RESEARCH ARTICLE

Enzyme bag for lactose-free milk obtaining

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ABSTRACT
Lactose is an important component of mammalian milk. It is a disaccharide also called "milk sugar". In healthy individuals, lactose enters the intestinal lumen and is being hydrolyzed to glucose and galactose by the enzyme lactase. There are people with reduced lactase activity and the disaccharide is hydrolyzed by the microorganisms in their gut. This leads to lactose malabsorption which leads to symptoms such as diarrhea, nausea, gas, and abdominal pain. The condition is called lactose intolerance. Those people with less or no amount of the enzyme lactase are forced to avoid lactose-containing foods or pre-process them in order to hydrolyze the milk sugar before consuming the product. This paper presents a method by which anyone could get lactose-free milk at home. The so-called "enzyme bag" resembles a tea bag that is dunked into a glass of milk. It uses the biodegradable, biocompatible, and non-toxic polysaccharide – chitosan as an inert and harmless carrier, and the immobilized enzyme beta-galactosidase. The process does not require special equipment - is performed at room temperature and is fast, easy, and convenient. The future application of this method allows a few tools and a few easy steps to solve the problem of a large number of people with lactose intolerance.

Key words: lactose, milk, lactose intolerance, enzyme immobilization

Introduction

Lactose intolerance is described as the impossibility of digesting lactose in the intestines due to low activity or lack of the enzyme lactase. Undigested lactose continues its pathway in the gastrointestinal tract and is fermented by intestinal bacteria causing a hyperosmotic load, leading to different symptoms e.g., abdominal pain, meteorism, nausea, diarrhea, and others (Storhaug et al., 2017). Unfortunately, there is no significant data showing epidemiological and clinical features of lactose intolerance in the Bulgarian population. When looking at the problem worldwide, about 70% of the world's population has lactose intolerance (Ugidos-Rodríguez et al., 2018).

There are sources of an enzyme that catalyzes the breakdown of lactose. The enzyme is β-galactosidase and it has a microbial origin. The enzyme reaction is also cleavage of the disaccharide lactose to the monosaccharides – glucose and galactose. The microbial sources of β-galactosidase are varied: bacteria (*Bacillus subtilis, Bifidobacterium bifidum, Escherichia coli, Streptococcus cremoris, Vibrio cholera, etc.*), fungi (*Aspergillus foelidis, Aspergillus oryzae, Mucor meihei, Penicillum canescens, etc.*), and yeast (*Candida pseudotropicalis, Saccharomyces lactis, Kluyveromyces bulgaricus, etc.*) (Panesar et al., 2010). In order to be used repeatedly and safely in the food industry, the enzyme is immobilized to a suitable polymeric carrier.

Enzyme carriers are divided into two big groups – organic and inorganic. The first group is natural like agarose, chitosan, collagen, and carbon; the second group could be represented by synthetic carriers like polystyrene, polyethylene glycol, and polyglycerol; or inorganic carriers like silica, nanofibers, and Lassy carbon (Sneha et al., 2019). Chitosan is a biopolymer with a very wide range of applications. It is a deacetylated form of chitin, which builds the exoskeleton of crabs, shrimps, snails, and clams. It consists of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine (Manigandan et al., 2018). In its chemical structure, chitosan has a positively charged amino group that is ready for coupling reactions (Azmana et al., 2021; Maleki et al., 2022). The immobilization technique is crucial for enzyme activity and possible steric constraints. The methods are physical or chemical and each of them has positives and negatives. In physical methods, the enzyme remains unchanged, but it is bound weakly to the carrier. This could lead to losses of the enzyme during repeated usage. Chemical methods offer a stronger bond between the enzyme and the carrier, but often the linkers are harmful to health and the structure of the enzyme could be altered (Sneha et al., 2019).

The enzyme carrier chosen for this study is chitosan due to its non-toxic and antibacterial effect, and also acceptable

price. In the industry, it is used in winemaking, the pharmaceutical industry, medicine, the food industry, and agriculture. Participating in the production of citrus juices, reduces their bitterness, improves the aromatic properties of wine, reduces the ripening time of dairy products, and improves their texture and taste. It has a non-toxic and antibacterial effect and can be used to treat meat products, fruits, and vegetables to extend their shelf life (Zhang et al., 2022). Anti-inflammatory, anti-fungal, anti-bacterial, and anti-tumor properties, as well as being biocompatible and biodegradable makes it useful in the medicine, pharmaceutical, and food industries (Azmana et al., 2021).

