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Cell Disruption Optimization and Covalent Immobilization of β-D-Galactosidase from *Kluyveromyces marxianus* YW-1 for Lactose Hydrolysis in Milk

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Abstract β-D-galactosidase (EC 3.2.1.23) from *Kluyveromyces marxianus* YW-1, an isolate from whey, has been studied in terms of cell disruption to liberate the useful enzyme. The enzyme produced in a bioreactor on a wheat bran medium has been successfully immobilized with a view to developing a commercially usable technology for lactose hydrolysis in the food industry. Three chemical and three physical methods of cell disruption were tested and a method of grinding with river sand was found to give highest enzyme activity (720 U). The enzyme was covalently immobilized on gelatin. Immobilized enzyme had optimum pH and temperature of 7.0 and 40 °C, respectively and was found to give 49% hydrolysis of lactose in milk after 4 h of incubation. The immobilized enzyme was used for eight hydrolysis batches without appreciable loss in activity. The retention of high catalytic activity compared with the losses experienced with several previously reported immobilized versions of the enzyme is significant. The method of immobilization is simple, effective, and can be used for the immobilization of other enzymes.

Keywords β-D-galactosidase · Cell-disruption · Wheat bran (WB) · Immobilization · Lactose hydrolysis · *Klyuveromyces marxianus YW-1*

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Introduction

β-D-galactosidase (EC 3.2.1.23) is a very important enzyme in the dairy industry. It is involved in the hydrolysis of lactose into glucose and galactose [1, 2]. Lactose is the main constituent of milk solids and accounts for the 40% of the solids in cow's milk [3]. The consumption of foods with a high content of lactose is problematic with a considerable percentage (70% of the world population) of people being intolerant. Lactose intolerance is imparted by the fact that these people genetically lack the lactose hydrolytic enzyme in their body or this enzyme naturally present in human intestine loses its activity during lifetime [4, 5]. This fact, together with the relatively low solubility and sweetness of lactose, has led to an increasing interest in the development of industrial processes to hydrolyze the lactose contained in dairy products.

β-D-galactosidase is mainly intracellular enzyme in bacteria and yeasts but in case of fungi, is an extracellular enzyme. β-D-galactosidase from *Escherichia coli* is the most extensively studied enzyme and its properties; its reaction mechanism and structure have already been determined [6], but this enzyme is not used in the food processing industry because of high cost and toxicity associated with this bacterial enzyme.

Industrial applications of lactose hydrolysis are limited by the fact that yeast produces lactase as an intracellular protein, which makes its extraction difficult. However, uses of certain chemical agents have been reported to increase the lactose permeability [7]. Cell disruption is necessary for the isolation of intracellular protein and a wide range of disruption methods can be used in the laboratory, both chemical (detergents, alkali, or enzymes) and mechanical (sonication, homogenizer) [8, 9]. Furthermore, the immobilization of intracellular enzyme improves its thermostability, protects from harsh environment, and eases downstream processing.

Immobilization of microorganisms and enzymes has been applied to many biochemical reactions and has been widely discussed in the literature. β-D-galactosidase preparations are widely used for hydrolysis of lactose in milk, milk products, and whey [10]. Lactose imparts a 'sandy' texture to many dairy products such as concentrated whey syrups, ice-cream, etc. [11] and is therefore best hydrolyzed to avoid this. Researchers over the years have studied the enzymic hydrolysis of lactose to develop a truly commercially usable immobilized enzyme for the food industry.

In the present work, *Kluyveromyces marxianus* YW-1, an isolate from whey, was used as a source of β-D-galactosidase production. Here, we have used wheat bran extract for the production of β-D-galactosidase, which is a novel attempt in *K. marxianus*. The chemical and physical treatments were applied to *K. marxianus* cells to increase its permeabilization. This paper therefore describes immobilization of β-D-galactosidase onto gelatin and its application in achieving lactose hydrolysis in milk.

Materials

Chemicals

All chemicals and reagents used were obtained from Qualigens Chemicals Ltd. (New Delhi, India). GOD-POD kit used for glucose estimation was procured from Mitra & Co., New Delhi, India. pNPG (4-nitrophenyl β-D-galactopyranoside) was from Fine Chemicals (Bombay, India).

