International Journal of
Dairy Science

ISSN 1811-9743

Academic
Journals Inc.

www.academicjournals.com

International Journal of Dairy Science 2 (2): 138-144, 2007
ISSN 1811-9743

Kinetic Analysis of Lactose Hydrolysis in Milk Using *Kluyveromyces marxianus* Cells Immobilized by Alginate and Agar Gel Entrapment

Parmjit S. Panesar
Biotechnology Research Laboratory, Department of Food Technology,
Sant Longowal Institute of Engineering and Technology, Longowal 148 106, India

Abstract: Lactose hydrolysis, which is of great concern due to nutritional, technological and environmental reasons, has been performed in skim milk using *Kluyveromyces maxrianus* cells. The yeast cells were permeabilized using ethanol in order to overcome the problem of poor permeability of cell membrane to lactose and subsequently immobilized by entrapment method in calcium alginate and agar-agar gel. The effects of gel concentration, temperature and treatment time on the performance of both the immobilized yeast cells preparations were investigated. Maximum hydrolysis (87.8%) of milk lactose was achieved with alginate entrapped yeast cells. The lactose hydrolysis reaction (in terms of loss of substrate as a function of time) using immobilized yeast cells can best be described as first order with half-life of ~ 44.4 minute for alginate gel in comparison to that of ~ 63 min for agar at 30°C in a batch process. Thus, former immobilization support/matrix is more efficient in lactose hydrolysis and demonstrated greater potential for future commercial application.

Key words: Yeast, permeabilization, immobilization, skim milk, lactose hydrolysis

INTRODUCTION

Lactose, the principal carbohydrate of milk, is not easily digested by a significant fraction of the global population. Due to lactose intolerance, the people suffer not only from mal-absorption but also from a general impairment of the normal digestive processes (Mahoney, 1997). 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 and Rubiolo, 1994). These nutritional and technological problems in milk products manufacture, has made lactose hydrolysis a subject of extensive study (Panesar *et al*., 2006).

The hydrolysis of lactose using β-D-galactosidase (EC 3.2.1.23), constitutes one of the most important applications of biotechnological processes. Lactose hydrolysis causes several changes of potential values on manufacture and marketing of dairy products (Coughlin and Charles, 1980). Additionally it can help to solve the problems related to the use of byproducts from cheese manufacturing industries avoiding serious pollution problems caused by their disposal. Thus in general, lactose hydrolysis provides several advantages like nutritional, because a significant fraction of the world population suffers from lactase deficiency; technological, because glucose and galactose are sweeter and more soluble than lactose and environmental, associated with whey disposal. Furthermore, glucose and galactose are more readily fermented than (Gekas and Lopez-Leiva, 1985).

Among the various β-D-galactosidase sources, the yeast *Kluyveromyces* sp. has emerged as an important source, since the yeast enzyme has an optimum pH suitable for lactose hydrolysis in milk. However, the industrial applications of processes based on the enzymatic hydrolysis of lactose are facing problems, since the enzyme is intracellular. The release of this enzyme from the yeast cells in good yield for purification is rather difficult (Joshi *et al*., 1989). Therefore, use of whole cells, as a

source of β-D-galactosidase is an interesting alternative, which can be further explored from the economic viewpoint. However, a major drawback in the use of whole yeast cells is the poor permeability to lactose but the use of permeabilized cells can alleviate such problems. Thus, permeablized cell technology can play a very important role in the production of lactose hydrolyzed milk and other bioconversions (Lee *et al*., 2004; Panesar *et al*., 2006; Vignoli *et al*., 2006). Furthermore, the application of immobilization technology in bioprocesses is of significant importance because of its several advantages over the free cell system Immobilization has been found to be the convenient method to make reuse of cells, to obtain higher cell densities in bioreactors and easier purification of the final product. Moreover, the continuous operation is more easily and efficiently controlled while using this technology (Brodelius and Vandamme, 1987).

