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Production of lactose-hydrolyzed milk using ethanol permeabilized yeast cells

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Abstract

To overcome the problem of enzyme extraction and poor permeability of cell membrane to lactose, experimentation was carried to permeabilize *Kluyveromyces marxianus* NCIM 3465 cells for their subsequent use for the production of lactose-hydrolyzed milk. Different process parameters, such as biomass load, temperature, agitation and treatment time, were optimized for maximum lactose hydrolysis in skim milk using these cells. The ethanol-permeabilized yeast cells gave 89% hydrolysis of milk lactose under optimized conditions.
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1. Introduction

Lactose, the main carbohydrate present in milk, is a disaccharide with a low relative sweetness and solubility, which is not easily digested by a significant fraction of the global population. Furthermore, lactose is a hygroscopic sugar and has a strong tendency to absorb flavours and odours and causes many defects in refrigerated foods such as crystallization in dairy foods, development of sandy or gritty texture and deposit formation (Carrara & Rubiolo, 1994). Treatment of milk and milk products with β-D-galactosidase to reduce their lactose content is the appropriate method for increasing their potential uses and to deal with the problems of lactose insolubility and lack of sweetness (Mahoney, 1997). Moreover, this treatment can make milk, a most suitable food, available to a large number of adults and children that are intolerant to lactose.

β-D-Galactosidase can be obtained from a wide variety of sources, such as microorganisms, plants and animals, however, according to the source, their properties differ markedly. The use of microbial lactases offers several advantages over other sources. The most widely used microbial sources are *Kluyveromyces* sp. and *Aspergillus* sp. Fungal enzymes generally have acidic pH optima in the range of 2.5–5.4, whereas yeast lactases are most active at pH values of 6.0–7.0. Consequently, fungal enzymes are most effective in acidic products such as, whey, and the yeast enzymes are most useful in treating products near to a neutral pH such as milk (Finocchiaro, Olson, & Richardson, 1980; Joshi, Gowda, Katwa, & Bhat, 1989).

It has been established that the industrial application of β-D-galactosidase has been hampered by the difficulty and expense of releasing active enzyme in good yield from the cells, and further, the cost of the purification processes, especially in the case of bacteria and yeast. Thus, the use of whole cells as a source of β-D-galactosidase has been found as an interesting alternative, which has not been fully

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explored. However, a major drawback in the use of whole cells is the poor permeability of the cell membrane to lactose (Joshi, Gowda, Katwa, & Bhat, 1987). The use of permeabilization technology, however, can overcome this problem and be helpful in the development of a low-cost technology for lactose hydrolysis. The present work was therefore carried out to apply permeabilization technology for the production of lactose-hydrolyzed milk using yeast cells.

2. Materials and methods

2.1. Microorganism

Kluyveromyces marxianus NCIM 3465 was procured from the National collection of Industrial Microorganisms, National Chemical Laboratory, Pune (India).

2.2. 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 a 250 ml flask, were inoculated with 20 h old inoculum, incubated at 30 °C temperature for 24 h under shaking conditions (100 rpm).

2.3. Permeabilization of yeast cells

The permeabilization of yeast cells was carried out using ethanol (15 min treatment time), following the method of Joshi et al. (1989).

2.4. Production of lactose-hydrolyzed milk

The permeabilized yeast cells were used for the lactose hydrolysis in 10% (w/v) skim milk at shake flask level. The boiled milk samples (50 ml of skim milk in 250 ml capacity conical flasks) were, after cooling, inoculated with a known weight of permeabilized yeast cells. The flasks were incubated at 30 °C under shaking conditions (100 rpm) 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.

2.5. Optimization of process parameters

The various process parameters, such as biomass load, temperature, agitation and treatment time were optimized by varying the respective parameters.

2.6. Enzyme assay

The assay for measurement of enzyme activity was followed by the method of Miller (1972). One unit of enzyme activity is defined as one micromole (μmol) of 2-nitrophenol liberated per min under standard assay conditions. All the enzyme assays were performed in triplicate and the mean values are reported.

2.7. Lactose estimation

The lactose estimation was carried out by following the procedure of Nickerson, Vujicic, and Lin (1976). To the 5 ml of prepared milk sample (treatment with zinc acetate–phosphotungstic acid reagent and sodium hydroxide) were added 5 ml of glycine–NaOH buffer and 0.5 ml each of methylamine–HCl and sodium sulfite solution. The sample mixture was thoroughly mixed and kept at 65 °C in a water bath for 25 min. After cooling, the sample mixture, immediately, in an ice-water bath for 2 min, the absorbance of the sample was taken at 540 nm on a spectrophotometer.

