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Lactose Hydrolysis in Whole Milk Using Immobilized *Kluyveromyces marxianus* Cells

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Abstract: To overcome the problem of enzyme extraction and poor permeability of cell membrane to lactose, permeabilization of *Kluyveromyces marxianus* cells was carried. Permeabilized whole cells can also be advantageous over more pure enzyme preparations due to increased stability maintained by the intracellular environment. In view of the advantages of immobilized cell system over free cell system, the permeabilized yeast cells were immobilized by entrapment in calcium alginate gel. Different process parameters (alginate concentration, bead size, biomass load, temperature, agitation, incubation time) were optimized to enhance the lactose hydrolysis. Maximum lactose hydrolysis (84.8%) was observed with yeast cells immobilized in 2% (w/v) alginate concentration after 150 min of treatment time. The developed system was highly stable and the alginate entrapped yeast cells can be recycled up to 7th cycle without any significant decrease in their ability to carry out the lactose hydrolysis.

Key words: Yeast, immobilization, alginate gel, lactose hydrolysis, whole milk

INTRODUCTION

Enzymatic hydrolysis of lactose with β-galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.23) constitutes one of most promising biotechnological applications in food industries. Lactose intolerance caused by intestinal insufficiency of the enzyme β-galactosidase and the environmental pollution caused by the discharge of large quantities of whey has made lactose hydrolysis a subject of extensive study. Lactose, which is principal carbohydrate of milk, can be hydrolyzed into its components (glucose and galactose) that exhibit better digestibility, higher solubility and sweetness (Mahoney, 1997; Panesar *et al*., 2006). Lactose hydrolyzed milk has demonstrated superior performance in the preparation of various dairy products (Law and Goodenough, 1991).

Microorganisms have been considered to be potentially most suitable source of β-galactosidase for industrial applications. Yeast (*Kluyveromyces*) has been considered as the most important source of β-galactosidase, as the neutral pH optima is well suited for hydrolysis of lactose in milk and are widely accepted as safe. However, yeast enzyme is intracellular and release of this enzyme with acceptable yield is difficult to achieve and rather expensive. Thus, the use of whole cells is an economically viable alternative to the purified enzyme (Joshi *et al*., 1989). However, a major drawback in this alternative is the poor permeability of cell membrane to lactose, which can be overcome through the use of permeabilization technology. Therefore, use of permeabilized cells is an interesting approach, which has been applied in whey sachharification and lactulose synthesis (Panesar *et al*., 2006; Lee *et al*., 2004).

Permeabilized microbial cells can be used as a crude enzyme preparation for industrial applications. Therefore, from economic view point the use of permeabilized whole cells, as a source of β-galactosidase is an interesting alternative. Permeabilized whole cells have been claimed to have an

advantage over more pure enzyme preparations due to increased stability, maintained by the intracellular environment; however, the low specific activity of the desired enzyme must be compensated. This can be done by immobilizing high biomass concentration. The application of immobilization technology is of significant importance in fermentation processes because of its advantages over free cell system, it permits higher cell densities in bioreactors, improves enzyme stability, makes reutilization and continuous operation possible and precludes the need to separate the cells from the milk following processing (Brodelius and Vandamme, 1987). The entrapment method has been accepted as an appropriate technique for the immobilization of β-galactosidase (Mammarella and Rubiolo, 2005). Keeping in view the above, the present investigation was carried out to develop immobilized cell system and to find out the optimal conditions for the maximal lactose hydrolysis in whole milk.

MATERIALS AND METHODS

Procurement of Macro Organism

Kluyveromyces marxianus was procured from National collection of Industrial Micro-organisms, National Chemical Laboratory, Pune (India).

Maintenance and Cultivation of the Culture

The culture was revived on maintenance medium containing (w/v) malt extract (0.3%), yeast extract (0.3%), peptone (0.5%) and glucose (1.0%). The culture was incubated at 30°C for 48 h and maintained for fortnightly intervals on agar slants at 4°C. The yeast was cultivated for the production of enzyme on the fermentation media composed of lactose (5%), peptone (0.5%), yeast extract (0.3%), ammonium sulphate (0.2%) and potassium dihydrogen orthophosphate (0.1%). The 50 mL fermentation media contained in 250 mL flask were inoculated with 20 h old inoculum, incubated at 30°C temperature for 24 h under shaking conditions (100 rpm).

