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PERMEABILIZATION OF A NEWLY ISOLATED *Kluyveromyces* sp. FOR THE PREPARATION OF WHOLE CELL BIOCATALYSTS WITH *β*-GALACTOSIDASE ACTIVITY

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ABSTRACT

The enzyme *β*-galactosidase catalyzes the hydrolysis of lactose and has wide range of potential applications in the dairy industry. Besides its application in lactose intolerance, the enzymatic hydrolysis of milk lactose also prevents lactose crystallization in frozen and condensed dairy products. Furthermore, innovative applications of *β*-galactosidase involve the production of biologically active prebiotics such as lactulose and galacto-oligosaccharide, which has applications in both food and pharmaceuticals field. However, the industrial applications of *β*-galactosidase are being hindered due to its intracellular location within the yeast cells. The permeabilization technology can be applied, to prevail the problem associated with permeability barrier of cell membrane to lactose and to prepare whole cell biocatalyst with high activities. Keeping in view of later, screening of different permeabilizing agents and optimization of various process parameters have been done using novel yeast isolate (*Kluyveromyces* sp.) to get maximum *β*-galactosidase activity. The permeabilization of yeast isolates for *β*-galactosidase was carried out using organic solvents individually or mixture of organic solvents (ethanol, acetone, n-propanol, iso-propanol, toluene, benzene etc.). Furthermore, the operating conditions (ratio of permeabilizing agents, temperature and treatment time) has also been optimized to get maximum *β*-galactosidase activity. Maximum permeabilization for *β*-galactosidase activity was observed with mixture of toluene (25%, v/v) and ethanol (50%, v/v) at temperature of 25 ^{o}C after treatment time of 15 min.

Keywords: *Kluyveromyces* sp.; *β*-galactosidase; ONPG assay; permeabilization; organic solvent; optimization.

INTRODUCTION

The enzyme *β*-galactosidase is not only used to catalyze the hydrolysis of lactose, but also in transgalactosylation reaction that produces different prebiotics like galacto-oligosaccharides and lactulose (Prenosil et al., 1989, Lee et al., 2004). It is very useful in the dairy industry and has been widely used to produce lactose-free dairy products for lactose intolerance individuals. This enzyme can be obtained from a variety of micro-organisms such as *Kluyveromyces lactis, K. marxianus, Aspergillus foelidis, Auerobasidium pullulans, Sulfolobus solfatarius, Bifidobacterium bifidum* etc. (Panesar et al., 2006). Among these the most commonly used microbial sources are *Kluyveromyces* sp. and *Aspergillus* sp. The industrial application of whole cells biocatalyst with *β*-D-galactosidase has been hindered by the difficulty of releasing active enzyme in good yield from the cells due to the intracellular location of enzyme. The cell disruption processes used for the extraction of intracellular products involve breakage of the cell envelope and releasing of all the intracellular constituents into surrounding medium. Further, the cost of the purification processes also becomes high. However, these types of problems can be solved using permeabilization technique. The permeabilization of yeast cells is a simple and rapid technique to permit the assay of enzymes under natural environmental condition.

Thus, cells permeabilization can be applied as an important tool in the biocatalytic process that can be an inexpensive alternative to purified enzyme systems (Panesar, 2008).

In this process, inspite of harsh treatment associated with disruption of cells, only cell structure is altered making it porous that allow the transfer of small molecules, such as substrates or products, to cross freely (Naglak et al., 1990). In the present investigation, permeabilization of isolated yeast strain was carried by using organic solvents individually or mixture of solvents (ethanol, acetone, n-propanol, iso-propanol, toluene, benzene etc.). Further, the optimization of process conditions such as ratio of toluene to ethanol, temperature and time for maximum permeabilization was also carried out.

MATERIALS AND METHODS

MICRO-ORGANISM

The *Kluyveromyces* sp., a *β*-galactosidase producing yeast strain, was isolated from by-products (whey) of dairy industry.

MATERIALS

The reagents used in the experiments were purchased from HiMedia Laboratories Pvt. Limited, Mumbai, India.

INOCULUM PREPARATION

Loopful of yeast from slant was added to sterile media containing (%, w/v) glucose (1.0), malt extract (0.3), yeast extract (0.3) and peptone (0.5), adjusted to pH 5.0 and incubated at temperature 30 ^{o}C for the incubation period of 20 hr.

