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Permeabilization of Yeast Cells with Organic Solvents for β-galactosidase Activity

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Abstract: Cell disruption techniques for intracellular product recovery operations result in the release of all cell constituents into surrounding medium, which complicates the downstream processing besides increase in the purification cost. However, these problems can be minimized using permeabilization technique. In the present investigation, the permeabilization of *Kluyveromyces marxianus* cells in relation to β-galactosidase (β-D-galactoside galactohydrolase, *E.C.* 3.2.1.23) activity was carried out using different organic solvents to avoid the problem of enzyme extraction. The permeabilization performance of the solvents was much dependent on their concentrations. Comparison of tested solvents has led to the selection of ethanol for maximal permeabilization of yeast cells. The optimum process conditions for permeabilization process were 50% (v/v) ethanol concentration, 25°C temperature and treatment time of 15 min. These permeabilized yeast cells displayed 90% lactose hydrolysis after 150 min of incubation time.

Key words: Yeast, β-galactosidase, solvents, permeabilization, lactose hydrolysis

INTRODUCTION

Biotechnology have made possible to produce a wide range of products using micro-organisms. Many microbial products are extracellular, however, much larger proportion of the potentially useful microbial products is retained within the cells. The cell disruption processes used to extract the intracellular products involve breakage of the cell envelope and release of all the intracellular constituents into surrounding medium (Chisti and Moo-Young, 1986) resulting the increase in cost of purification processes. Moreover, the release of the enzyme from the yeast cells in good yield is rather difficult and requires prolonged treatment with chemicals (e.g., toluene) at higher temperatures (Joshi *et al*., 1989). However, these types of problems can be avoided using permeabilization technology (Hettwer and Wang, 1986; Joshi *et al*., 1989). Living cells primarily control solute uptake by means of plasma membrane. The permeability of this membrane can be altered without the total destruction of cell integrity, by a treatment commonly known as cell permeabilization.

Permeabilization technology offers several advantages over cell disruption techniques (Hettwer and Wang, 1986). In this, the cell structure is altered to make it porous to allow small molecules, such as substrates or products to cross freely and the cells are spared from the harsh treatment associated with disruption of cells (Naglak *et al*., 1990). The treatment is simple, rapid and allows the assays of enzymes under conditions similar to those prevailing *in vivo* with regard to concentration and

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interaction of macromolecules. Permeabilized cells can be considered as source of insoluble enzymes which could have similar uses to enzymes immobilized by conventional methods (Somkuti and Steinberg, 1994).

β-Galactosidase most commonly known as lactase has been suggested for the production of lactose hydrolyzed milk for lactose intolerant persons and hydrolysis to obtain glucose and galactose (Mahoney, 1997; Panesar *et al*., 2006). Among the various organisms, the yeast *Kluyveromyces* has been reported as the most important source for the production of β-galactosidase, 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 limited being the enzyme intracellular, making its extraction difficult and expensive. Therefore, use of permeabilized cells is an interesting alternative, which can be further explored. Earlier focus was on the enzymic hydrolysis by β-galactosidase from various micro-organisms in free or immobilized form. However, in recent years the use of permeabilized cells has received more attention. The permeabilization of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub sp. *bulgaricus* cultures has been carried out using ethanol (Somkuti *et al*., 1998). In recent studies, permeabilization technique has also been applied to *Z. mobilis* for the production of sorbitol (Vignoli *et al*., 2006).

Considerations in the efficacy and cost have led to the predominant use of organic solvents (Champluvier *et al*., 1988) however, much research is needed on the factors affecting the performance of these solvents during yeast cell treatment. In the present investigation, different chemical agents were compared for their effectiveness in permeabilization of the yeast cells for β-galactosidase activity and their subsequent utilization in lactose hydrolysis.

MATERIALS AND METHODS

Procurement and Cultivation of Micro-organism

Kluyveromyces marxianus NCIM 3465 was procured from the National collection of Industrial Micro-organisms, National Chemical Laboratory, Pune (India). 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 fermentation media was inoculated with 20 h old inoculum, incubated at 30°C temperature for 24 h.

