ABSTRACT

Lactulose is non-digestible sugar containing fructose and galactose linked through glycosidic bond. As a prebiotic sugar, it can be applied in commercial infant formulas and various dairy products because it has ability to promote the proliferation of intestinal Bifidobacterium, which is considered as a very important humanizing factor. Lactulose has attracted much attention due to its many industrial uses, including treatment of constipation and hepatic encephalopathy.

Biosynthesis of lactulose can be carried out by both chemical and enzymatic transformation of lactose. But at commercial level, it is produced by alkaline isomerization of lactose. However, this method consists of expensive separation and purification steps to remove byproducts that cause difficulty in waste management and product purification. Thus, an eco-friendly approach, i.e., an enzymatic transformation process seems to have considerable potential in industrial lactulose production.

In this direction, this present research work was carried out to develop a convenient process for lactulose production by using an enzyme β-galactosidase. But, the industrial application of β-galactosidase has been hampered by the cost related to extraction and purification of enzyme. Thus, the use of β-galactosidase as a whole cell biocatalyst is an effective way to lower the production cost because complex purification is not required. For whole cell biocatalyst, isolation of yeast culture has been carried having high potential to produce β-galactosidase. However, the permeability barrier of the cell envelope for substrates and products often causes very low reaction rates in whole cells, especially yeast cells, therefore to overcome these technical hindrances, permeabilization cell technique has been applied. To improve the economics of the process, immobilization cell technology has also been applied for
lactulose production. Further, whey, a dairy by-product remaining after cheese production rich in lactose content has also been explored to produce lactulose using immobilized cell technology, which can make the process cost effective.

The present work has been compiled into five chapters. Chapter one includes the introduction of the presented work, in which after introducing the lactulose, its production methods and applications, the objectives of the presented work has been discussed. In the second chapter, study begins with the literature review on lactulose and the different process technologies for its production using both chemical and enzymatic methods. Furthermore, under enzymatic methods for lactulose production, discussion is not only limited to the biotransformation of lactose to lactulose by using different forms of enzyme like free, immobilized, whole cells, but also detail about the microbial production of β-galactosidase, permeabilization of microbial cells, immobilization techniques has been discussed. In the end of this chapter, the potential applications of lactulose have also been summarized. Material and methods used to fulfill the objectives of the research works are discussed in third chapter. It includes methodology on the isolation of β-galactosidase producing yeast cells, optimization of media and process parameters for the production of β-galactosidase, permeabilization of yeast cells to get maximum β-galactosidase activity, optimization of processes for biotransformation of lactose to lactulose using both free and immobilized cell system. Along with these, different analytical techniques applied during the course of the present investigation to estimate β-galactosidase activity, lactose concentration, lactulose concentration, protein, fat, mineral, etc. have also been given. Chapter four includes results and discussion about the isolation, permeabilization, immobilization and the biotransformation of lactose to lactulose using both free and immobilized cell system.
In first section of the chapter fourth, an efficient novel β-galactosidase producing yeast strain was isolated from whey samples collected from milk processing industries. The identification of these novel yeast isolate was done on the basis of the amplification of partial 18S rRNA, ITS1, 5.8S rRNA, ITS2 and partial 28S rRNA gene fragment using high-fidelity PCR Polymerase. Based on nucleotides homology and phylogenetic analysis, the isolated yeast culture was identified as Kluyveromyces marxianus.

In the second section of the fourth chapter, the optimization of media and process conditions was carried out for enhancing the production of β-galactosidase from isolated yeast culture. The maximum β-galactosidase activity (1.711 IU/mgDW) was obtained from the isolated K. marxianus strain grown in an optimized medium containing lactose 5.0% (w/v), yeast extract 0.3% (w/v), urea 0.1% (w/v), magnesium sulphate heptahydrate 0.05% (w/v), pH 5.5, inoculum size 6% (v/v) of 20 h old culture, agitation rate 100 rpm, temperature 30 °C and incubation time of 28 h. Response Surface Methodology (RSM), was applied to evaluate the effect of various factors on the optimization of different process parameters to get maximum β-galactosidase production. The recommended optimum operating conditions for β-galactosidase production to achieve maximum enzyme activity were lactose concentration 5.0% (w/v), yeast extract 0.38% (w/v), urea 0.12% (w/v), magnesium sulphate heptahydrate 0.05% (w/v), pH 5.34, temperature 30 °C and incubation time 27 h. At these process conditions, the predicted value of enzyme activity was found to be 1.75 IU/mgDW.