SUBMERGED FERMENTATION OF YEAST ISOLATE FOR β-GALACTOSIDASE PRODUCTION

The yeast inoculum of 6.0% (v/v) were inoculated into the media containing (%, w/v) lactose (5.0), yeast extract (0.38), magnesium sulphate (0.05), urea (0.12), adjusted to pH 5.34 and incubated at temperature 30 ^{o}C for the incubation time of 27 hr.

MEASUREMENT OF ENZYME ACTIVITY

β-Galactosidase activity assay was carried out using the method of Miller (1972). The release of O-nitro phenol from O-nitrophenyl-*β*-D-galactopyanoside was measured at 420 nm with the help of spectrophotometer (DR 5000, HACH, Germany). One unit of enzyme activity is equivalent to one micromole of O-nitrophenol liberated per min under standard assay conditions. All the enzyme assays were performed in triplicate.

SCREENING OF PERMEABILIZATION AGENTS

The permeabilization of yeast cells was carried out by using various chemical agents (n-butanol, n-propanol, iso-propanol, acetone, ethanol, benzene, tritonX-100, cetyltrimethylammonium bromide and toluene) for *β*-galactosidase activity. The mixture of permeabilizing agents (ethanol and acetone, ethanol and toluene, ethanol and n-propanol, ethanol and iso-propanol, ethanol and n-butanol, ethanol and benzene) has also been tested for the permeabilization of yeast cells.

OPTIMIZATION OF PROCESS PARAMETERS FOR β-GALACTOSIDASE ACTIVITY

The various process parameters such as ratio of permeabilizing agents, temperature and treatment time have been optimized for the permeabilization of yeast cells and to get maximum *β*-galactosidase activity. The range of process parameter applied for were; 10:90-60:40 ratio of toluene (25%, v/v) to ethanol (50%, v/v), temperature (10-40 ^{o}C) and treatment time of 5-30 min.

RESULTS AND DISCUSSION

The effect of different chemical agents and process parameters on the permeabilization of yeast cells for *β*-galactosidase activity has been discussed below:

EFFECT OF DIFFERENT PERMEABILIZING AGENTS ON β-GALACTOSIDASE ACTIVITY

The permeabilization of yeast cells were carried out using various organic solvents (ethanol, acetone, n-propanol, iso-propanol, toluene, benzene etc.). Among these organic solvents the maximum enzyme activity (1.597 IU/mgDW) was observed with the treatment of 50% (v/v) ethanol (Fig 1a-c). Similarly, ethanol (50%, v/v) has also been found to be an effective permeabilizing agent to permeabilized yeast cells for the lactose hydrolysis and lactulose synthesis (Panesar et al., 2006, Lee et al. (2004), whereas, 70% (v/v), ethanol was found as an efficient permeabilizing agent for *K. fragilis* (Gonzalez-Siso and Suarez-Doval, 1994). Further, an increase in enzyme activity was observed by combining the ethanol (50%, v/v) with other organic solvents like acetone (30%, v/v), n-propanol (20%, v/v), iso-propanol (40%, v/v), toluene (25%, v/v), n-butanol (10%, v/v). Amongst the various combination of permeabilizing agents used, the maximum enzyme activity of 1.631 IU/mgDW was observed with mixture (50:50) of toluene (25%, v/v) and ethanol (50%, v/v). However, the minimum enzyme activity (1.602 IU/mgDW) was observed with ethanol and n-butanol mixture. The ratio of toluene to ethanol, treatment time and temperature has also been optimized to get maximum *β*-galactosidase activity. From the results, it was concluded that the enzyme activity in with the enzyme activity increase with an increase in percentage concentration of toluene in ethanol upto 40%, and beyond that decrease in activity was seen.