Chitosan is easily available from nature and being an inexpensive biopolymer makes it economically advantageous (Manigandan et al., 2018). The number of properties it possesses and the fact that it is easily accessible and has a low cost allows for many future applications.

In this study, different carriers suitable for the food industry are compared. An unconventional representation of the enzyme is proposed. An enzyme bag, similar to a standard tea bag, is used to make lactose-free milk. The stability of the enzyme in the presented form was investigated.

Materials and Methods

Material

Chitosan (high molecular weight), carboxymethyl cellulose sodium salt (average MW ~250 000 Da) (CMC), iron (III) chloride, glutaraldehyde, glucose oxidase from *Aspergillus niger* 220 U/g (GOx), peroxidase from horse radish 10 000 U/vial (HRP), lactose, *o*-toluidine were purchased from Sigma-Aldrich (Germany). Beta-galactosidase was Co-lactase® (produced from *Aspergillus oryzae*, 4 500 FCC/mL) from Maxima Healthcare Ltd., UK. Gelatin (Dr. Ötker), vegetable oil, UHT milk, and hen's eggs were delivered from the local market.

Enzyme carrier obtaining

The first step for spherical beads of CMC obtaining was dissolving 2.5 g of the dry substance in 50 mL of distilled H_2O (dH_2O) with constant stirring using a magnetic stirrer until the mixture was completely homogenized (3 h). The obtained CMC was with a concentration of 5.0%. The second step was bead formation. A syringe with a capacity of 2 mL was filled with the reaction mixture. Using the syringe, the mixture was, drop by drop, carefully put into an aqueous solution of 5.0% $FeCl_3$, forming spherical beads. On the next day, the beads were washed with dH_2O until the liquid becomes discolored and were stored in dH_2O. The method was a modified version of Akalin & Pulat (2018). Beads of 3.6% CMC were also prepared by the method described above.

Chitosan beads were prepared by the modified method of Barbusinski et al. (2018). The method started with dissolving 1 g chitosan in 50 mL of 1% CH_3COOH in a water bath at 50°C and continuously stirring using a glass stirrer. After that, a syringe with a capacity of 2 mL was filled with the reaction mixture. Using the syringe, the mixture was, drop by drop, carefully put into a solution containing 80 mL 10% NaOH and 20 mL 96% C_2H_5OH, forming spherical beads. The newly formed beads were left in the ethanol-containing solution for 90 min and then washed in dH_2O until the litmus test was neutral. They were stored in dH_2O until use.

Gelatin beads were prepared by combining 10 g dry substance in 50 mL 50°C dH_2O and stirring until dissolving. Then, a syringe of 2 mL was loaded, and drop by drop the mixture was left in vegetable oil for bead formation. The gelatin beads were stored in the oil.

The white and brown egg shells were washed and put in boiling water (30 min) for sterilization and 30 min in acetone. Then, the eggshell was loaded in a dryer overnight (60°C).

All of the materials used for carriers were selected to be approximately 4 – 5 mm in size. After that, they were modified and the enzyme β-galactosidase was immobilized. The enzyme producer was selected previously (Krasteva et al., 2021).

Enzyme immobilization onto carriers

Before immobilization of the enzyme each of the carriers was dried with filter paper and an equal weight of them was prepared in separate beaker glasses. The crosslinking agent was 5% glutaraldehyde in 50 mM phosphate buffer (PB) pH 8. The used ratio carrier: crosslinker was 1g : 2mL. The modification was performed for 2 h at room temperature (RT) on a laboratory rotator. The modified carriers were washed 10 times with 50 mM PB pH 8 and 20 times with 10 mM PB pH 7.4. That was enough for glutaraldehyde residual removal (Chengolova & Ivanov, 2022).

The liquid suspended from the modified carriers was poured out and 10 mg/mL β-galactosidase in 10 mM PB pH 7.4 was added in a ratio of 1 g carrier to 2 mL enzyme. The immobilization was performed overnight at 4°C. On the next day, the excess of the β-galactosidase was collected for further Bradford protein analysis (Kielkopf et al., 2020). The carriers were washed 5 times with 10 mM PB pH 7.4 and were stored in the same buffer before analyses.