In immobilization technology, the nature of matrix used plays a very important role in the success and commercialization of the developed technology. The substantial concern with use of immobilized yeast cells is reduction of internal mass transfer resistance during the process (Nedović *et al*., 2000). Internal mass transfer relates to transfer of substrates and products within the carrier, i.e., through the polymeric carrier matrix and aggregates of immobilized cells inside the carrier (Nedović *et al*., 2001). Keeping in view, the present studies was carried out to compare the kinetics of lactose hydrolysis using permeabilized yeast cells immobilized in Ca-alginate and agar gel.

MATERIALS AND METHODS

Procurement of Micro-organism

Kluyveromyces marxianus NCIM 3465 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 biomass on fermentation media composed of lactose (5%), peptone (0.5%), yeast extract (0.3%), ammonium sulphate (0.2%) and potassium dihydrogen orthophosphate (0.1%).

Permeabilization of Yeast Cells

The permeabilization of yeast cells was carried out following the method of Joshi *et al*. (1989) with slight modifications. The cells were harvested from 5 mL of broth by centrifugation (5000 rpm ×5 min at 4°C) and washed twice with phosphate buffer (0.1 M, pH 7.0). Ethanol (50%) was used as permeabilization agent and was added to the yeast biomass. The contents were mixed on a vortex mixture and incubated for 15 min, under shaking conditions. After this, the cells were re-centrifuged and washed twice with the phosphate buffer.

Immobilization of Yeast Cells

Sodium alginate and agar-agar gel were used as immobilization matrices for the entrapment of permeabilized yeast cells. The procedure of Marwaha *et al*. (1984) with slight modifications was used for immobilization of yeast cells in alginate. The permeabilized yeast cells were mixed thoroughly with sodium alginate (at the specified concentrations) and the resultant slurry was extruded as drops through a sterilized glass 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. The permeabilized yeast cells were immobilized in the agar gel in form of cubes. Different concentrations

of agar-agar gel were melted and then cooled to 45-50°C. Then, the permeabilized yeast cells were thoroughly mixed with agar and the resultant slurry was poured into petriplates. After solidification, cubes (4 mm) were made by cutting the gel.

Production of Lactose Hydrolyzed Milk

The immobilized yeast biomass was used for the lactose hydrolysis in 10% (w/v) skim milk at flask level. Boiled milk samples (50 mL of skim milk in 250 mL capacity conical flasks) after cooling were inoculated with the alginate beads/agar cubes containing 160 mg dry wt permeabilized yeast cells. The flasks were incubated at 30°C under shaking conditions (80 rpm) for 3 h. The samples were taken at specific time intervals and analyzed for lactose content.

Lactose Estimation

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

RESULTS AND DISCUSSION

Effect of Gel Concentration on the Hydrolysis of Milk Lactose Using Immobilized Yeast Cells

Yeast biomass immobilized using different concentrations (2.0-3.0%, w/v) of sodium alginate, was used for the lactose hydrolysis (Fig. 1). The beads of uniform size and shape were formed and were very stable due to the formation of a strong Ca-alginate complex. Maximum lactose hydrolysis of 87.8% was observed with cells immobilized in 2.0% (w/v) alginate. At higher gel concentrations, a decrease in the lactose hydrolysis was found, which may be due to the diffusional resistance with the increased gel concentration. The alginate beads were found very stable during the course of experimentation and no deformation/disintegration of beads was observed during the studies.

The entrapment of permeabilized yeast cells was carried out in agar-agar (2.0, 2.5 and 3.0%, w/v) in the form of cubes. This immobilized cell preparation of agar cubes was used for lactose hydrolysis and samples were analyzed for lactose content (Fig. 1). The maximum hydrolysis of milk lactose (78.3%) was observed with cubes of 2.0% (w/v) agar. At higher concentration, a decrease in lactose hydrolysis was recorded. These agar-cubes were stable during the course of experimentation.

Two types of diffusional resistances are reported during immobilization processes. External diffusional limitations arising from the fact that substrates must be transported from the bulk solution to the immobilized biocatalyst's surface across a boundary layer of water. Internal diffusional limitations stemming from the fact that substrates must diffuse inside the immobilized particle. These diffusional limitations have been reported to reduce the catalytic efficiency of immobilized biocatalyst (Klibanov *et al*., 1983; Pilkington *et al*., 1998).

