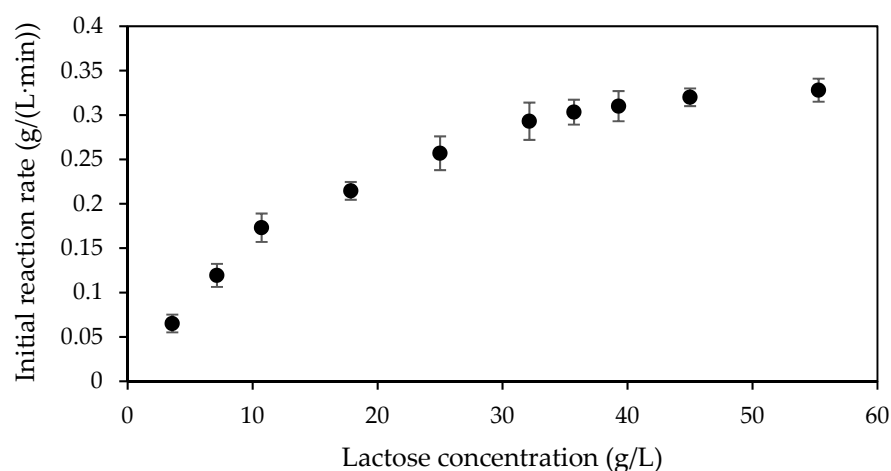
Figure 9. Kinetics of encapsulated NOLA (C_{enzyme} inside capsules is 0.8 g/L, capsule diameter is 4.02 mm, $V_{capsules}/V_{solution}$ = 0.4, 15 °C, pH 6.6).

Like the native NOLA, the encapsulated biocatalyst is also competitively inhibited by galactose (Figure 10). On the basis of 32 reactions, the average value of the K_i constant (Equation (1)) was determined as 7.40 g/L (Table 3). This value is considerably lower than for the native preparation.

Figure 10. Galactose inhibition on encapsulated NOLA—Equation (1), C_{enzyme} inside capsules is 0.8 g/L, capsule diameter is 4.02 mm, $V_{capsules}/V_{solution}$ = 0.4, 15 °C, pH 6.6.
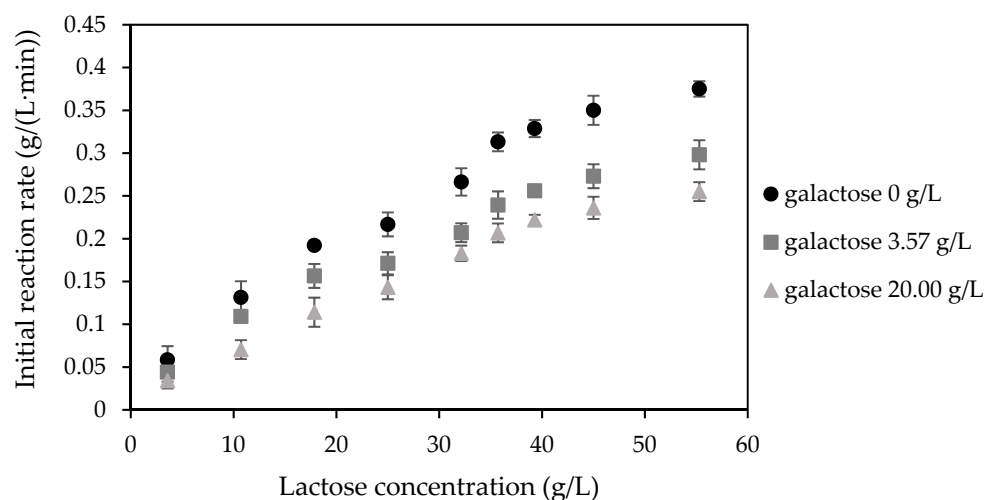
Table 3. The galactose effect—the values of the inhibition constant Equation (1); encapsulated NOLA (C_{enzyme} inside capsules is 0.8 g/L, capsule diameter is 4.02 mm, $V_{capsules}/V_{solution}$ = 0.4, 15 °C, pH 6.6); k_3 = 0.6 ± 0.03 L/min, K_m = 21.19 ± 1.02 g/L.