Methods

Microbial Strain and Culture Conditions

K. marxianus YW-1 [Microbial Type Culture Collection (MTCC) Chandigarh, India, accession number MTCC 5201] [12] isolated from whey was used for the production of β-D-galactosidase. The culture was maintained at pH 7.0 at 30 °C for 24 h on a medium containing malt extract (0.3%), yeast extract (0.3%), peptone (1%), and glucose (1%). The β-D-galactosidase was produced in an optimized fermentation medium (50 ml) containing wheat bran extract (6%, *w*/*v*), lactose (3%, *w*/*v*), yeast extract (1%, *w*/*v*), and L-glutamic acid (0.2%, *w*/*v*) with pH 4.5 for 30 h at 30 °C. To study the effect of enzyme production, different carbon sources (1%, *w*/*v*) were added to the basic medium before autoclaving.

Production of β-D-Galactosidase

The production of β-D-galactosidase was carried out on a lab scale (1.5 L) in a stirred tank fermentation bioreactor (previously used for production of other enzymes [13], Biolab, B Braun, Germany) fitted with a Rustom type impellor with six blades and working volume of 1 L with aeration and agitation at 0.75 vvm and 200 rpm, respectively. To control the foam formation, sterilized silicon oil (0.002%, *w*/*v*) was added at the beginning of 30-h fermentation.

β-D-Galactosidase Activity Assay

The assay was performed as per method of Allan and Balasubramanyan [14]. The reaction mixture contained 0.1 M sodium citrate buffer pH 4.5 (0.8 ml), 5.0 mM pNPG (0.1 ml), and enzyme solution (0.1 ml) and was incubated at 37 °C for 30 min before being stopped by adding 0.1 M NaOH (2 ml). The liberated 4-nitrophenolate anion was measured at 420 nm and its concentration calculated from a plot constructed for standard sodium 4-nitrophenolate solutions. One unit of enzyme activity is defined as 1 μmol of 4-nitrophenolate liberated per minute at pH and 28 °C. All the enzyme assays were performed in triplicate and the mean values are reported.

Extraction of β-Galactosidase from *K. marxianus* YW-1

Various methods were tried to maximize extraction yield of β-D-galactosidase from *K. marxianus* YW-1.

Chloroform-Ethanol

Method of Champluvier et al. [15] was used with little modification. The cells were harvested from 100 ml broth by centrifugation (3,000×*g*, 4 °C, 15 min) and washed twice with PPb-Mn buffer (50 mM KH_2PO_4 adjusted with KOH to the desired pH 6.5 and supplemented 1:1 with 0.1 M $MnCl_2$ to give a final concentration of 50 mM $MnCl_2$). This cell biomass was resuspended in the same buffer (1.5 mg dry weight ml^{-1}). The cell suspension 10 ml of 1:4 of the solution (chloroform/ethanol) was added and the whole incubated at 30 °C for 30 min. The resultant cell suspension was centrifuged (3,000×*g*, 4 °C, 15 min) and washed repeatedly with PPb-Mn buffer (100 ml). Cell biomass was resuspended in same buffer and analyzed for enzyme activity.

SDS-Chloroform

The cells with β-D-galactosidase activity were lysed with SDS and chloroform. An aliquot of cell biomass (100 ml) was diluted in Z-buffer pH 7.0 (composition (gl^{-1}): $Na_2HPO_4.7H_2O$, 16.1; $NaH_2PO_4.H_2O$, 5.5; KCl, 0.75; $MgSO_4.7H_2O$, 0.246; and 2-mercaptoethanol, 2.7 ml) to a final volume of 10 ml. The cells were lysed by the addition of chloroform (50 μl) and sodium dodecyl sulphate (0, 15, and 20 μl). The reaction mixture was pre-incubated at 28 °C for 5 min and then the β-D-galactosidase enzyme activity was assayed.

Liquid Nitrogen

The cells were harvested from 100 ml broth by centrifugation (3000×*g*, 4 °C, 15 min) and washed with 50 mM PPb-Mn buffer (pH 6.5). The resultant pellet after centrifugation was crushed in liquid N_2 in a mortar and pestle and this process was repeated once again. The resultant solid was dissipated in 2–4 ml PPb-Mn buffer and re-centrifuged, the supernatant being assayed for enzyme activity.