Since, the beads/cubes formed by using 2% (w/v) alginate and agar-gel supported the maximum lactose hydrolysis and showed no change in their stability during the course of experimentation, this concentration was selected for further experimentation.

Effect of Temperature on Hydrolysis of Milk Lactose Using Immobilized Yeast Cells

To find out the optimal temperature for lactose hydrolysis, skim milk and immobilized yeast cells mixture was incubated at different temperatures (25-40°C) and the observations recorded are presented in Fig 2. Both the immobilized preparations displayed maximum hydrolysis of milk lactose at 30-35°C, however with further increase in temperature, a decrease in lactose hydrolysis was observed. At optimal temperature range, the maximum lactose hydrolysis of 87.8 and 78.3% was observed with yeast cells immobilized in alginate and agar gel, respectively. Considering the enzymatic characteristic of maximum hydrolytic rate at a specific temperature, 30-35°C was considered as its optimal reaction temperature. The temperature above the optimal range can affect the enzyme activity through thermal inactivation of the enzymes.

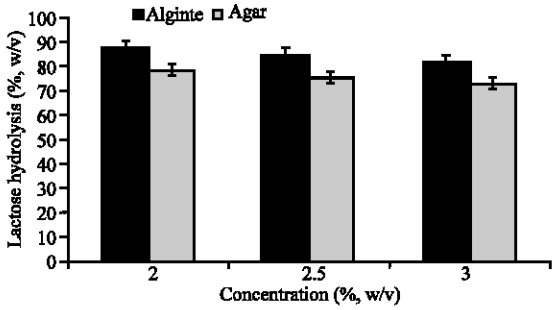

Fig. 1: Effect of gel concentration on the hydrolysis of milk lactose using immobilized yeast cells (at 30°C). Bars indicate the standard deviation from triplicate determinations

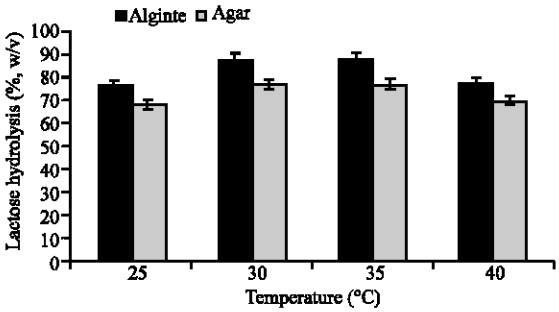

Fig. 2: Effect of temperature on the hydrolysis of milk lactose using immobilized yeast cells (2% gel concentration). Bars indicate the standard deviation from triplicate determinations

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

From the above observations, a temperature range of 30-35°C was considered optimal, however, a temperature of 30°C was used in further studies.

Effect of Treatment Time on Hydrolysis of Milk Lactose by Immobilized Yeast Cells

To investigate the effect of treatment time (30-180 min), the skim milk and entrapped yeast cells mixture was incubated at 30°C. A progressive increase in the hydrolysis of milk lactose with the increase in incubation period was observed up to 150 min incubation time and thereafter no improvement in this function was recorded (Fig. 3). Maximum lactose hydrolysis of 87.8% was observed with alginate entrapped yeast cells after 150 min of treatment time, thereafter no improvement in the hydrolysis was recorded. Whereas, a lactose hydrolysis of 78.3% was recorded after 150 min of treatment time with agar entrapped yeast cells. No improvement in lactose hydrolysis with further increase in incubation may be attributed to the product inhibition (Mahoney, 2003). Thus the optimal reaction cycle for the hydrolysis of milk lactose was considered 150 min.

Different workers have reported different optimal incubation periods for the hydrolysis of lactose. Batsalova *et al*. (1987) have reported 75% lactose hydrolysis after 5-6 h using immobilized β-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-galactosidase on thiosulfonate supports (Ovsejevi *et al*., 1998).