3. Results and discussion

3.1. General

The effect of the following process parameters was monitored to optimize the lactose hydrolysis in skim milk during the course of the present investigation.

3.2. Permeabilization of yeast cells

To find out the effectiveness of ethanol as a permeabilizing agent, yeast cells were treated with different ethanol concentrations (20–70%, v/v). The results (Fig. 1) showed a progressive increase in the enzyme activity up to 50% (v/v); however, a decrease in enzyme activity was observed with further increase in the concentration. The ethanol concentration of 50% (v/v) displayed maximum enzyme activity (1.54 IU/mg DW). However, low enzyme activity was recorded with other ethanol concentrations used. It has been observed that permeabilization increases with the chemical concentration up to a critical value, where a maximum enzyme activity can be observed. At higher concentrations of the agent, the enzyme activity decreases, which may be attributed to the leakage of the enzyme from the cells or cell lysis. At low concentrations, the lower enzyme activity may be due to an insufficient amount of the agent for effective permeabilization.

Kluyveromyces cells are known to possess a lactose carrier protein (lactose permease) on their cell membrane that mediates the transport of lactose across the cell membrane (Dickson & Barr, 1983). Yet, availability of substrate seems to be the limiting factor in expressing the full enzymatic activity of whole cells. In permeabilization, the cell envelope is altered to allow small molecules, such as substrates, products, or coenzymes, to cross freely. The permeabilizing

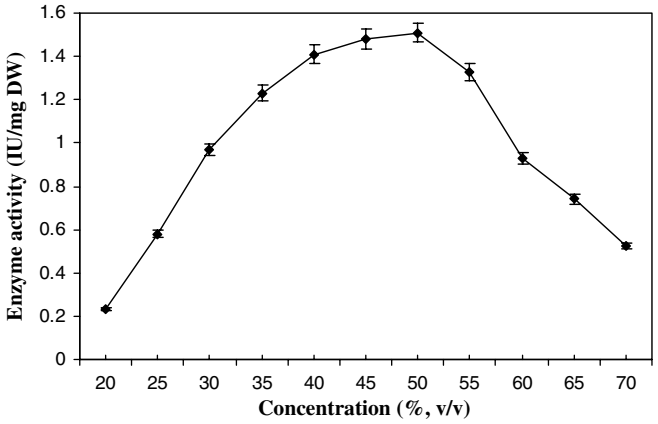

Fig. 1. Effect of ethanol concentration on the permeabilization of yeast cells. Bars indicate the standard deviation from triplicate determinations.

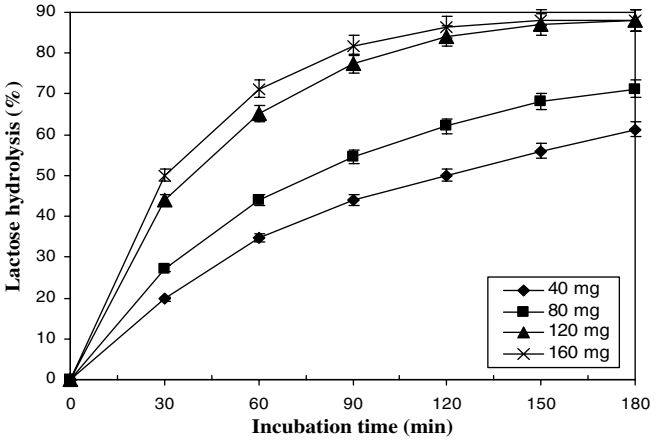

Fig. 2. Hydrolysis of milk lactose by yeast cells with cell load as a function. Bars indicate the standard deviation from triplicate determinations.

agent may disrupt the membrane structures to allow the passive passage of low molecular weight solutes in and out of cells, including lactose and its products of hydrolysis. These agents act on the cell membranes by decreasing the phopholipid content (Siso et al., 1992). Organic solvents, as permeabilizing agents, have shown results of up to 30% decrease in phopholipid content of yeasts (Decleire, De-Cat, & Van-Huynh, 1987). Recently, Lee, Kim, and Oh (2004) have shown that ethanol-permeabilized *K. lactis* cells can be used for lactulose synthesis from lactose and fructose.

Keeping in view the foregoing points, a 50% (v/v) concentration of ethanol was selected for the permeabilization of yeast cells.