Galactose (g/L)	K_i (g/L)
3.57	7.25 ± 0.22
7.14	7.79 ± 0.51
14.29	6.33 ± 0.44
20.00	8.24 ± 0.13

On the basis of 21 reactions, the average value of the inhibition constant (K_i) for glucose was determined as 10.54 g/L, while activation constant (K_a) was 4.11 g/L. The constant values k_3 and K_m were within the error range of the values determined from the initial reaction rates. Experimental values and calculated with the above values of constants are presented in Table 4.

Table 4. The glucose effect on the reaction rate (g/(L·min))—experimental data and values calculated using Equation (2); C_{enzyme} inside capsules is 0.8 g/L, capsule diameter is 4.02 mm, $V_{capsules}/V_{solution}$ = 0.4, 15 °C, pH 6.6; k_3 = 0.6 ± 0.03 L/min, K_m = 21.19 ± 1.02 g/L.

Lactose (g/L)	Glucose 0.36 g/L		Glucose 1.43 g/L		Glucose 3.57 g/L	
	Exper.	Calcul.	Exper.	Calcul.	Exper.	Calcul.
5	0.048	0.067	0.053	0.067	0.058	0.053
15	0.111	0.157	0.132	0.158	0.131	0.137
25	0.157	0.217	0.186	0.217	0.192	0.200
35	0.183	0.258	0.211	0.258	0.217	0.249
45	0.216	0.289	0.244	0.289	0.266	0.288
50	0.243	0.302	0.300	0.302	0.313	0.305
55	0.282	0.313	0.308	0.313	0.329	0.320

The effect of galactose inhibition in encapsulated NOLA is stronger than glucose influence; thus, higher mono sugar concentrations decrease the reaction rate (Figure 11).
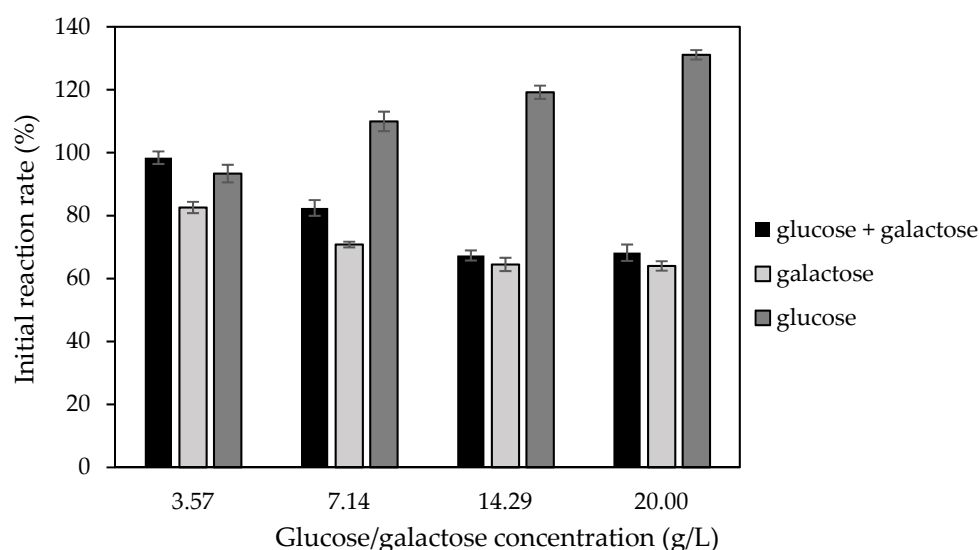
Figure 11. The impact of glucose and galactose on encapsulated NOLA (39.29 g/L lactose, C_{enzyme} inside capsules is 0.8 g/L, capsule diameter is 4.02 mm, $V_{capsules}/V_{solution}$ = 0.4, 15 °C, pH 6.6).

3. Discussion

The presented work focused on low-temperature lactose hydrolysis by the commercial β-galactosidase NOLA, both in native and encapsulated form. Temperature values during lactose hydrolysis must be closely related to the properties of dairy products. In the case described, 15 °C was used—one of the higher temperatures used for storing and transporting milk, but acceptable and allowing relatively rapid enzymatic catalysis.