Homogenization

The pellet biomass (100 ml broth) was suspended in citrate buffer (pH 4.5, 5 mM), chilled and homogenized using a power driven glass-Teflon homogenizer at 500–1,500 rpm for 5–10 s per stroke. The resultant cell debris was removed by centrifugation (3,000×*g*, 5 min) and the enzyme activity of the supernatant was assayed.

Sonication

The broth biomass (100 ml) was centrifuged 3,000×*g* for 20 min and the resultant pellet was suspended in 50 mM PPb-Mn buffer pH 6.5 (5 ml). The cell suspension was subjected to sonication (Soniprep 150 sonicator, Sanyo, Japan) in cycles of 40 s burst with 20 s intermittent cooling. The disrupted cells suspension was centrifuged and enzyme activity was assayed.

Grinding with River Sand

Finally, our earlier method optimized for inulinase extraction was used with little modification for the release of β-D-galactosidase from *K. marxianus* [12]. Coarse river sand boiled in distilled water for 15–20 min was washed twice with diethyl ether and once with absolute alcohol and dried in an oven (60 °C, 20 min). The pellet biomass (~1.05 g) was dissolved in 50 mM sodium pyrophosphate buffer pH 7.2 to form cell slurry. Sand (1 gm/10 ml cell slurry) was added to the cell slurry and the mixture was pre-chilled and ground for 15–20 min in a pestle and mortar after which 50 mM PPb-Mn buffer pH 6.5 (50 ml) was added. On centrifugation, sand and cell debris were removed and the supernatant was assayed for enzyme activity.

Estimation of Lactose

Lactose estimation was performed as per the method of Parry and Doan [16]. Saturated picric acid (1 ml) was added to milk sample (1 ml) followed by addition of 25% *w*/*v* sodium carbonate (1 ml) and the mixture was kept in boiling water bath for 20 min. Thereafter it was

cooled to room temperature and the volume was made up to 20 ml. The OD of the resultant solution was measured at 520 nm. The concentration of lactose in milk sample was calculated from the standard curve using lactose as standard. Glucose estimation was done by using GOD-POD (glucose oxidase-peroxidase) kit (Mitra & Co., New Delhi, India).

Immobilization

The enzyme, β-D-galactosidase, was covalently immobilized on gelatin with little modification [17]. The fibers were formed by pumping through a syringe needle (16 mm) a solution composed of alginate (0.5 g), gelatin (1 g), glycerol (4 ml), 25 mM sodium acetate buffer pH 4.5 (6 ml), and β-D-galactosidase (150 U) into 50-mM calcium chloride (50 ml) solution prepared in 25 mM calcium acetate buffer pH 4.5 containing 5% (*w*/*v*) glutaraldehyde. During fiber formation, the calcium chloride solution was not agitated. The fibers were hardened for 1 h and cut into long pieces washed with 25 mM calcium acetate buffer (pH 4.5) and stored in same buffer until used.

Immobilization yield is defined as the ratio of β-D-galactosidase activity of the immobilized enzyme to that of the β-D-galactosidase activity of free enzymes. The activity of the immobilized enzyme was measured with p-NPG and with lactose solution (100 ml, 4.8% lactose) at 37 °C. The aliquots were removed at intervals to measure the degree of lactose hydrolysis.

The activity of immobilized enzyme was investigated for various parameters like enzyme concentration, pH (acetate and phosphate buffer), temperature, reusability, and lactose hydrolysis in milk.

Reusability of Immobilized ß-Galactosidase The immobilized β-D-galactosidase was reused to evaluate hydrolysis of lactose in fresh milk (100 ml). After each assay, fibers were taken out and stored in acetate buffer pH 4.6 for 4 h. This procedure was repeated for nine successive cycles. The activity determined for the first time was considered as control (100%) for the calculation of remaining percent activity after each use.

Results and Discussions

In the fermentation medium, wheat bran (WB) was used to economize the production of β-D-galactosidase from *K. marxianus* YW-1. Enzyme activity was observed in the supernatant obtained after the separation of yeast biomass from the fermented broth. Maximum β-D-galactosidase production (6.2 IU/ml) was observed in a bioreactor in 30 h, when all parameters optimized at shake flask level were used in the bioreactor run. The maximum enzyme production could be due to continuous supply of oxygen to yeast culture and also uniform distribution of medium components by agitation. An increase in enzyme production was recorded on using optimized concentration of wheat bran (6.0%, *w*/*v*) and thereafter, a decline in enzyme production was observed (data not shown). Maximum enzyme production was observed at 6.0% (*w*/*v*) wheat bran concentration (results submitted elsewhere). Kapoor et al. [18] also reported secretion of high levels of xylanase by *Bacillus pumilus* on using wheat bran in a fermentation medium. Similarly, other workers have found wheat bran useful in maximizing production of various enzymes such as alkaline protease [19] and tannase [20].