Permeabilization of Yeast Cells

Permeabilization of yeast cells was carried out following the method of Joshi *et al.* (1989) with slight modifications. Yeast cells were harvested from broth by centrifugation and washed twice with phosphate buffer (0.1 M, pH 7.0). Ethanol (50%) was mixed with yeast biomass and the mixture was incubated (15 min) under shaking conditions. The treated cells were centrifuged and washed twice with phosphate buffer.

Immobilization of Yeast Cells

Immobilization of yeast cells was carried out using alginate gel (Marwaha and Kennedy, 1984). The permeabilized yeast cells were mixed with sodium alginate (2.0%, w/v) and the resultant slurry was extruded as drops through a sterilized syringe, into calcium chloride (0.075 M) solution. The beads were left suspended in calcium chloride solution for 5 h to allow complete gelation. The beads were washed with sterilized distilled water prior to their use to remove excess of calcium ions and un-entrapped cells.

Production of Lactose Hydrolyzed Milk

The above immobilized yeast cells were used for the lactose hydrolysis in whole milk at flask level. The boiled milk samples (50 mL milk) after cooling were inoculated with alginate beads containing a known weight of permeabilized yeast cells. The flasks were incubated at 30°C under shaking conditions for 3 h (unless otherwise specified). The samples were taken at specific time intervals and analyzed for lactose content. All the experiments were performed in triplicate and the mean values are reported.

Optimization of Process Parameters

Various process parameters (alginate concentration, bead size, biomass load, temperature, agitation and treatment time) were optimized by varying the respective parameters.

Lactose Estimation

The lactose estimation was carried out following the procedure of Nickerson *et al*. (1976).

RESULTS AND DISCUSSION

To develop an efficient immobilization cell system using yeast cells for lactose hydrolysis, the effect of the following parameters was investigated.

Hydrolysis of Milk Lactose as a Function of Alginate Concentration

Different concentrations (1.5-3.0%, w/v) of sodium alginate were used for the immobilization of yeast biomass. The beads of uniform size and shape were formed at a gel concentration of 2.0-3.0% (w/v). However, the beads of 1.5% (w/v) gel concentration were soft and started disintegrating during the course of experimentation, which may be attributed to the formation of unstable complex of Ca-alginate gel. The initial lactose hydrolysis rate was nearly linearly proportional to the incubation time, however, it tends to be much slower after 90 min, reaching maximum at 150 min (Fig. 1). Maximum lactose hydrolysis (72.7%) was observed with yeast cells immobilized in 2.0% (w/v) alginate. At higher alginate gel concentrations tested, a decrease in the lactose hydrolysis was found, which may be due to the diffusional resistance with increased gel concentration. The use of low concentration of alginate will be advantageous from economic viewpoint as well.

Since, the beads formed by using 2% (w/v) alginate were stable and supported the maximum lactose hydrolysis in whole milk, this concentration was selected for further experimentation.

Hydrolysis of Milk Lactose as a Function of Bead Size

The yeast cells were immobilized in beads of variable sizes (2.51-4.49 mm) to find out the optimal bead size. The yeast cells entrapped in bead size of 2.51-2.87 mm supported maximum lactose hydrolysis (79.5%). Further increase in bead size resulted in lower lactose hydrolysis and minimum lactose hydrolysis was observed with 4.50 mm bead size (Table 1). However, the beads less than 2.51 mm were deformed, which may be attributed to the difficulty in manual extrusion of viscous gel through the syringe.

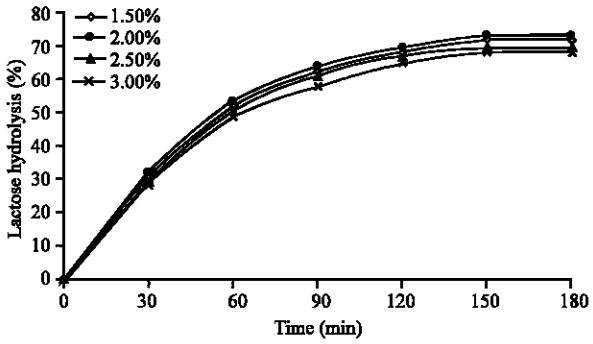

Fig. 1: Hydrolysis of milk lactose by immobilized yeast cells as a function of alginate concentration (1.5, 2.0, 2.5 and 3.0%)

Table 1: Hydrolysis of milk lactose by immobilized yeast cells as a function of bead size

Bead size (mm)	Lactose hydrolysis (%)
2.51-2.58	79.51
2.82-2.87	79.53
3.11-3.18	77.27
3.42-3.47	75.76
4.43-4.49	77.23

Table 2: Hydrolysis of milk lactose by immobilized yeast cells as a function of biomass load

Biomass load (mg dry wt.)	Lactose hydrolysis (%)
117	79.54
158	84.78
196	84.77
237	84.65

Table 3: Hydrolysis of milk lactose by immobilized yeast cells as a function of temperature

Temperature (°C)	Lactose hydrolysis (%)
25	72.73
30	84.79
35	84.76
40	73.48

The increase in the hydrolysis of milk lactose with decrease in bead size may be attributed to the greater surface area of small beads. It is well known that higher surface/volume ratio is correlated with smaller diameters of beads and with faster movement of molecules from or into a bead (Carrara and Rubiolo, 1994).