Due to the reusability of the preparation, the current technological trends encourage immobilised enzyme application. Nonetheless, the proposition of NOLA encapsulation is entirely new. The literature reports present various examples of β-galactosidase immobilisation. Among them, κ-carrageenan beads [28], chitosan-hydroxyapatite beads [29], fibres composed of alginate and gelatin hardened with glutaraldehyde [30], cellulose acetate-polymethylmethacrylate membrane [31], and glass beads [32] have been proposed. The utilisation of sodium alginate in the dairy industry context is justified due to the natural source of the main compound and mild gelation conditions. However, in some cases, calcium ions negatively affected enzyme activity [5,17,33–35]. There was no decrease in NOLA activity after incubation in the gelation bath in the presented immobilised preparation.

The produced 4 mm diameter capsules are easy to separate, e.g., using sieves. The stability of the encapsulated enzyme allows six cycles of total lactose hydrolysis within a cycle-dependent time of 2–4 h.

They are several kinetic model descriptions of enzymatic lactose hydrolysis catalysed by β-galactosidase. Depending on the source of β-galactosidase, the classic Michaelis–Menten model as well as an equation with product inhibition (by galactose or glucose) are presented. In this study, the competitive inhibition by galactose and simultaneous competitive inhibition and activation by glucose was suggested. The first observation is known from previous reports [21,36], but the positive glucose effect has not been previously described. Equation (3) describes all these phenomena. The values of its constants are summarised in Table 5.

$$r = \frac{k_3 \cdot C_{enzyme} \cdot \left(1 + \frac{C_{glucose}}{K_{a(glucose)}}\right) \cdot C_{lactose}}{K_m \cdot \left(1 + \frac{C_{glucose}}{K_{i(glucose)}}\right) \cdot \left(1 + \frac{C_{galactose}}{K_{i(galactose)}}\right) + C_{lactose}} \tag{6}$$

Table 5. The values of constants of Equation (6); 15 °C, pH 6.6.

	Native NOLA	Encapsulated NOLA
k_3 (1/min)	3.28	0.46
K_m (g/L)	30.00	21.19
$K_{a(glucose)}$ (g/L)	26.28	7.40
$K_{i(glucose)}$ (g/L)	15.79	10.54
$K_{i(galactose)}$ (g/L)	0.76	4.11

In contrast, the kinetic parameters for native cold-active β-galactosidase obtained from *Arthrobacter* sp. for strain BgaS and its mutants were specified at 15 °C as 0.17–4.35 g/L and 44.4–3015.6 L/min, for K_m and k_3, respectively. Additionally, the K_i value for galactose inhibition was in the range of 2.49–8.21 g/L [37]. Furthermore, for chitin-immobilised β-galactosidase isolated from *K. marxianus*, the kinetic parameters were determined as 6.26 g/L, 12.67 g/L, and 0.24 μmol/min IU, respectively, for K_m, K_i *(galactose)*, and k_3 [19]. According to the latest reviews, regardless of the β-galactosidase isolation source, the value of K_m may be more or less than K_i. Additionally, the negative galactose effect is more substantial in fungal preparations than yeast and bacterial [18]. The available reports, which present encapsulated β-galactosidase into chitosan and alginate-gelatin with glutaraldehyde, can be characterised by K_m values such as 3.25 and 16.7 g/L at 35–37 °C, respectively [30,38].

Enzyme immobilisation brings undoubted benefits into the area of industrial biocatalysis. The most important is enzyme reuse. In the presented research, encapsulated NOLA can be utilised for six cycles. In subsequent cycles, NOLA activity decreased. The last cycle time should be extended to about 4 h. These results are satisfactory, especially in the case of temperature value during lactose conversion, 15 °C, which corresponds to the temperature of milk transportation and storage. For comparison, acid whey bioconversion catalysed by NOLA can last even 2 h at 40 °C [28].

Enzyme encapsulation can be related to diffusion hindrance, both substrate and reaction products. The obtained results indicate that diffusion effects on hydrolysis during encapsulated NOLA action can be excluded. According to [39], during lactose hydrolysis in the packed-bed reactor, the intraparticle diffusion resistance and external mass transfer resistance affect the internal effectiveness factor. In the presented study, the effect of diffusion was investigated in a mixing reactor proposed as a process solution.