Permeabilization of Yeast Cells

The permeabilization of yeast cells was carried out using eight different organic solvents. 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). Different concentration of permeabilization agents (benzene, n-butanol, n-propanol, triton X-100, iso-propanol, toluene, ethanol and acetone) were added to the yeast biomass. The contents were mixed on a vortex mixture and incubated for specified time intervals, under shaking conditions. After this, the cells were re-centrifuged and washed twice with the phosphate buffer and analysed for enzyme activity.

Lactose Hydrolysis

The permeabilized yeast cells were used for the lactose hydrolysis in 5% (w/v) lactose solution (pH 7.0, 0.1 M phosphate buffer). The permeabilized yeast cells (120 mg dry wt) were added to the lactose solution (50 mL of lactose solution in 250 mL capacity conical flasks). The flasks were incubated at 30°C under shaking conditions (100 rpm) and samples were analyzed for lactose content.

Enzyme Assay

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

Lactose Estimation

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

RESULTS AND DISCUSSION

The permeabilization of yeast cells was carried out to find out the best chemical agent for maximal β-galactosidase activity. The results obtained from the different experimental sets are presented and discussed below.

Screening of Permeabilization Agents

The yeast cells were treated with eight different organic solvents to find out their optimal concentration for the permeabilization. The concentrations of benzene, n-butanol, n-propanol, triton X-100 was varied from 5-40% (v/v) and the results are presented in Fig. 1. To find out the optimum concentration of acetone and ethanol for the effective permeabilization of yeast cells, its concentration was varied from 20-55% (v/v). In another set of experiments, the effectiveness of iso-propanol and toluene as permeabilization agent was tested by applying their different concentrations from (15-50%, v/v) to the yeast cells (Fig. 2).

It has been observed from the data (Fig. 1-2) that permeabilization of the yeast cells 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 less enzyme

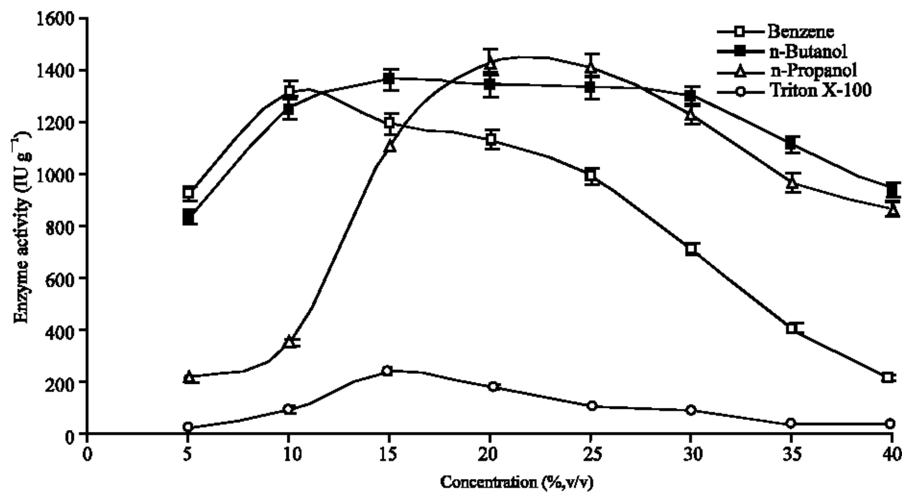

Fig. 1: Effect of varying concentrations of benzene, n-butanol, n-propanol and triton X-100 on permeabilization of yeast cells. Bars indicate the standard deviation from triplicate determinations