Although, few units of β-D-galactosidase were present in the supernatant culture because most of the enzyme lies intracellular. It has been reported that β-D-galactosidase is

mainly intracellular enzyme in yeasts [21]. Thus optimization of disruption procedure was investigated for maximizing extraction of enzyme from *K. marxianus* YW-1.

Cell Disruption

Comparing the different chemical (ethanol:chloroform, 4:1; SDS and chloroform; and liquid nitrogen) and physical methods (homogenization, sonication, and grinding with river sand) for the extraction of β-D-galactosidase, the physical methods of cell disruption were found to work better than the chemical methods, as revealed by the data in Table 1. Although chloroform: ethanol mixture and sonication resulted in appreciable enzyme activities, 599 and 478 U, respectively, the method of grinding with river sand gave highest enzyme activity (720 U/g) of all the methods (Table 1). Numanoglu and Sungur [22] investigated the disruption of *Kluyveromyces lactis* and observed physical method of blending with glass beads to be better than the chemical lysis. In our prior attempt, grinding with river sand appeared to be efficient for disruption of *K. marxianus* YS-1 for the production of inulinase [12].

The physical method (grinding with river sand) appeared more cost-effective and with low risk of toxicity associated when compared with chemical methods. Hence, for further experiments a physical method was selected to disrupt *K. marxianus* cells.

Immobilization

The enzyme was immobilized on glutaraldehyde cross-linked gelatin under mild conditions which resulted in an insoluble structure accompanied by color change due to the formation of an aldimine linkage. The amount of immobilized enzyme was calculated by the proportion of the enzyme which came out of the enzyme immobilization solution. Consequently, the immobilization strategy achieved 57% yield, which is defined as the ratio of the activity of the immobilized enzyme to the activity of the free enzyme used. The rationale of using glutaraldehyde was based on our earlier successes where glutaraldehyde or glyoxyl supports for immobilizing rhamnosidase greatly reduced noncompetitive inhibition [23]. Other workers too found success on immobilizing β-D-galactosidase via the glyoxyl or the glutaraldehyde groups [24]. In another study, β-D-galactosidase from *K. lactis* was immobilized onto graphite surface using glutaraldehyde as the cross-linking reagent with the activity yield of 25% [25]. Moreover, it is also known that β-D-galactosidase has an affinity to gelatin [17] and bone powder [26].

The enzyme ß-galactosidase when entrapped in chitosan gel beads showed 59% immobilization efficiency and while testing for hydrolytic activity in a packed bed reactor retained 56% of enzyme activity [27]. Recently, ß-glactosidase has been immobilized on the

Table 1 Disruption of *K. marxianus* YW-1 for the release of β-D-galactosidase.

Cell disruption		Enzyme activity (U/mg)
Type	Method	
Chemical	Permeabilization by mixture of chloroform and ethanol (4:1)	599
	Lysis with SDS and chloroform	Nil
	Lysis with liquid nitrogen	215
Physical	Homogenization	36
	Sonication	478
	Grinding with river sand	720

surface of concanavalin-A-layered calcium alginate-starch beads [28]. The leaching of the enzyme from support could be prevented by cross-linking the immobilized enzyme with glutaraldehyde.

Optimization of Enzyme Concentration

On increasing β-D-galactosidase concentration (25, 50, 100, 150, and 175 U) during the immobilization process, hydrolysis of lactose (4.8%) concomitantly increased. Lactose hydrolysis was 95% with 150 U of immobilized enzyme and it did not improve further upon increasing the immobilized enzyme concentration (175 U). In all probability, the non-improvement in lactose hydrolysis may be due to attainment of equilibrium rather then the end-product inhibition. Hence, in subsequent experiments, an optimum load of β-D-galactosidase (150 U) was used (Table 2) as it appeared cost-effective thus economizing the production process.