The need to hydrolyse lactose is a challenge in particular to obtain an allergy-free product. Nevertheless, we note that there is another aspect to the hydrolysis of lactose to monosaccharides. Sugar supplementation of dairy products is a commonly used phenomenon, especially during production of yoghurts, milk drinks, and puddings, to achieve the appropriate taste and texture, colour, and viscosity of milk-related foodstuffs [1,8]. This is an undesirable approach, especially in the diet of children and obese people. The solution can bring lactose hydrolysis. The sweetness index of the lactose-free products is defined as 3.7 times greater than in the same products, including lactose. Moreover, the effect of this observation is compared to the 2.5% *w/v* sucrose addition.

4. Materials and Methods

4.1. Materials

The commercial β-galactosidase NOLA™ Fit 5500 (NOLA) was obtained from Chr. HANSEN (Hørsholm, Denmark), sodium alginate, and HEPES buffer were purchased from Sigma-Aldrich (Munich, Germany). The analytical glucose test was obtained from Biomaxima (Lublin, Poland). The other reagents were purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland).

4.2. Methods

4.2.1. Kinetic Characteristic of NOLA in Native Form

Lactose decomposition was carried out in plastic test tubes at a volume of 15 mL. The reaction mixture (the final volume of 5 mL) consisted of NOLA at an appropriate concen-

tration in the range of 0.26–7.15 g/L and 5.0–55.0 g/L lactose solution in 0.1 M HEPES buffer, pH 6.6. The reaction was carried out at 15 °C. The moment of enzyme addition to the lactose solution was recognised as the beginning of the reaction. The reaction progress was determined by the glucose concentration measurements, which were determined spectrophotometrically at 500 nm by the Biomaxima analytical glucose test. A 10 μL aliquot of the sample was added to 1 mL of analytical reagent. After mixing, the solution was incubated for 5 min at 37 °C. Next, the spectrophotometric measurement was performed. The initial reaction rate was calculated until 5% of lactose mass was hydrolysed.

4.2.2. The Effect of Glucose and Galactose on NOLA Activity

To assess the impact of glucose and galactose on NOLA activity, exogenous sugars at the concentration of 0.25–28.0 g/L were added to the 55.0 g/L lactose solution. The reaction was performed at 1.5 g/L NOLA, 15 °C. This strategy was used for native and encapsulated preparation.

4.2.3. Kinetic Measurements

The kinetic parameters were obtained for three different enzyme concentrations 1.5, 1.2, and 0.8 g/L at 5.0–55.0 g/L lactose in 0.1 M HEPES buffer, pH 6.6, at 15 °C. The product inhibition was determined at glucose and galactose concentrations in the range of 0.5–28.0 g/L. The final reaction volume was 5 mL. For calculations, OriginPro 2021 (Trail version) was used.

4.2.4. NOLA Encapsulation

The alginate capsules with NOLA were prepared according to [26]. The reagents were dissolved in 0.1 M HEPES buffer pH 6.6. The tested enzyme concentrations, determined by the Lowry method [40], were in the range of 0.45–1.5 g/L. To avoid enzyme diffusion during capsule creation, the crosslinking bath included the same enzyme concentration as in the dropped solution. In this solution, the capsules were stored until they were applied in reaction.

4.2.5. The Activity of Encapsulated NOLA

Lactose hydrolysis was carried out in thermostated stirred-tank reactors of a total volume of 50 mL, at 15 °C and with a stirring velocity of 230 rpm. The volume of the reaction mixture was 10 mL, and the number of capsules was calculated each time to obtain the appropriate enzyme concentration in the total reaction volume (final concentration of 0.45–1.5 g/L). Lactose solution at the desired concentration in the range of 3.57–39.29 g/L was prepared in 0.1 M HEPES buffer solution. The moment of capsule addition to the lactose solution was recognised as the beginning of biocatalysis. The reaction progress was determined through measurements of glucose concentration, which was determined spectrophotometrically at 500 nm using a glucose analytical reagent.

4.2.6. NOLA Stability at the Presence of Calcium Ions

To determine NOLA stability in the presence of Ca^{2+}, the enzyme was incubated in 15% $CaCl_2$ solution. At specific intervals (0.5–360 h), the activity of incubated NOLA was determined. For this purpose, the sample of NOLA at a concentration of 0.5 g/L was added to 1.61 g/L lactose solution at 15 °C. The NOLA stability in the presence of Ca^{2+} was determined based on the changes of the initial reaction rate values before and after incubation.