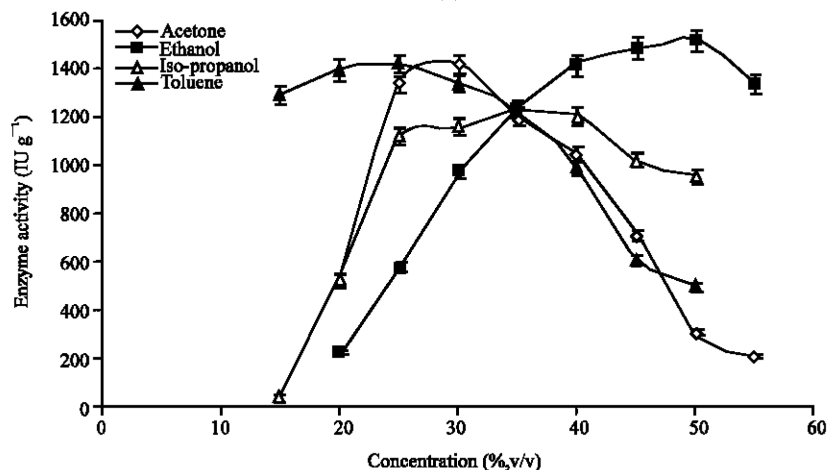

Fig. 2: Effect of varying concentrations of acetone, ethanol iso-propanol and toluene on permeabilization of yeast cells. Bars indicate the standard deviation from triplicate determinations

activity may be due to the insufficient amount of the agent for effective permeabilization. Similar observations have been made by earlier researchers during use of cetyltrimethylammonium bromide as permeabilization agent (Joshi *et al*., 1987).

Moreover, the data reveals that benzene, n-butanol, n-propanol, iso-propanol, acetone, toluene and ethanol were effective in carrying out the permeabilization of yeast cells, however, n-hexane, triton X-100 was found less effective. Different optimal concentration of benzene, n-butanol, n-propanol, iso-propanol, toluene, ethanol and acetone were 10, 15, 20, 35, 25, 50 and 30% (v/v) respectively. Amongst the various permeabilizing agents used, the maximum enzyme activity of 1510 IU g^{-1} (dry wt.) was observed with 50% (v/v) ethanol, followed by n-propanol (Fig. 3).

Ethanol (70%) has also been found as an efficient permeabilization agent for *K. fragilis* (Gonzalez-Siso and Suarez-Doval, 1994). The maximum efficacy has also been reported with 30% isopropanol, 40% tert-butanol and 70% ethanol for the permeabilization of *Kluyveromyces bulgaricus* IRC 101 cells (Decleire *et al*., 1987). However, in present studies, maximum permeabilization was observed with 50% ethanol. The different optimal concentrations of permeabilization agents for the effective permeabilization of yeast cells has been reported by earlier researchers may be due to the different yeast strains and process conditions used for their permeabilization.

Kluyveromyces cells are known to posses a lactose carrier protein (lactose permease) on their cell membrane that mediates the transport of lactose across the cell membrane (Dickson and 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 chemical agents may have disrupted 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. It has been reported that the permeabilizing agents act on the cell membranes by decreasing the phospholipid content (Siso *et al*., 1992).

Effect of Temperature on the Permeabilization

To find out the effect of temperature on the permeabilization of yeast cells using ethanol as a permeabilization agent, the temperature was varied from 10-40°C (Fig. 4). The enzyme activity