Kinetic parameters The effect of lactose concentration (1–5 mg) on β-D-galactosidase was investigated in triplicate and results were plotted as Lineweaver and Burk (not shown here). K_m (Michaelis constant) for free and immobilized β-D-galactosidase was 22 and 11 mM, respectively. The decrease in K_m values as a result of immobilization indicates better enzyme activity which has also been observed by earlier workers [22]. The V_{max} values obtained for the immobilized enzyme was about 25% of that obtained with free enzyme, which can be evaluated as a good observation. However, β-D-galactosidase from *K. lactis* immobilized on cellulose-gelatin showed a smaller value of K_m (11.8 mM) than that estimated for the soluble enzyme (13.3 mM) [22].

Optimization of pH and Temperature

Variation of the pH (4.0, 5.0, 6.0, 7.0, and 8.0) of immobilized enzyme (150 U) catalyzed hydrolysis of 4.8% (*w*/*v*) lactose showed that lactose hydrolysis increased up to pH 7.0 and thereafter started decreasing at higher pH. As pH 7.0 was found to give maximum hydrolysis it was selected for further experiments (Fig. 1), it also being the pH suitable for use in the hydrolysis of lactose in milk.

Incubation of immobilized β-D-galactosidase at pH 7.0 but at various temperatures (30, 40, and 50 °C) with 4.8% (*w*/*v*) lactose solution showed that maximum hydrolysis was obtained at 40 °C, showing improvement from 30 °C but again decreased at increasing the reaction temperature to 50 °C (Fig. 1). Ladero et al. [29] also observed the optimum pH and temperature for the same enzyme to be 7.0 and 40 °C, respectively. Olusanya and Olutiola [30] reported the optimum pH and temperature for lactose hydrolysis by β-D-galactosidase to be 7.0 and 40 °C. However, other workers on immobilizing β-D-galactosidase to different supports displayed different optimum pH (6.5) and optimum temperature (50°C) [31].

Table 2 Effect of immobilized β-D-galactosidase concentration on lactose hydrolysis.

Immobilized enzyme (U)	Lactose hydrolysis (%)
25	16
50	46
100	80
150	95
175	95

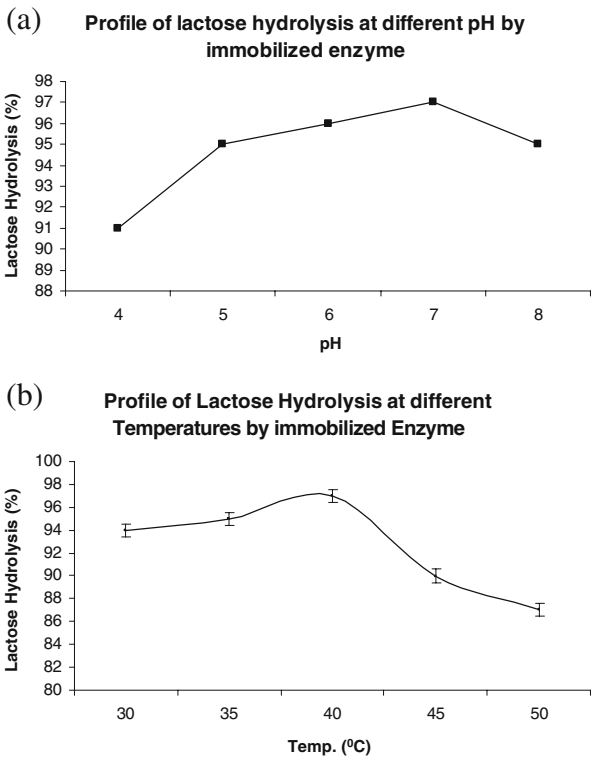

Fig. 1 Profile of lactose hydrolysis at different pH (**a**) and temperature (**b**) by immobilized β-D-galactosidase enzyme

Lactose Hydrolysis by Immobilized Enzyme in Milk

Milk sample (100 ml) was incubated with immobilized β-D-galactosidase (150 U) at 40 °C to investigate lactose hydrolysis at 2-h interval. A progressive increase in the hydrolysis of milk lactose with increase in incubation period was observed up to 4 h of incubation time and thereafter there was no improvement in the function (Fig. 2). The maximum lactose hydrolysis (49%) was observed after 4-h incubation time. The lack of improvement in lactose hydrolysis with further increase in incubation time may be attributed to end product inhibition. Similarly, Martins et al. [32] observed that the β-D-galactosidase activity in *K. marxianus* CBS6556 is decreased by high concentrations of galactose. Also, Ladero et al. [29] observed a yield of 43% on using immobilized enzyme for the hydrolysis of lactose and they determined kinetically that enzyme from yeast was inhibited by galactose. On comparing enzymes from thermophilic bacteria and a yeast, inhibition by galactose is more acute for thermophilic enzymes than for the enzyme from *Kluyveromyc*es sp.