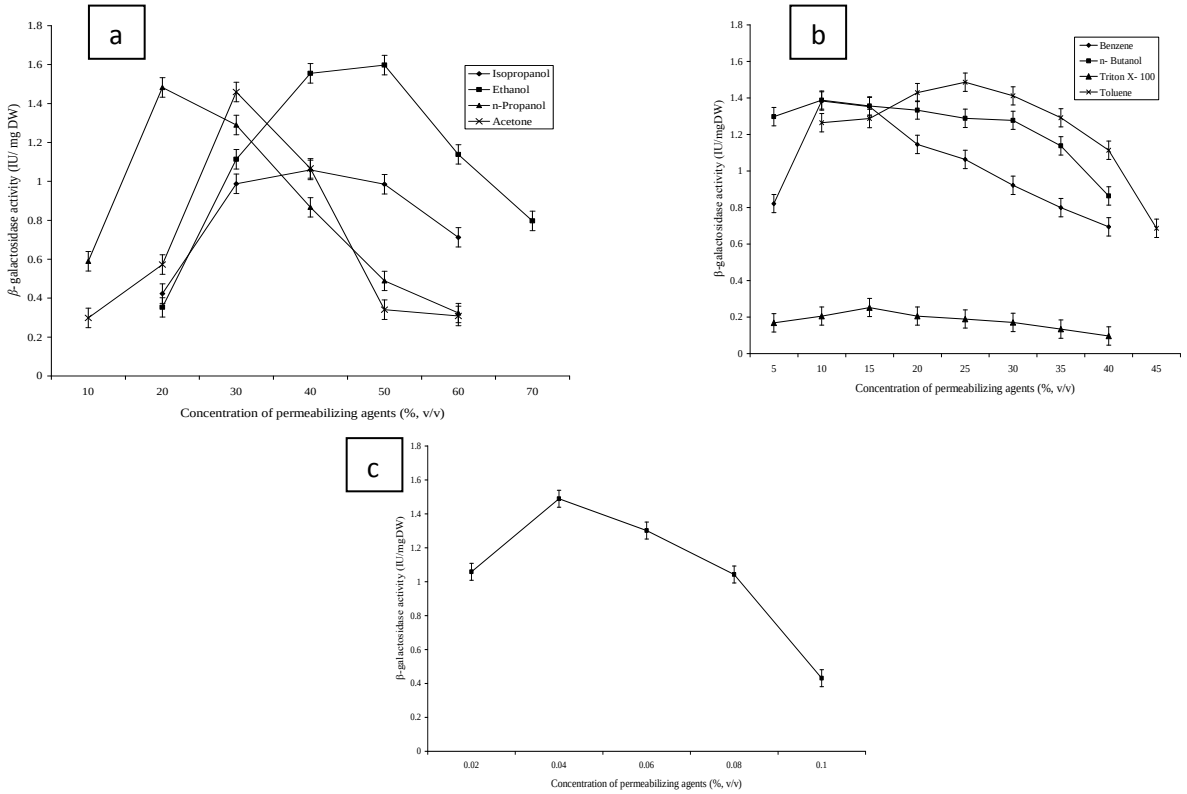

Fig. 1 Effect of different permeabilizing agents a) iso-propanol, ethanol, n-propanol and acetone b) benzene, n-butanol, triton X-100, toluene c) cetyltrimethylammonium bromide (CTAB); on *β*-galactosidase activity

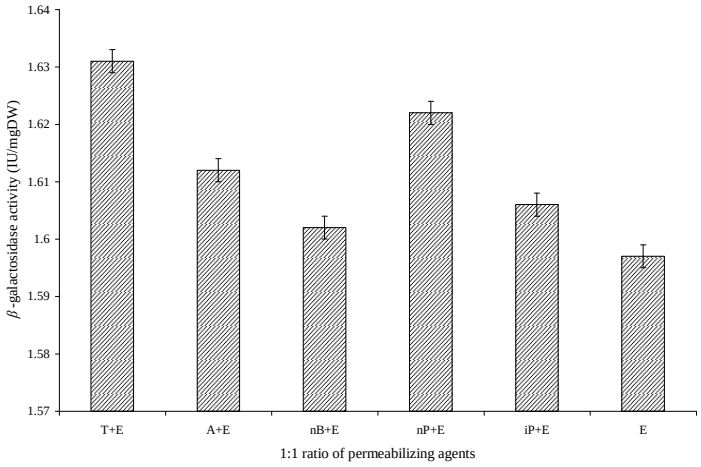

Fig. 2 Effect of a mixture of permeabilizing agents on *β*-galactosidase activity [Abbreviation: ethanol (E), acetone (A), toluene (T), n-butanol (nB), n-propanol (nP) and iso-propanol (iP)