In the third section of the fourth chapter, the permeabilization of yeast isolate for β-galactosidase was carried out using organic solvents individually and using mixture of organic solvents. Among the tested organic solvents (ethanol, acetone, n-propanol, iso-propanol, toluene, benzene, ethanol and acetone, ethanol and toluene,
ethanol and n-propanol, ethanol and iso-propanol, ethanol and n-butanol, ethanol and benzene), mixture of ethanol and toluene showed maximum permeabilization of yeast cells. Therefore, the mixture of ethanol and toluene was used for the optimization of process conditions for cell permeabilization and the optimized process parameters for the maximum permeabilization of yeast cells were found to be 40:60 ratio of toluene (25%, v/v) and ethanol (50%, v/v), 25 °C temperature and treatment time of 15 min. At these process conditions, maximum enzyme activity of 1.658 IU/mgDW was observed. RSM was also applied for the optimization of different process parameters for the permeabilization of yeast cells. The recommended process variables were found to be 50:50 ratio of toluene (25%, v/v) and ethanol (50%, v/v), temperature of 25 °C and treatment time of 12 min to get maximum β-galactosidase activity of 1.71 IU/mgDW. These permeabilized isolated K. marxianus cells were later used for lactulose production.

In the fourth section of results and discussion chapter, different process conditions (substrate concentration, biomass concentration, pH, reaction temperature, and reaction time) were optimized for maximum lactulose production using permeabilized K. marxianus cells. The optimum conditions were 2 g (DW/L) biomass, 40% (w/v) lactose, 20% (w/v) fructose, temperature of 50 °C and pH 7.0. Under these conditions, the permeabilized cells resulted in the lactulose production of 21.36 g/L after reaction time of 3 h.

In the fifth section of the fourth chapter, different immobilizing matrices such as sodium alginate, k-carrageenan, chitosan, agarose, agar-agar, pectin, alginate-pectin, alginate-carrageenan, alginate-chitosan, alginate-agarose, alginate-agar, alginate-xanthan and alginate-gelatin were tested for the immobilization of permeabilized yeast cells. Among these, the beads prepared from alginate and carrageenan was found to be
the best in terms of strength, cell holding capacity, pH and thermal stability. The optimization of various process parameters (beads size, biomass concentration, pH, reaction temperature and time) has been carried out for production of lactulose using alginate-carrageenan beads. The best result was found with the bead size of 2.65-2.75 mm under the optimum reaction conditions of 3.0 g (DW/L) biomass, 40% (w/v) lactose & 20% (w/v) fructose at temperature of 50 °C and pH 7.0. Under these conditions, the permeabilized immobilized yeast cells resulted in production of approximately 31.12 g/L lactulose after reaction time of 4 h. The recycling of beads was done to check the stability of alginate-carrageenan beads upto 10th cycle. From the results, it was concluded that there is no marked decrease in lactulose production ability from immobilized system upto the 7th cycle and after that a significant decrease in the lactulose production was observed. The physico-chemical parameters like hardening, thermal stability and characterization of functional groups, swelling behaviours and pH resistance of beads were also studied to check the stability of hybrid beads (alginate-carrageenan) after each cycle of lactulose production. The results of reusability experiments demonstrated that the production efficiency of lactulose at 10th batches was approximately 51% to that of 1st batch.

In the sixth section of the fourth chapter is devoted to test the above findings into real system involving whey (dairy by-product) as a raw material for the production of lactulose using alginate-carrageenan beads. The optimum conditions for lactulose production using immobilized cells (4.0 gDW/L) were 40% (w/v) lactose, 20% (w/v) fructose, temperature of 50 °C and pH 7.0. Under these conditions, the immobilized yeast cells resulted in production of 24.16 g/L lactulose after reaction period of 3 h. The stability of hybrid beads for the production of lactulose using whey was checked by recycling of beads upto 10 cycles. It was found that there was no significant decrease in
ability of immobilized cell system to synthesize lactulose up to the 6th cycle from whey and after that decrease in the lactulose production was observed and the production of lactulose recorded at 10th batches was 13.65 g/L.

Based on above findings, the results have been summarized and conclusions have been drawn in fifth chapter. The novel yeast isolate having high potential to produce β-galactosidase was successfully used for the biotransformation of lactose to lactulose as both permeabilized and immobilized cell system. The developed permeabilized cell technology for lactulose synthesis can help to solve the problem associated with the extraction and purification to make it cost effective as compared with the recent commercial methods. To further improve the economics of the process, immobilization technology has been applied for lactulose production. The optimized parameters using immobilized cells have resulted in good yield of lactulose. Further, lactulose produced from whey (dairy by-product) by using immobilized cell technology, not only could solve the disposal problem of whey but also give an economical biotransformation. The developed technology has shown promising results at laboratory scale and can be further helpful in the production of lactulose at large scale from industrial point of view.