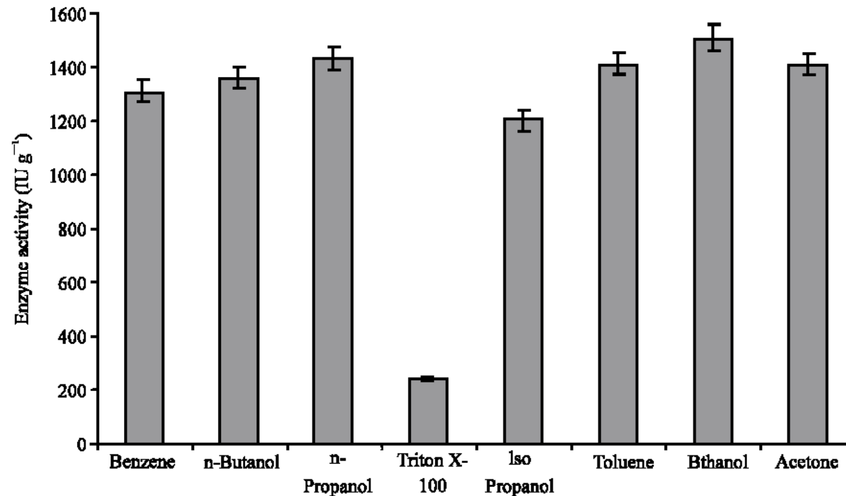

Fig. 3: Comparison of various permeabilization treatments on yeast cells. Bars indicate the standard deviation from triplicate determinations

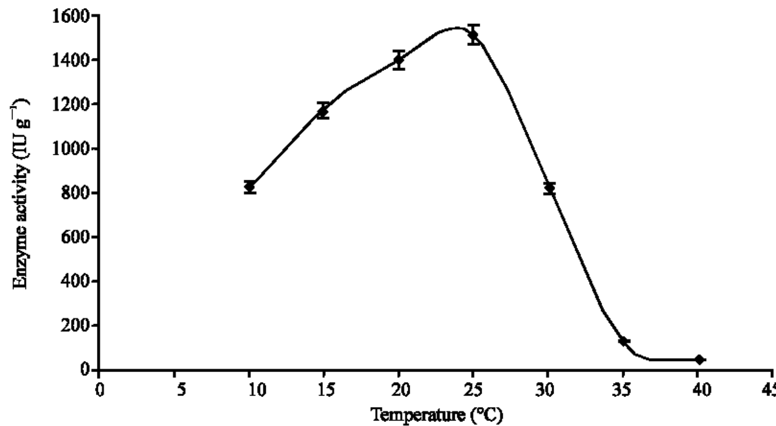

Fig. 4: Effect of temperature on ethanol permeabilization of yeast cells. Bars indicate the standard deviation from triplicate determinations

increased with increase in temperature up to 25°C, however, further increase in temperature has shown a severe decrease in enzyme activity. The enzyme activity of 1510 IU g^{-1} (dry wt.) was observed at 25°C. The low levels of enzyme activities detected at higher temperatures indicate an adverse effect of higher temperature on the permeabilization process, which may be due to the partial inactivation of the enzyme. Since, optimal permeabilization of yeast cells was observed at 25°C, it was selected for further experimentation. Similar temperature (25°C) has also been used for the permeabilization of *Kluyveromyces bulgaricus* IRC 101 (Decleire *et al*., 1987). However, Joshi *et al*. (1989) have reported 26°C as the optimal temperature for the permeabilization of *K. fragilis* NRRL Y-1196 cells using digitonin.

Effect of Treatment Time on the Permeabilization

To optimize the treatment time for the effective permeabilization of yeast cells using ethanol, the mixture containing yeast cells and permeabilizing agent was incubated at 25°C for different time

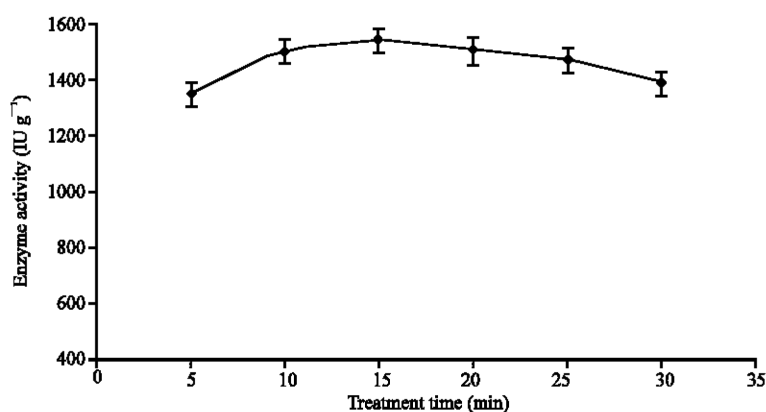

Fig. 5: Effect of treatment time on ethanol permeabilization of yeast cells. Bars indicate the standard deviation from triplicate determinations