Lactose free milk obtaining

The enzyme bag was prepared by placing 12 g chitosan beads with immobilized β-galactosidase in a gauze and tying it with a thread so that approximately 15 cm of the thread remains free. The enzyme bag thus prepared looked like a tea bag. It was loaded in 200 mL UHT milk from the local market at room temperature. Three different fat contents were used and compared: 0.1%, 1.5%, and 3.2%. Standard lactose

solution with 4.7% lactose in 10 mM PB saline pH 7.4 was used as a control. Samples (150 μL) from the milk with a dipped enzyme bag were collected every 5 min.

The changes in the lactose amount during the lactose-free milk obtaining were determined due to the equation: 1 molecule glucose + 1 molecule galactose = 1 molecule lactose. The initial lactose content in the used UHT cow milk was 4.7% regardless of fat concentration (Costa et al., 2019; Mohamed et al., 2021). The increasing glucose concentration was a sign of decreasing lactose concentration. The glucose concentration was determined every 5 min after the enzyme reaction started by a modified method of Latha & Pari (2004). The analysis was performed using Eppendorf vials. Each vial contained 0.8 mL 0.9% NaCl, 0.3 mL 5% $ZnSO_4$, and 0.3 mL 0.3M NaOH. There was a sediment of ZnO. After that, 73 μL of the sample (milk) were added and the mixture was incubated for 10 min, RT, and centrifuged at 12 000 rpm for 5 min. Meanwhile, the working reagent for the color reaction was prepared: 0.4 mL 2 mg/mL GOx, 0.2 mL 2 mg/mL HRP, 0.2 mL 1% *o*-toluidine, and 39.2 mL 0.1M acetate buffer pH 4.8. After the end of the centrifugation, 0.3 mL of the supernatant were collected in a new vial and 0.8 mL of the working reagent for the color reaction was added. The mixture was incubated for 30 min and the absorbance of the samples was read at 625 nm (Jenway 7205 UV/Vis spectrophotometer). The exact concentration of glucose in the sample was determined from a pre-prepared glucose standard.

Results and Discussions

The Four different carriers were compared before lactose-free milk obtaining (Figure 1). The CMC beads were prepared at two different concentrations of the initial substance - 3.6% and 5%. Part of them was left to air dry for 2 weeks and after that, they were rehydrated. The beads from CMC were not able to regain their volume and appearance. Chitosan beads were more stable after drying and rehydration. They were 2 mm at the dried state and after rehydration – back to 4 mm. The situation with gelatin was similar to CMC. It reduced its volume to 3mm, but it was not able to regain its original volume after rehydration. As it was expected, the eggshell remained stable under those conditions.

All of the carriers were modified with glutaraldehyde and an equal enzyme amount was added for immobilization. The immobilized β-galactosidase was calculated from the difference between the amount of enzyme input and the amount of enzyme remaining as a residue in the liquid after immobilization. The results after enzyme immobilization and stability of the carrier (after drying and rehydration) are presented in Table 1. The highest percent immobilized enzyme was with CMC and chitosan, white eggshell also showed good results. CMC was prepared at two different concentrations - 5.0% and 3.6%. Better immobilization was obtained with the lower concentration. The immobilized β-galactosidase was 6.75 mg enzyme per 1 g 3.6% CMC beads. However, a disadvantage remains that upon drying and rehydration, the CMC beads cannot regain their original properties and volume (initial diameter 4 mm and final diameter 1 mm). This would prevent obtaining bigger quantities of them and storing them for a longer period of time.

White and brown eggshells showed different affinities to enzyme immobilization. White eggshells immobilized a higher amount of the added β-galactosidase (61.4%), and brown eggshells had a lower ability (49.2%). This was probably due to the mineral composition of the eggs. Brown eggs had a higher concentration of Ca, Na, Cu, Zn, and Al, and also the eggs with darker color had higher weight (Drabik et al., 2021). Consequently, measured by weight, the pieces of white eggshell would be a larger number than the pieces from brown eggshell, and provide a larger contact surface. In terms of stability, the shell was stable in long-term storage, but they were brittle, had sharp edges, and was not convenient to work with.

