Lactulose, a synthetic disaccharide composed of two sugar molecules fructose and galactose bonded together with $\beta$-1, 4-glycosidic bond. This compound cannot be decomposed by human digestive enzymes and stimulate the proliferation of health beneficial bacteria of intestine tract. Due to wide range of applications of lactulose, it can be useful in both food and pharmaceutical industries. In food industries, it is applied in a wide variety of foods as a bifidus factor or as a functional ingredient for intestinal regulation and can be beneficial to the health of consumers. Additionally, lactulose can be used as a sweetener for diabetics, as a sugar substitute in confectionery products, beverages, infant milk powders, bakery products, yoghurts, dairy desserts and in various liquid or dried food preparations, which are routinely manufactured for old people. In the pharmaceutical field, lactulose is used mainly for the treatment of constipation, hepatic encephalopathy, complication of liver disease, anti-endotoxin effects, to maintain blood glucose and insulin level.

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 has several disadvantages, like high level of lactulose degradation and colored by-products in the reaction mixture, which includes expensive separation and purification steps to remove byproducts. Therefore, an alternative environmentally friendly approach, i.e. an enzymatic transformation process seems to be a useful approach for lactulose production. In lactulose biosynthesis, $\beta$-galactosidase enzyme which has both hydrolysis and
transgalactosylation activities works as the catalyst. But, the industrial applications of β-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 intracellular β-galactosidase as a whole cell biocatalyst is an effective way to lower the β-galactosidase production cost because complex purification is not required. However, the permeability barrier of the cell envelope for substrates and products often causes very low reaction rates in whole cells, especially yeast cells. To overcome this, many chemical agents can be used to increase the lactose permeability of microbial cells. Furthermore, immobilization technology has been found to be convenient method to make reuse of cells, higher cell densities in bioreactors and easier purification of final product. Thus, the present investigation has been carried out based on the isolation, permeabilization and immobilization of yeast cells for the production of lactulose.

For the isolation of yeast cultures and to find out the efficient novel β-galactosidase producer strain, whey samples were collected from different milk/dairy plant operating in different states of India (Punjab, Haryana, Madhya Pradesh, and Bihar). More than 50 strains of yeast were isolated and tested for the β-galactosidase activity. Among these yeast isolates, WI15 displayed maximum enzyme activity (1.129 IU/mgDW). These values were found higher than the yeast cultures (\textit{Kluyveromyces marxianus} MTCC 1388, \textit{K. marxianus} NCIM 3551, \textit{K. marxianus} NCIM 3566, \textit{K. marxianus} NCIM 3465) procured from culture collection centers under similar conditions. The identification of these novel yeast isolates 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*.

The optimization of media and process conditions has been carried out for enhancing the production of $\beta$-galactosidase from isolated yeast culture. The maximum $\beta$-galactosidase activity activity (1.711 IU/mgDW) for isolated *K. marxianus* strain was observed with optimized medium containing lactose 5.0% (w/v), yeast extract 0.3% (w/v), magnesium sulphate heptahydrate 0.05% (w/v), urea 0.1% (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), which is the combination of mathematical and statistical techniques was applied to evaluate the effect of various factors on the optimization of different process parameters to get maximum $\beta$-galactosidase production. The recommended optimum operating conditions for $\beta$-galactosidase production to achieve maximum enzyme activity were lactose concentration 5.0% (w/v), yeast extract 0.38% (w/v), magnesium sulphate heptahydrate 0.05% (w/v), urea 0.12% (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.

The permeabilization of yeast isolate for $\beta$-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), 25.0 °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 25.0 °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 employed for lactulose production.

Different process conditions (substrate concentration, biomass concentration, pH, reaction temperature and reaction time) have been optimized for maximum lactulose production. 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. The application of immobilization cell technology have significant advantages over free cell system, it permits higher cell densities in bioreactors, decreases product inhibition, improves enzyme stability, makes reutilization and continuous operation and precludes the need to separate the cells upon completion of process. To overcome the problems associated with permeabilized cell technology and further make the process economical, the immobilized cell technology has also been applied for the production of lactulose.

Different immobilizing matrices such as sodium alginate, *k*-carrageenan, chitosan, agarose, agar-agar and pectin were tested for the immobilization of permeabilized yeast cells. Among these, the maximum lactulose production (26.37 g/L) was observed with 2.0% (w/v) sodium alginate. Further, the hybrid bead technology was also developed to further enhance the stability of beads. Different hybrid beads
using mixture of immobilization matrices (alginate and pectin, alginate and carrageenan, alginate and chitosan, alginate and agarose, alginate and agar, alginate and xanthan, alginate and gelatin) were tested for the lactulose production. The results indicated that hybrid beads made from alginate-carrageenan, and alginate-xanthan mixture resulted into approximate similar lactulose production of 26.38 g/L and 26.25 g/L lactulose, respectively.

The physico-chemical characterization (morphological, hardening, thermal stability and characterization of functional groups, swelling behavior and flow properties) of beads made from alginate, alginate-carrageenan and alginate-xanthan have been carried out. To check the stability, recycling of beads was carried out as a function of enzyme activity at each cycle during the production of lactulose. From the results, it was observed that the alginate and alginate-xanthan beads were stable only upto 5<sup>th</sup> cycle and 6<sup>th</sup> cycle, respectively but the alginate-carrageenan beads were stable upto 7<sup>th</sup> cycle in terms of $\beta$-galactosidase activity. Thus, alginate-carrageenan beads can be the best matrices for the biosynthesis of lactulose, due to its high thermal stability, cell entrapment efficiency and pH resistant ability.

Further, optimization of various process parameters (beads size, biomass concentration, pH, reaction temperature and time) has been carried out for the 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, 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 period of 4 h. The recycling of beads was done to check the stability of alginate-carrageenan beads upto 10<sup>th</sup> cycle. From the results, it was concluded that there is no marked decrease in
lactulose production ability from immobilized system up to 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 alginate-carrageenan beads after each cycle of lactulose production. The results of reusability experiments demonstrated that the production of lactulose at 10th batches was approximately 51% to that of 1st batch. Hence, it was concluded that optimized immobilization of yeast cells in alginate-carrageenan can be useful for continuous lactulose production.

Further, to make the process economical, whey (dairy by-product) has been used as a raw material for the production of lactulose using alginate-carrageenan beads. The optimum reaction conditions for lactulose production using immobilized cells (4.0 gDW/L) were 40% (w/v) lactose and 20% (w/v) fructose at 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 has also been checked by recycling of beads up to 10 cycles. From the results, 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.

Based on above findings, it can be summarized that the novel yeast isolate (K. marxianus) has high potential to produce β-galactosidase. The isolated yeast strain was successfully used for the biotransformation of lactose to lactulose as both permeabilized and immobilized cell system. The developed technologies for production of lactulose can help to solve the problem associated with the extraction and purification, thus make the process cost effective as compared with the recent
commercial methods. But, under the optimized process conditions, immobilized cell system resulted in good yield of lactulose as compared to that of permeabilized cell system. Furthermore, lactulose produced from whey lactose using immobilized cell technology make the biotransformation process more economical. Thus, the developed technology will provide very useful guideline for future development of immobilized cell technology for the production of lactulose at large scale from industrial point of view.