3.3. Hydrolysis of milk using permeabilized yeast cells

3.3.1. Hydrolysis of milk lactose with biomass load as a function

The effect of permeabilized cell concentration on the lactose hydrolysis was investigated by adding the different cell concentrations (40–160 mg DW) to the skim milk. The lactose hydrolysis at lower cell concentrations appeared to be nearly linearly proportional to the time of incubation (Fig. 2). However, at higher cell concentrations (120–160 mg DW), only the initial rate of hydrolysis was linearly proportional to the incubation time and tended to be much slower at 90 min and thereafter. However, the maximum lactose hydrolysis (88%) was observed after 3 h of incubation period with 120 mg DW yeast biomass. Further increase in cell biomass did not show any improvement in lactose hydrolysis.

The increase in the lactose hydrolysis with the higher biomass concentration (120 mg DW) may be due to the increased availability of enzyme from yeast biomass. The lack of further increase in lactose hydrolysis at the subsequent biomass level (160 mg DW) may be attributed to the substrate limitations or product inhibition (Mahoney, 2003).

Joshi et al. (1987) have used 1 g/50 ml of cetyltrimethylammonium bromide-permeabilized *K. fragilis* NRRL Y-1196 cells for lactose hydrolysis of skim milk. Cell concentrations of 1% and 2% (w/v) of digitonin-permeabilized yeast cells were optimal for lactose hydrolysis in sweet whey and milk, respectively (Joshi et al., 1989).

Since, 120 mg DW of yeast biomass supported the maximum hydrolysis of lactose after 3 h of treatment time, it was selected for further experimentation.

3.3.2. Hydrolysis of milk lactose with temperature as a function

To find out the optimal temperature for lactose hydrolysis, skim milk and permeabilized yeast cells mixture was incubated at different temperatures (25–40 °C). A temperature range of 30–35 °C showed maximum levels of lactose hydrolysis (Fig. 3); however, further increase in temperature had an inhibitory effect on the hydrolysis. At optimal temperature range (30–35 °C), the maximum lactose hydrolysis of 88% was achieved after 180 min of incubation time. This may be attributed to the higher rate of enzyme reaction at optimal temperature. It has been well documented that below and above the optimal temperature, the rate of enzymatic reaction is affected.

Similarly, Champluvier, Kamp, and Rouxhet (1988) have used 30 °C for the lactose hydrolysis in 5% lactose solution using *K. lactis* CBS 683-permeabilized cells. However, a temperature of 37 °C has also been used for lactose hydrolysis in milk and sweet whey, using permeabilized *K. fragilis* NRRL Y-1196 cells (Joshi et al., 1987, 1989).

From the above observations, a temperature range of 30–35 °C was considered optimal for hydrolysis of milk lactose using permeabilized yeast cells, however, a temperature of 30 °C was selected for further experimentation.

3.3.3. Hydrolysis of milk lactose with agitation as a function

To study the effect of agitation on the hydrolysis of milk lactose, the mixture of skim milk and permeabilized

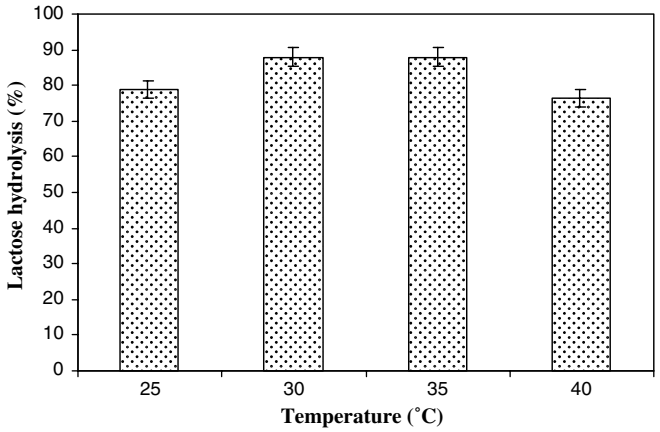

Fig. 3. Hydrolysis of milk lactose by yeast cells with temperature as a function. Bars indicate the standard deviation from triplicate determinations.

yeast cells was incubated under stationary conditions in a BOD incubator and shaking conditions (60–140 rpm) on a rotary shaker. The maximum lactose hydrolysis (88%) was recorded under shaking condition at 80 rpm; however, a decrease in lactose hydrolysis was observed at lower agitation rate (Fig. 4). Furthermore, at higher agitation rate, a slight decrease in the lactose hydrolysis was recorded. The minimum hydrolysis of milk lactose was observed under stationary conditions. The increase in lactose hydrolysis with agitation mode of treatment may be attributed to the uniform distribution of yeast biomass in the milk, resulting in better interaction between the substrate and the enzyme, and also to the increase in oxygen transfer rate.