The gelatin beads were with lower enzyme immobilization capacity. Probably, due to the bead formation method with vegetable oil. The method was easy to apply, and well-shaped beads were obtained, but the oil used made them largely hydrophobic and this prevented immobilization of the enzyme. Therefore, only 2.5% of the enzyme was immobilized. In terms of stability, they were stable during

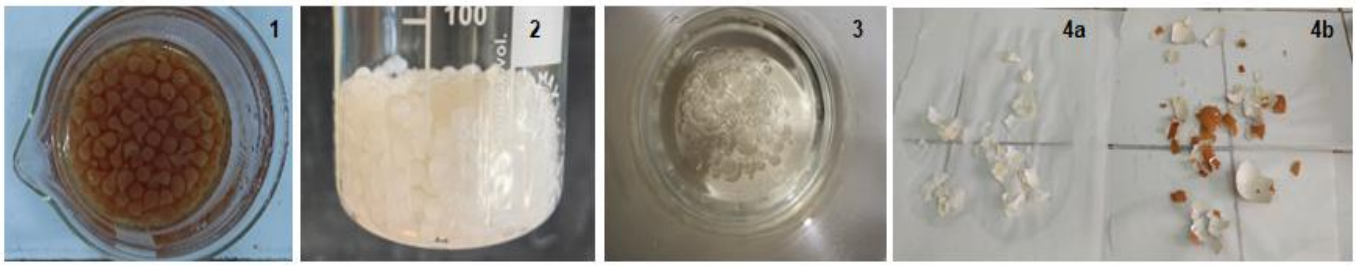

Figure 1. *Carriers for enzyme immobilization: (1) 5% CMC beads, (2) chitosan beads, (3) gelatin beads, (4a) white egg shell, (4b) brown egg shell.*

long-term storage (initial diameter 4 mm and final diameter 3 mm).

The best results were with chitosan beads. Their enzyme immobilization capacity was the highest (69.0%). Also, they were easy to use and stable (initial diameter 4 mm and final diameter 4.5 mm). Based on the obtained results, further work was continued with the chitosan beads.

The enzyme bag was prepared using chitosan beads with immobilized β-galactosidase. The enzyme bag was loaded in UHT milk and samples were collected every 5 min to observe the enzyme reaction (Figure 2). A color reaction with glucose oxidase, peroxidase, and o-toluidine was used to evaluate the enzymatic reaction in time (Figure 3). Lactose in the milk is broken down by the enzyme immobilized on the chitosan beads, producing equimolar amounts of glucose and galactose. The established increase in the concentration of glucose in the reaction mixture (milk) indicated that the enzyme successfully carries out its specific reaction.

The same enzyme bag was used twice. The first use was on the day of obtaining the bag, and the second use was a month later. A comparison of the activity of the β-galactosidase is shown in Figure 4. It was observed that the enzyme retained its activity unchanged even after a month of storage. After each use, the enzyme bag was thoroughly washed with dH_2O until a clear washing dH_2O was obtained, and for storage, it was placed in a beaker with dH_2O, tapped, and put at 4°C.

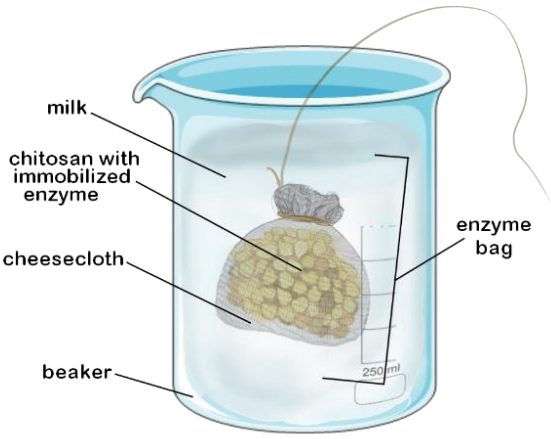

Figure 2. *Enzyme bag with immobilized β-galactosidase onto chitosan beads loaded in UHT milk.*

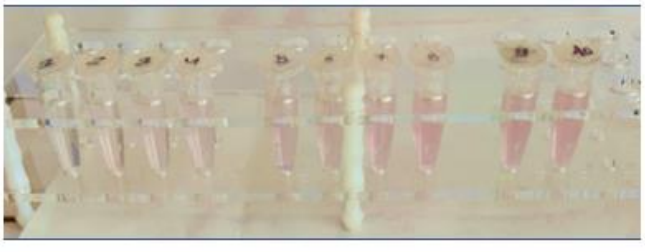

Figure 3. *Color reaction with o-toluidine for glucose concentration determination.*