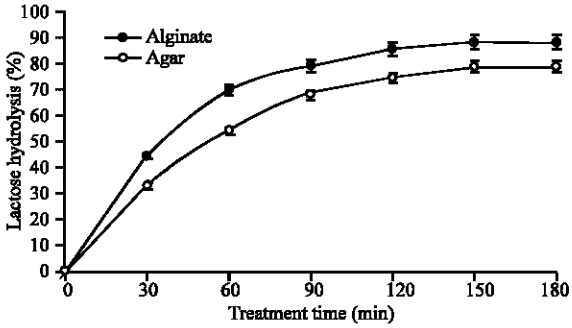

Fig. 3: Effect of treatment time on the hydrolysis of milk lactose using immobilized yeast cells (at 30°C with 2% gel concentration). Bars indicate the standard deviation from triplicate determinations

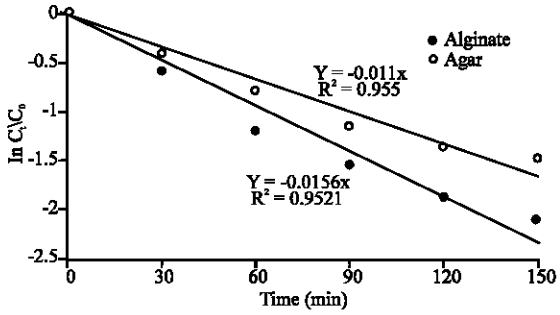

Fig. 4: Determination of first order rate kinetics for the hydrolysis of milk lactose (a plot of ln (C_t/C_0) versus t) using yeast cells entrapped in alginate and agar matrices at 30°C by integral method of analysis

Reaction Kinetics of Lactose Hydrolysis by Immobilized Yeast Cells

The apparent rate constant (min^{-1}) associated with the conversion of lactose was determined assuming first-order kinetics. The Integral method of analysis has been applied to find out the reaction kinetics for the lactose hydrolysis reaction (disappearance of substrate as a function of time). For the first-order reaction kinetics (i.e., an exponential decrease of substrate concentration as a function of time), the relationship between substrate concentration (C) and time (t) being:

$$C_t = C_0 e^{-kt} \quad (1)$$

where C_t is the substrate concentration at any time t, C_0 is the initial the substrate concentration (i.e., at time t = 0) and k is the rate constant. Equation (1) can be re-expressed as:

$$\ln (C_t/C_0) = -kt \quad (2)$$

Therefore, for first-order reaction kinetics, a plot of ln (C_t/C_0) versus time (t) gives a straight line with a slope of -k (Fig. 4). Linear regression analysis of Fig. 4 data gives k-values of 0.0156 min^{-1} (for alginate as immobilization matrix) with R^2 value of 0.9521 and 0.011 min^{-1} (for agar as immobilization matrix) with R^2 value of 0.955.

Half life for a reaction is the time it takes for substrate concentration to decrease from C_0 to ½ C_0 is given by for the first order kinetics:

$$kt_{1/2} = -\ln\left(\frac{1}{2}C_0/C_0\right) = -\ln\frac{1}{2} = \ln 2 \quad (3)$$

and Eq. 3 can be re-expressed as:

$$t_{1/2} = \frac{(\ln 2)}{k} \quad (4)$$

Therefore the half-life for the lactose hydrolysis using yeast cells entrapped in alginate matrix was ~44.4 min and that for agar matrix was ~ 63 min.

So it is clear from the above that the yeast cells immobilized in Ca-alginate gel were more efficient in lactose hydrolysis and demonstrated greater potential for future commercial application. The calculated k-values and half-life ($t_{½}$) values for lactose hydrolysis using immobilized yeast cells indicate that choice and characteristics of immobilization matrix are very important during this process.

CONCLUSIONS

The selection of support/matrix during the immobilization process plays a very important role in the success of a process. Maximum hydrolysis (87.8%) of milk lactose was achieved with alginate entrapped yeast cells. Calculated reaction rate and half-life ($t_{½}$) values of alginate entrapped cells indicate that yeast cells entrapped in alginate matrix can hydrolyze lactose at significantly higher rate in comparison to that of yeast cells immobilized in agar matrix (~1.5 times faster under the same conditions). The developed technology has wide potential application in lactose hydrolysis of milk and associated industries and requires further investigation on a larger scale.

REFERENCES

Ates, S. and U. Mehmetoglu, 1997. A new method for immobilization of β-galactosidase and its utilization in a plug flow reactor. Process Biochem., 32: 433-436.