4.2.7. NOLA Reuse

The possibility of encapsulated NOLA reuse was determined in cycles. Each cycle was monitored to the 97% of lactose decomposition by encapsulated NOLA. The reaction mixture was consisted of 39.29 g/L lactose and 1.5 g/L NOLA at 15 °C, pH 6.6.

4.2.8. Diffusion Restrictions

To check the diffusion resistances, the capsules with 1.5 g/L NOLA concentration and diameters in the range of 2.32–4.02 mm were used. The reaction mixture consisted of 39.29 g/L lactose in 0.1 M HEPES buffer solution, pH 6.6, at 15 °C.

4.2.9. Determination of Encapsulated NOLA Kinetic Parameters

The kinetic parameters for encapsulated NOLA were determined in the presence of lactose at the concentration range of 3.57–55.29 g/L. The NOLA concentration inside capsules was 0.8 g/L and the capsule diameter was 4.02 mm. The product inhibition was determined at the presence of exogenous glucose and galactose in the concentration of 3.57–20.0 g/L. The reaction volume was 10 mL. For calculations, OriginPro 2021 (Trail version) was used.

5. Conclusions

From an industrial point of view, the efficient lactose hydrolysis catalysed by NOLA at 15 °C is noteworthy. This is due to the encapsulated preparation properties: low temperature of biocatalysis, highly active and stable enzyme, using the natural compound as alginate to create a hydrogel network, and kinetic properties indicating a positive glucose effect. Additionally, the highest value of sweetener index related to lactose-free milk reduces the necessity of exogenous sweetener addition, which is valuable especially in milk desserts. Furthermore, NOLA activity at 15 °C creates the opportunity for new technological process construction in which lactose can be efficiently hydrolysed during milk transportation and storage. Moreover, the encapsulated preparation can be easily separated between cycles due to capsule size. This approach allows combining the economic benefits of low-temperature catalysis and the high quality of the lactose-free products.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. McCain, H.; Kaliappan, S.; Drake, M. Invited review: Sugar reduction in dairy products. *J. Dairy Sci.* **2018**, *101*, 8619–8640. [CrossRef]
2. Saqib, S.; Akram, A.; Halim, S.A.; Tassaduq, R. Sources of β-galactosidase and its applications in food industry. *3 Biotech* **2017**, *7*, 79. [CrossRef]
3. Velázquez, A.L.; Vidal, L.; Alcaire, F.; Varela, P.; Ares, G. Significant sugar-reduction in dairy products targeted at children is possible without affecting hedonic perception. *Int. Dairy J.* **2020**, *114*, 104937. [CrossRef]
4. Dekker, P.J.T.; Koenders, D.; Bruins, M.J. Lactose-Free Dairy Products: Market Developments, Production, Nutrition and Health Benefits. *Nutrients* **2019**, *11*, 551. [CrossRef]
5. Harju, M.; Kallioinen, H.; Tossavainen, O. Lactose hydrolysis and other conversions in dairy products: Technological aspects. *Int. Dairy J.* **2012**, *22*, 104–109. [CrossRef]
6. Pawlos, M.; Znamirowska, A.; Kluz, M.; Szajnar, K.; Kowalczyk, M. Low-lactose fermented goat milks with bifidobacterium animalis SSP. lactis BB-12. *J. Microbiol. Biotechnol. Food Sci.* **2020**, *9*, 751–755. [CrossRef]
7. Zagorska, J.; Ciprovica, I.; Straumite, E.; Majore, K. Acceptance of low-sugar yoghurt among Latvian teenagers. *Agron. Res.* **2020**, *18*, 1897–1905. [CrossRef]
8. Luzzi, G.; Steffens, M.; Clawin-Rädecker, I.; Hoffmann, W.; Franz, C.M.A.P.; Fritsche, J.; Lorenzen, P.C. Enhancing the sweetening power of lactose by enzymatic modification in the reformulation of dairy products. *Int. J. Dairy Technol.* **2020**, *73*, 502–512. [CrossRef]
9. Trusek, A.; Dworakowska, D.; Czyzewska, K. 3D enzymatic preparations with graphene oxide flakes and hydrogel to obtain lactose-free products. *Food Bioprod. Process.* **2020**, *121*, 224–229. [CrossRef]

10. Botvynko, A.; Bednářová, A.; Henke, S.; Shakhno, N.; Čurda, L. Production of galactooligosaccharides using various combinations of the commercial β-galactosidases. *Biochem. Biophys. Res. Commun.* **2019**, *517*, 762–766. [CrossRef]