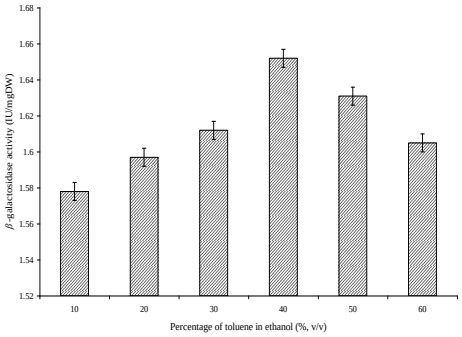

Fig. 3 Effect of percentage of toluene in ethanol on *β*-galactosidase activity

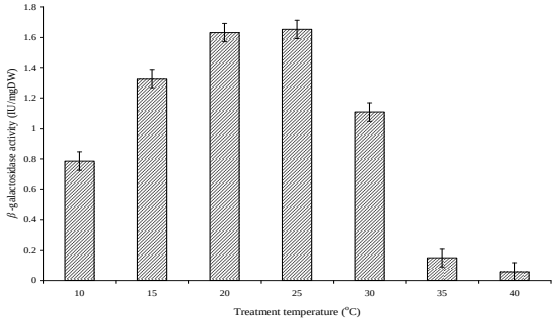

Fig.4 Effect of treatment temperature on *β*-galactosidase activity

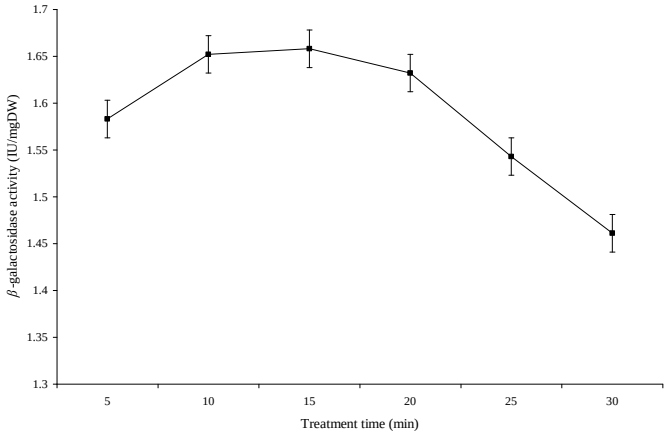

Fig. 5 Effect of treatment time on *β*-galactosidase activity

EFFECT OF TREATMENT TEMPERATURE ON β-GALACTOSIDASE ACTIVITY

Further, to find out the effect of temperature on the permeabilization of yeast cells using mixture of toluene and ethanol, the temperature was varied from 15-40 ^{o}C (Fig. 4). From the results, it was observed that the enzyme activity increase with an increase in treatment temperature upto 25 ^{o}C, however, further increase in temperature showed decrease in enzyme activity. The enzyme activity of 1.652 IU/mgDW was observed at the treatment temperature of 25^{o}C. At the high level of treatment temperature 40 ^{o}C, decrease in enzyme activity (0.056 IU/mgDW) was seen; it might be due to the partial inactivation of the enzyme.

However, Joshi et al. (1989) have reported 26 ^{o}C as optimal temperature to permeabilize *K. fragilis* NRRLY 1196, whereas, Flores et al. (1994) found an effective treatment temperature of 30^{o}C for the permeabilization of *K. lactis* CBS 683.

EFFECT OF TREATMENT TIME ON β-GALACTOSIDASE ACTIVITY

The treatment time also play an important role for the effective permeabilization of yeast cells using a mixture of toluene and ethanol. The mixture of yeast cells and permeabilizing agent was incubated for different time interval of 5-30 min at temperature of 25^{o}C (Fig. 5). From the results of experiments, it was found that the *β*-galactosidase activity of yeast cells increased with the increase in treatment time upto 15 min, beyond which decrease in enzyme activity was observed, may be due to the partial inactivation of enzyme or the cell lysis with the increase in the incubation time with organic solvents (toluene and ethanol). The maximum enzyme activity of 1.658 IU/gmDW was found at the treatment time of 15 min. However, the optimum treatment time of 14 min has been reported in case of permeabilization of *Kluyveromyces marxianus* var. *lactis* NCIM 3566 with cetyltrimethylammonium bromide (Kaur et al., 2009), whereas the optimal treatment time was extended upto 30 min for the permeabilization of *K. fragilis* NRRL Y-1196 cells with digitonin as the permeabilizing agents (Flores et al., 1994).

CONCLUSIONS

From the results of above experiments, it can be concluded that a mixture of toluene and ethanol is an effective permeabilizing agent for the permeabilization *Kluyveromyces* sp. isolate. The optimum operating conditions for permeabilization process were 40:60 ratio of toluene (25%, v/v): ethanol (50%, v/v), 25 ^{o}C temperature and process duration of 15 min. The use of permeabilized cells can help to solve the problems/costs associated with intracellular located enzyme extraction and purification from yeast cells and in the development of a low-cost and simple technology for lactose hydrolysis and prebiotic production (galacto-oligosaccharides and lactulose).

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