intervals (5-30 min). The results (Fig. 5) revealed that the enzyme activity of yeast cells increased with the increase in incubation time up to 15 min, beyond this a decrease in this function was observed. The maximum enzyme activity was 1540 IU g^{-1} at 15 min incubation. The decrease in the enzyme activity at higher treatment time may be due to the partial inactivation of enzyme or cell lysis with increased incubation in ethanol. Therefore, 15 min exposure of yeast cells to ethanol at 25°C is sufficient to yield maximum enzyme activity and it was used in further studies.

During the experimentation, it was observed that the concentration of the permeabilizing agent and temperature are two critical factors for the effective permeabilization of yeast cells. The enzyme activity decreased beyond a certain concentration of permeabilization agent, which may be due to the inactivation of the enzyme or the lysis of cells at higher concentration.

The studies have shown that ethanol at a concentration of 50% (v/v) is the most efficient permeabilizing agent for yeast cells. The optimal temperature and incubation time recorded for the effective permeabilization of yeast cells for β-galactosidase activity are 25°C and 15 min, respectively and these were used in subsequent experimentation. 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 (Siso *et al*., 1992).

Hydrolysis of Lactose Using Permeabilized Cells

The ethanol permeabilized yeast cells and the lactose solution was incubated to find out the hydrolysis of lactose (Fig. 6). A progressive increase in lactose hydrolysis with the increase in incubation period was observed up to 150 min and thereafter no improvement in this function was recorded. The maximum lactose hydrolysis of 90% was observed with 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 (Mahoney, 2003). Thus, ethanol-permeabilized yeast cells successfully carried out the lactose hydrolysis, which can be applied for the lactose hydrolysis in milk and whey saccharification (Becerra *et al*., 2001). 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 the persons which are lactose intolerant. Recently, the permeabilized cells have also been suggested for lactulose synthesis from lactose and fructose (Lee *et al*., 2004).

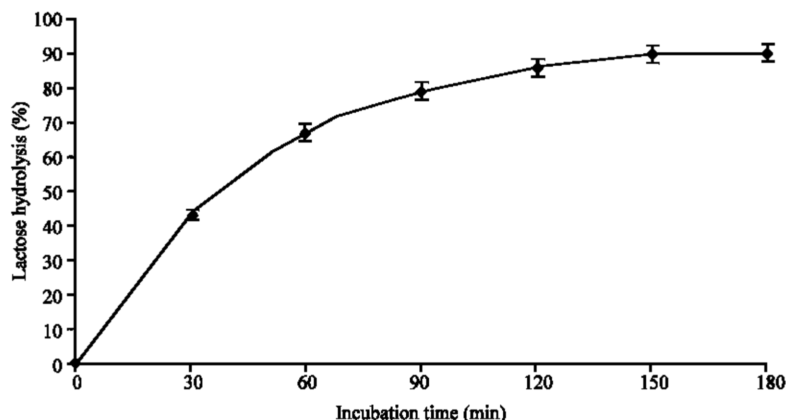

Fig. 6: Hydrolysis of lactose by permeabilized yeast cells as a function of incubation time. Bars indicate the standard deviation from triplicate determinations

CONCLUSIONS

Among the different chemical agents tested, ethanol is the most effective agent for the permeabilization of *K. marxianus* NCIM 3465 cells for β-galactosidase activity. The optimal conditions for the permeabilization of yeast cells were 50% ethanol concentration, 25°C temperature and 15 min of treatment time. The ethanol-permeabilized yeast cells successfully hydrolyzed the lactose, which can be applied for the lactose hydrolysis in milk and whey. The use of permeabilized cells can help to overcome the problems associated with enzyme extraction and purification from yeast cells and in the development of a low-cost technology for lactose hydrolysis. In addition, ethanol has ready availability, low price and is a component of many fermented foods/beverages allowing in principle, the use of permeabilized cells in food applications.

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