Batsalova, K., K. Kunchev, Y. Popova, A. Kozhukharova and N. Kirova, 1987. Hydrolysis of lactose by β-galactosidase immobilized in polyvinyl-alcohol. Applied Microbiol. Biotechnol., 26: 227-230.

Brodelius, P. and E.J. Vandamme, 1987. Immobilized cell systems. In: Biotechnology. Rehm, H.J. and G. Reed (Eds.), VCH Publs., Weinheim, Germany, pp: 406-464.

Carrara, C.R. and A.C. Rubiolo, 1994. Immobilization of β-galactosidase on chitosan. Biotechnol. Prog., 10: 220-224.

Coughlin, R.W. and M. Charles, 1980. Applications of lactase and immobilized lactase. In: Immobilized Enzymes for Food Processing. Pitcher, W.H. (Ed.), CRC Press, Florida, USA., pp: 153-179.

Gekas, V. and M. Lopez-Leiva, 1985. Hydrolysis of lactose: A literature review. Process Biochem., 20: 2-12.

Joshi, M.S., L.R. Gowda, L.C. Katwa and S.G. Bhat, 1989. Permeabilization of yeast cells (*Kluyveromyces fragilis*) to lactose by digitonin. Enzyme Microb. Technol., 11: 439-343.

Klibanov, A.K., 1983. Immobilized enymes and cells as practical catalysts. Science, 219: 722-727.

Lee, Y.J., C.S. Kim and D.K. Oh, 2004. Lactulose production by β-galactosidase in permeabilized cells of *Kluyveromyces lactis*. Applied Microbiol. Biotechnol., 64: 787-793

Mahoney, R.R., 1997. Lactose: Enzymatic modification. In: Advanced Dairy Chemistry. Fox, P.F. (Ed.), Chapmann and Hall, London, pp: 77-125.

Mahoney, R.R., 2003. Enzymes exogenous to milk in dairy, β-D-galactosidase. *In* Encyclopaedia of Dairy sciences. Vol. 2. Roginski, H., J.W. Fuquay and P.F. Fox (Eds.), Academic Press, London, pp: 907-914.

Marwaha, S.S. and J.F. Kennedy, 1984. Ethanol production from whey permeate by immobilized yeast cells. Enzyme Microb. Technol., 6: 18-22.

Nedović, V.A., A. Durieux, L. Van Nedervelde, P. Rosseels, J. Vandegans, A.M. Plaisant and J.P. Simon, 2000. Continuous cider fermentation with co-immobilized yeast and *L. oenos* cells. Enzyme Microb. Technol., 26: 834-839.

Nedović, V.A., B. Obradović, I. Leskošek-Ėukalović, O. Trifunović, R. Pešić and B. Bugarski, 2001. Electrostatic generation of alginate microbeads loaded with brewing yeast. Process Biochem., 37: 17-22.

Nickerson, T.A., I.F. Vujicic and A.Y. Lin, 1976. Colorimetric estimation of lactose and its hydrolytic products. J. Dairy Sci., 59: 386-390.

Ovsejevi, K., V. Grazu and F. Batista Viera, 1998. β-galactosidase from *Kluyveromyces lactis* immobilized on to thiosulfinate/thiosulfonate supports for lactose hydrolysis in milk and dairy by-products. Biotechnol. Technol., 12: 143-148.

Panesar, P.S., R. Panesar, R.S. Singh, J.F. Kennedy and H. Kumar, 2006. Microbial production, immobilization and applications of β-D-galactosidase. J. Chem. Technol. Biotechnol., 81: 530-543.

Pilkington, P.H., A. Margaritis, N.A. Mensour and I. Russell, 1998. Fundamentals of immobilised yeast cells for continuous beer fermentation: A review. J. Inst. Brewing, 104: 19-31.

Vignoli, J.A., M.A.C. Celligoi, R.S.F. da Silva and M. de Barros, 2006. The production of sorbitol by permeabilized and immobilized cells of *Z. mobilis* in sucrose. Braz. Arch. Biol. Technol., 49: 683-687.