Similarly, many workers have reported the suitability of gentle shaking for lactose hydrolysis using permeabilized yeast cells (Bachhawat, Gowda, & Bhat, 1996; Joshi et al., 1987; Siso et al., 1992).

As the maximum lactose hydrolysis was observed at 80 rpm, it was selected for further studies.

3.3.4. Hydrolysis of milk lactose with time-course as a function

To investigate the effect of treatment time (30–180 min), the skim milk and permeabilized yeast cells mixture was incubated under the above optimized conditions. A progressive increase in the hydrolysis of milk lactose with increase in incubation period was observed up to 150 min of incubation time and thereafter no improvement in this function was recorded (Fig. 5). The maximum lactose hydrolysis of 89% was observed with 120 mg DW yeast biomass after 150 min of incubation period. The lack of improvement in lactose hydrolysis with further increase in incubation time may be attributed to the product inhibition.

Bachhawat et al. (1996) have reported 120–150 min as the optimal treatment time for the maximum lactose hydrolysis in sweet whey using permeabilized *K. fragilis* NRRL Y-1196 cells. However, an incubation time of 120 min has been reported optimal for lactose hydrolysis in skim milk using permeabilized *K. fragilis* NRRL Y-1196 cells (Joshi et al., 1987).

From the foregoing account, it can be concluded that ethanol is an effective agent for the permeabilization of *K. marxianus* NCIM 3465 cells. The ethanol-permeabilized yeast cells successfully hydrolyzed the lactose in the skim milk. Lactose hydrolysis up to 89% can be obtained with 120 mg DW of permeabilized yeast cells at 30 °C under shaking conditions (80 rpm) after 150 min of treatment time. The developed technology can be successfully applied for the production of lactose-hydrolyzed milk. The hydrolysis of lactose in milk and milk products to reduce their lactose content can increase their potential uses and could make milk, a most suitable food, available to persons who are lactose-intolerant. Much work has been carried using the purified β-D-galactosidase for the production of lactose hydrolysed milk; however, the use of whole cells as a source of β-D-galactosidase is as an interesting alternative approach, which should be further explored by applying permeabilization technology. The use of permeabilized cells

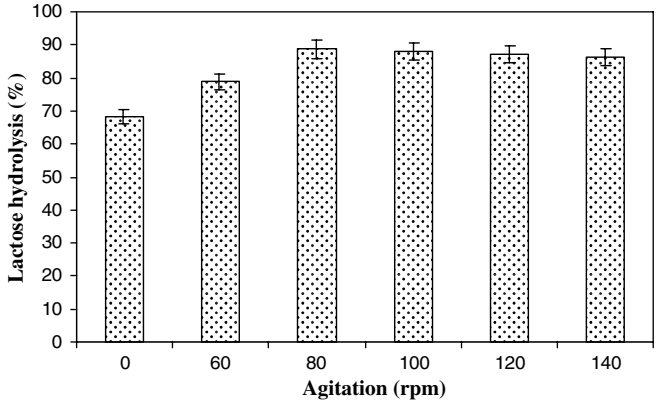

Fig. 4. Hydrolysis of milk lactose by yeast cells with agitation as a function. Bars indicate the standard deviation from triplicate determinations.

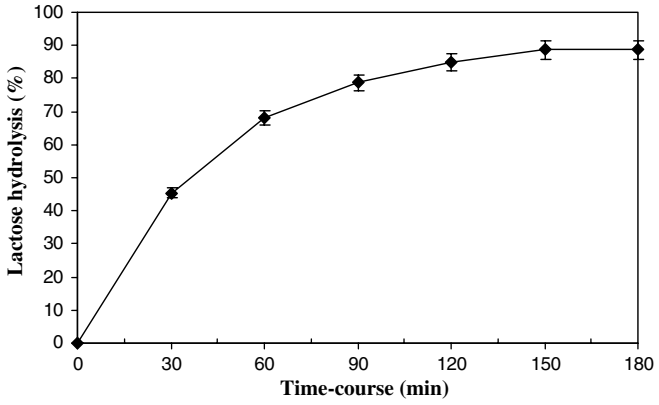

Fig. 5. Hydrolysis of milk lactose by yeast cells with time-course as a function. Bars indicate the standard deviation from triplicate determinations.

can help to overcome the problems/costs associated with enzyme extraction and purification from yeast cells and development of a low-cost technology for 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. Therefore, the use of ethanol-permeabilized yeast cells has good potential in the production of lactose-hydrolyzed milk, which can further be explored to scale up the process.

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