The bead sizes of 2.51-2.87 mm resulted in maximum level of lactose hydrolysis, however, the bead size of 2.82-87 mm was selected for further experimentation.

Hydrolysis of Milk Lactose as a Function of Yeast Biomass Load

Since the bioconversions are influenced by the cell load of the beads, different yeast biomass (117-237 mg dry wt) was immobilized in alginate gel to study the effect of concentration of entrapped biomass on lactose hydrolysis. An increase in the lactose hydrolysis with increasing concentration of biomass was observed up to 158 mg (dry wt.), which supported 84.8% lactose hydrolysis (Table 2). Higher cell concentrations have not shown any further improvement in the lactose hydrolysis, which may be attributed to the substrate limitations or product inhibition (Mahoney, 2003).

Hydrolysis of Milk Lactose as a Function of Temperature

The effect of temperature on the lactose hydrolysis in whole milk by alginate entrapped yeast cells was monitored at 25-40°C. Maximum hydrolysis of milk lactose was observed at 30-35°C, however with further increase in temperature, a decrease in the lactose hydrolysis was observed (Table 3). At optimal temperature range, the maximum lactose hydrolysis of 84.8% was recorded. Considering the enzymatic characteristic of maximum hydrolytic rate at a specific temperature, 30-35°C was considered as its optimal reaction temperature. The temperature below and above the optimal range, tends to inactivate the biocatalyst, thus affecting the hydrolytic rate.

Thermal stability of β-D-galactosidases differs from one enzyme source to another i.e., thermal stability of yeast, mould and bacterial enzyme is different. Ates and Mehmetoglu (1997) have suggested 30°C as the optimal temperature for carrying out the lactose hydrolysis using immobilized *A. oryzae* β-D-galactosidase. The lactose hydrolysis has also been performed in milk and dairy by-products at 25°C using immobilized *K. lactis* β-D-galactosidase on thiosulfonate supports (Ovsejevi *et al.*, 1998).

Table 4: Hydrolysis of milk lactose by immobilized yeast cells as a function of agitation

Temperature (°C)	Lactose hydrolysis (%)
0	63.38
60	72.71
80	81.25
100	84.85
120	84.5

Hydrolysis of Milk Lactose using as a Function of Agitation

The effect of agitation on lactose hydrolysis was determined by keeping the suspension of immobilized yeast cells and milk, under stationary condition in an incubator and shaking conditions (60-120 rpm). A minimum level of lactose hydrolysis was observed at stationary mode, however, an increase in the hydrolysis of milk lactose was observed up to 100 rpm (Table 4). No further improvement was observed at higher agitation rate. These observations suggest that diffusion of substrate from the bulk solution to the catalytic particle is a hydrolysis rate limiting factor, which can be minimized by enhancing the stirring speed (Klibanov, 1983). The maximum amount of lactose reduction was recorded at 100 rpm and it was used in further experimentation.

The use of agitation mode for the lactose hydrolysis has been supported by a number of workers (Batsalova *et al.*, 1987; Szczodrak, 2000). The agitation speed of 150 rpm has been used for lactose hydrolysis in whey permeate using immobilized β-D-galactosidase from *K. fragilis* (Szczodrak, 2000).

Hydrolysis of Milk Lactose as a Function of Time-Course

To find the optimal reaction time for maximum hydrolysis of milk lactose using immobilized yeast cells, the lactose hydrolysis was monitored at different incubation periods. A progressive increase in the lactose hydrolysis was observed up to 150 min of incubation and thereafter no improvement in the hydrolysis was recorded (Fig. 2). Maximum lactose hydrolysis (84.8%) was recorded at 150 min of treatment time, thereafter no increase in this function was observed. Thus the optimal reaction cycle for the lactose hydrolysis of whole milk was considered 150 min.