11. Pulicherla, K.; Ghosh, M.; Rao, K.R.S.S.; Kumar, P.S. Psychrozymes—The Next Generation Industrial Enzymes. *J. Mar. Sci. Res. Dev.* **2011**, *1*, 1. [CrossRef]

12. Panesar, P.S.; Kumari, S.; Panesar, R. Potential Applications of Immobilized β-Galactosidase in Food Processing Industries. *Enzym. Res.* **2010**, *2010*, 473137. [CrossRef] [PubMed]

13. Cantone, S.; Ferrario, V.; Corici, L.; Ebert, C.; Fattor, D.; Spizzo, P.; Gardossi, L. Efficient immobilisation of industrial biocatalysts: Criteria and constraints for the selection of organic polymeric carriers and immobilisation methods. *Chem. Soc. Rev.* **2013**, *42*, 6262–6276. [CrossRef]

14. Eş, I.; Vieira, J.D.G.; Amaral, A.C. Principles, techniques, and applications of biocatalyst immobilization for industrial application. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 2065–2082. [CrossRef] [PubMed]

15. Labus, K.; Drozd, A.; Trusek-Holownia, A. Preparation and characterisation of gelatine hydrogels predisposed to use as matrices for effective immobilisation of biocatalysts. *Chem. Pap.* **2015**, *5*, 523–530. [CrossRef]

16. Nedovic, V.; Kalusevic, A.; Manojlovic, V.; Levic, S.; Bugarski, B. An overview of encapsulation technologies for food applications. *Procedia Food Sci.* **2011**, *1*, 1806–1815. [CrossRef]

17. Labus, K.; Wolanin, K.; Radosiński, Ł. Comparative Study on Enzyme Immobilization Using Natural Hydrogel Matrices—Experimental Studies Supported by Molecular Models Analysis. *Catalysts* **2020**, *10*, 489. [CrossRef]

18. Jurado, E.; Camacho, F.; Luzón, G.; Vicaria, J. A new kinetic model proposed for enzymatic hydrolysis of lactose by a β-galactosidase from Kluyveromyces fragilis. *Enzym. Microb. Technol.* **2002**, *31*, 300–309. [CrossRef]

19. Illanes, A.; Wilson, L.; Tomasello, G. Effect of modulation of enzyme inactivation on temperature optimization for reactor operation with chitin-immobilized lactase. *J. Mol. Catal. B Enzym.* **2001**, *11*, 531–540. [CrossRef]

20. Ladero, M.; Santos, A.; García-Ochoa, F. Kinetic modeling of lactose hydrolysis with an immobilized β-galactosidase from Kluyveromyces fragilis. *Enzym. Microb. Technol.* **2000**, *27*, 583–592. [CrossRef]

21. Chen, K.-C.; Houng, J.-Y.; Ling, A.C. Product inhibition of the enzymatic hydrolysis of lactose. *Enzym. Microb. Technol.* **1985**, *7*, 510–514. [CrossRef]

22. Boyacı, I.H.; Baş, D.; Dudak, F.C.; Topçu, A.; Saldamlı, I.; Şeker, U.; Özgür, Ş.; Tamerler, C.; Boyaci, I.H.; Saldamli, I. Statistical Modeling of β-galactosidase Inhibition During Lactose Hydrolysis. *Food Biotechnol.* **2006**, *20*, 79–91. [CrossRef]

23. Suri, S.; Kumar, V.; Prasad, R.; Tanwar, B.; Goyal, A.; Kaur, S.; Gat, Y.; Kumar, A.; Kaur, J.; Singh, D. Considerations for development of lactose-free food. *J. Nutr. Intermed. Metab.* **2019**, *15*, 27–34. [CrossRef]

24. Güven, R.G.; Kaplan, A.; Güven, K.; Matpan, F.; Dogru, M. Effects of various inhibitors on β-galactosidase purified from the thermoacidophilic Alicyclobacillus acidocaldarius subsp. Rittmannii isolated from Antarctica. *Biotechnol. Bioprocess Eng.* **2011**, *16*, 114–119. [CrossRef]