Recycling of Immobilized Enzyme

The immobilized β-D-galactosidase (150 U) was reused up to nine times to hydrolyze lactose in fresh milk (100 ml) in 4 h. The lactose hydrolysis remained stable for eight batches and decreased thereafter indicating either clogging or decreased activity of immobilized enzyme. The stagnation of activity of immobilized enzyme can be attributed

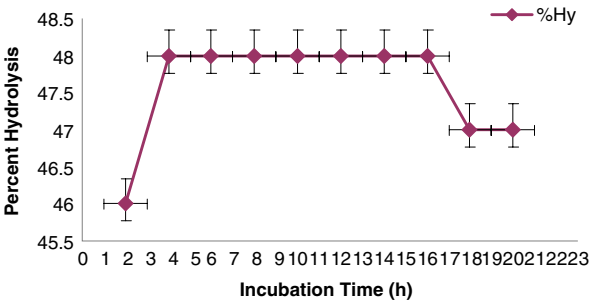

Fig. 2 Lactose hydrolysis by immobilized β-D-galactosidase enzyme in milk

to several factors, such as protein conformational changes induced by the support, steric hindrances, and diffusional effects. The factors may operate simultaneously or separately, alternating the microenvironment around the enzyme [33]. The immobilized β-D-galactosidase activity stability was an encouraging observation, but does require further experimentation before resuming scale-up studies (Fig. 3). In concurrence to our results, Numanoglu and Sungur [22] found that immobilized enzyme retained more than 80% of its original activity after eight uses.

Zhou and Chen [25] on immobilizing β-D-galactosidase on graphite surface by glutaraldehyde observed 70% lactose hydrolysis after 3 h. The immobilized enzyme showed a good storage and operational stability.

Dickson and Martin reported a decrease in β-D-galactosidase in the stationary phase of *K. lactis* culture even when lactose was detected in the culture medium [34]. They observed that decrease in activity could be due to several reasons: increasing concentration of an inhibitor, such as glucose, or exhaustion of activator, such as lactose, or even some proteolysis of the enzyme.

Although immobilization reduced the activity of the enzyme, this procedure has increased enzyme reusability, a process much suitable for food processing industry. Reusability of immobilized β-D-galactosidase for the hydrolysis of lactose in milk of course increases productivity of the system.

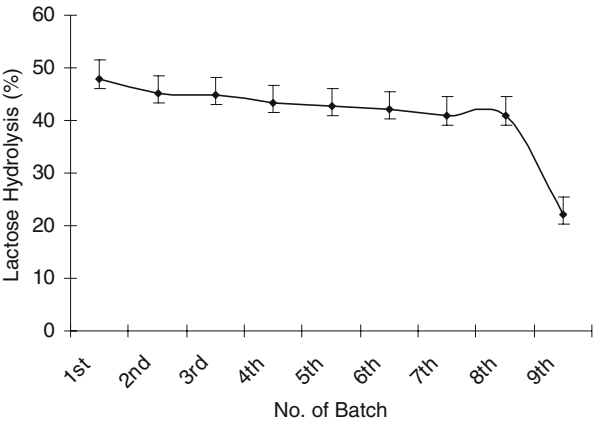

Fig. 3 Reusability of immobilized β-D-galactosidase enzyme

Conclusion

In this work, β-D-galactosidase from *K. marxianus* YW-1 was produced in a bioreactor containing wheat bran medium. Chemical and physical methods were successfully applied for cell permeabilization. Finally, the physical method of cell disruption was preferred because of its simplicity, safety, and high activity for food industry. The enzyme immobilization method developed for this work is a simple, inexpensive, and promising one. The immobilized enzyme was stable and active in operational conditions, with a considerable lactose conversion in milk. This work shows the potential of this method in industrial application.

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