The lack of further increase in lactose hydrolysis may be attributed to the substrate limitations or product inhibition (Mahoney, 2003). The presence of fat has also been reported to impair the performance of immobilized preparations in bioconversions (Roy and Gupta, 2003). Different researchers have reported different optimal incubation periods for the hydrolysis of lactose. Batsalova *et al.* (1987) have reported 75% lactose hydrolysis in whey after 5-6 h using immobilized fungal β-D-galactosidase. However, 85-90% lactose hydrolysis in milk and dairy by-products after 2.5 h of incubation has also been reported using immobilized *K. lactis* β-D-galactosidase on thiosulfonate supports (Ovsejevi *et al.*, 1998).

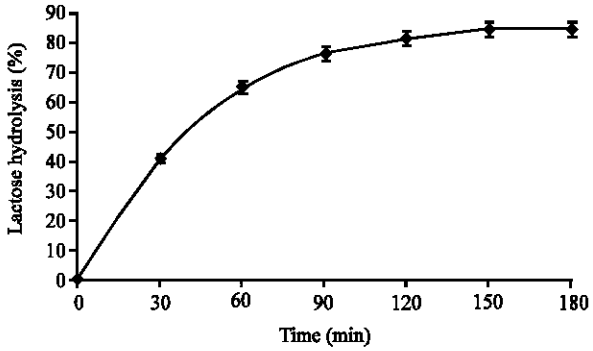

Fig. 2: Hydrolysis of milk lactose by immobilized yeast cells as a function of time-course. Bars indicate the standard deviation from triplicate determinations

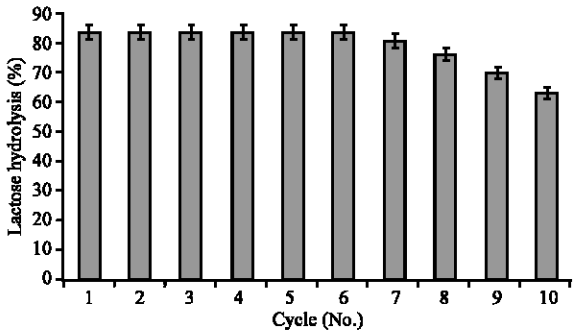

Fig. 3: Hydrolysis of milk lactose with recycling of immobilized yeast cells as a function. Bars indicate the standard deviation from triplicate determinations

Recycling of Alginate Entrapped Yeast Cells for Hydrolysis of Milk Lactose

The alginate beads containing yeast cells were recycled to test the stability of alginate entrapped yeast cells for repeated use in lactose hydrolysis of whole milk. After every cycle of 150 min, the lactose hydrolyzed milk was removed and replaced by fresh whole milk. The reuse of immobilized yeast cells up to 6th cycle showed no change in their ability to carryout the lactose hydrolysis, but thereafter, a slight decrease (81.8%) was found at the 7th cycle (Fig. 3). However, a sharp decrease was observed during the 8th and 9th cycle and the lactose hydrolysis of 75.7 and 71.2%, respectively was recorded.

The decrease in the lactose hydrolysis after 7th cycle may be attributed to the initiation of degeneration of entrapped yeast biomass and subsequent decrease in its ability to carry out the lactose hydrolysis. The shrinkage/deformation and loss of hardness of beads were observed during subsequent cycles. Thus, the alginate entrapped yeast cells can be successfully used for lactose hydrolysis in whole milk up to 7th cycle. A blocked TSI-agarose-β-galactosidase derivative has been reused for five cycles for the lactose hydrolysis of whey (Ovsejevi *et al*., 1998). In whey, the immobilized enzyme was reused up to 5 cycles without any marked decrease, however a significant decrease in the hydrolysis during subsequent cycles has also been reported (Szczodrak, 2000).

CONCLUSIONS

Kluyveromyces marxianus cells entrapped in 2% (w/v) alginate and a bead size of 2.82-87 mm displayed maximal lactose hydrolysis in whole milk at 30-35°C under shaking conditions (100 rpm) after 150 min of treatment. The alginate entrapped yeast cells can be recycled up to 7 th cycle without any significant change in their ability to carry out the lactose hydrolysis. The use of ethanol as a permeabilization agent has many additional advantages including its ready availability, low price and the fact that it is a component of many fermented foods/beverages allowing in principle, the use of permeabilized cells in food industries. The application of alginate gel for cell entrapment is very prospective due to its good stability and acceptability in the food industry. The convenient, easy to manufacture and relatively inexpensive method for the immobilization, resulting in high lactose hydrolysis and good operational stability seems to lend hope to the possibility of using such preparations for lactose hydrolysis in milk, milk products and whey.

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