25. Zhang, Z.; Zhang, F.; Song, L.; Sun, N.; Guan, W.; Liu, B.; Tian, J.; Zhang, Y.; Zhang, W. Site-directed mutation of β-galactosidase from Aspergillus candidus to reduce galactose inhibition in lactose hydrolysis. *3 Biotech* **2018**, *8*, 452. [CrossRef] [PubMed]

26. Czyzewska, K.; Trusek, A. Encapsulated catalase from Serratia genus for H2O2 decomposition in food applications. *Pol. J. Chem. Technol.* **2018**, *20*, 39–43. [CrossRef]

27. Trusek-Holownia, A.; Noworyta, A. Efficient utilisation of hydrogel preparations with encapsulated enzymes—A case study on catalase and hydrogen peroxide degradation. *Biotechnol. Rep.* **2015**, *6*, 13–19. [CrossRef]

28. Samadov, R.; Ciprovica, I.; Zolnere, K.; Cinkmanis, I. The optimization of acid whey permeate hydrolysis for glucose-galactose syrup production. In Proceedings of the 13th Baltic Conference on Food Science and Technology, Jelgava, Latvia, 2–3 May 2019; pp. 254–257. [CrossRef]

29. Elnashar, M.M.; Awad, G.E.; Hassan, M.E.; Eldin, M.S.M.; Haroun, B.M.; El-Diwany, A.I. Optimal Immobilization ofβ-Galactosidase ontoκ-Carrageenan Gel Beads Using Response Surface Methodology and Its Applications. *Sci. World J.* **2014**, *2014*, 571682. [CrossRef]

30. Harsa, S.T. β-Galactosidase Immobilization on Chitosan-Hydroxyapatite Complex: Effects of Immobilization Conditions. *J. Nutr. Health Food Eng.* **2014**, *1*, 13–23. [CrossRef]

31. Tanriseven, A.; Doğan, Ş. A novel method for the immobilization of β-galactosidase. *Process. Biochem.* **2002**, *38*, 27–30. [CrossRef]

32. Ansari, S.A.; Satar, R.; Zaidi, S.K.; Ahmad, A. Immobilization of Aspergillus oryzae β-Galactosidase on Cellulose Acetate-Polymethylmethacrylate Membrane and Its Application in Hydrolysis of Lactose from Milk and Whey. *Int. Sch. Res. Not.* **2014**, *2014*, 1–6. [CrossRef]

33. Eskandarloo, H.; Abbaspourrad, A. Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. *Food Chem.* **2018**, *251*, 115–124. [CrossRef] [PubMed]

34. Bollella, P.; Hibino, Y.; Kano, K.; Gorton, L.; Antiochia, R. The influence of pH and divalent/monovalent cations on the internal electron transfer (IET), enzymatic activity, and structure of fructose dehydrogenase. *Anal. Bioanal. Chem.* **2018**, *410*, 3253–3264. [CrossRef] [PubMed]

35. Deshpande, S.S.; Cheryan, M. Effects of Phytic Acid, Divalent Cations, and Their Interactions on α-Amylase Activity. *J. Food Sci.* **1984**, *49*, 516–519. [CrossRef]

36. Zolnere, K.; Liepins, J.; Ciprovica, I. The impact of calcium ions on commercially available β-galactosidase. In Proceedings of the 11th Baltic Conference on Food Science and Technology, Jelgava, Latvia, 27–28 April 2017; pp. 27–30.

37. Coker, J.A.; Brenchley, J.E. Protein engineering of a cold-active β-galactosidase from Arthrobacter sp. SB to increase lactose hydrolysis reveals new sites affecting low temperature activity. *Extremophiles* **2006**, *10*, 515–524. [CrossRef]
38. Freitas, F.F.; Marquez, L.D.S.; Ribeiro, G.P.; Brandão, G.C.; Cardoso, V.L.; Ribeiro, E.J. Optimization of the immobilization process of β-galatosidade by combined entrapment-cross-linking and the kinetics of lactose hydrolysis. *Braz. J. Chem. Eng.* **2012**, *29*, 15–24. [CrossRef]
39. Al-Muftah, A.E.; Abu-Reesh, I.M. Effects of internal mass transfer and product inhibition on a simulated immobilized enzyme-catalyzed reactor for lactose hydrolysis. *Biochem. Eng. J.* **2005**, *23*, 139–153. [CrossRef]
40. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [CrossRef]