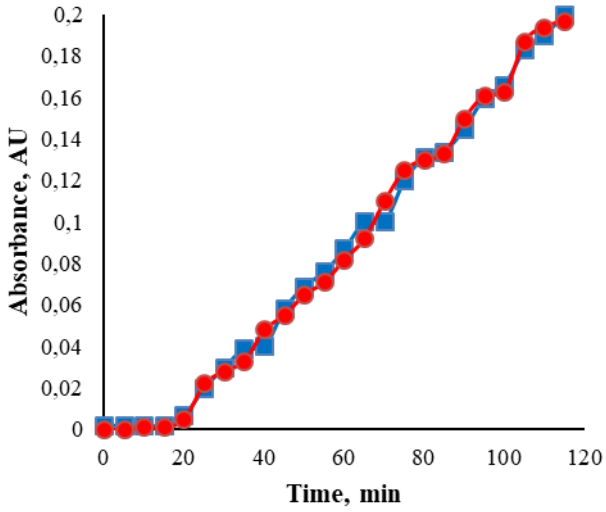

Figure 4. *Lactose-free milk obtaining with enzyme bag: (■) new bag, and (●) old bag.*

The effect of milk fat content was also tracked. For this purpose, samples of the milk were taken with a loaded enzyme bag at the 40th min. A test was made to establish the glucose concentration. The results are presented in Figure 5. No significant difference in enzyme activity was observed in milk at 0.1% and 1.5% fat content. A slight decrease is observed in milk 3.2% fat.

The activity of β-galactosidase immobilized onto different carriers had been determined by other authors too. Arsalan et al. (2020) used β-galactosidase for the same producer (*Aspergillus oryzae*) as that in this study. Their enzyme was immobilized onto silver nanoparticles and also reported for high activity. The experiment was for a comparison of the activity of the immobilized enzyme and free enzyme stored at 4°C for 30 days. The immobilized enzyme had 77% activity, and the free one had only 26%. The results were similar to those presented in the current study.

The compatibility of the enzyme and chitosan as its carrier had also been studied by other authors. Gaur et al. (2006) used β-galactosidase from *Aspergillus oryzae* and immobilized it with chitosan. After immobilization, the enzyme half-life and thermostability were enhanced. The authors studied the oligosaccharide synthesis by the enzyme and reported that the immobilized β-galactosidase obtained a higher yield (17.3%) compared to free β-galactosidase (10.0%) in 120 min. Consequently, the advantages of the β-galactosidase produced from *Aspergillus oryzae* were discussed and studied by other authors too.

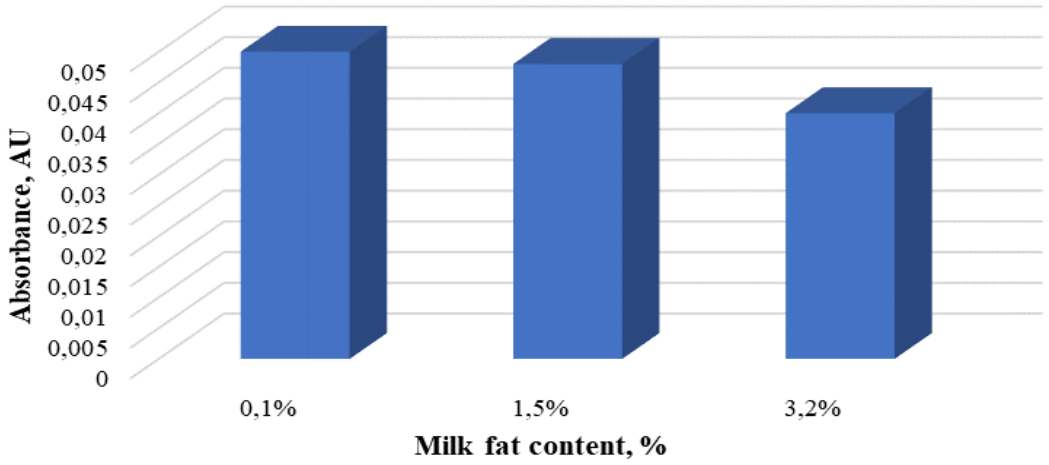

Figure 5. *Effect of milk fat content using an enzyme bag of chitosan beads with immobilized β-galactosidase .*

The chitosan, used as a carrier, showed good results and did not negatively affect the enzyme activity. The β-galactosidase-chitosan combination provides promising properties that can find application in the food industry.

Conclusion

An enzyme bag for lactose-free milk obtaining was prepared and presented. Different enzyme carriers that are harmless to consumers and the environment were studied and compared. Chitosan showed the best results. Chitosan beads with immobilized β-galactosidase, which were prepared as an enzyme bag, offer an easy and convenient way to obtain lactose-free milk. The storage conditions and also the milk fat content had no significant changes in the enzyme activity. The presented enzyme bag can be used repeatedly by any consumer. It is pocket-sized and easily stored in the refrigerator.

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