2. REVIEW OF LITERATURE

2.1 Pre-genomic studies in *Z. mobilis*

During the pre-genomic period, much of the research was focused upon biochemical and molecular aspects, fermentation studies, heterologous expression of key ethanologenic enzymes, process development, saccharification, co-culture studies and strain improvement. Gunasekaran *et al.,* (1986) demonstrated production of bioethanol by 4 different strains of *Z. mobilis* by using natural substrates like cane juice and molasses. Kamini and Gunasekaran (1987, 1989) have shown that synchronized ethanol production from lactose by strains of *Z. mobilis* 1960, B 4286 by coculturing with *K. fragilis* 665. Gunasekaran and Kamini (1991) demonstrated elevated ethanol production from lactose by immobilized cells of *Z. mobilis* B 4286 cocultured with *K. fragilis* 665. Nellaiah *et al.,* . (1988) has reported the production of 94.0 g l^{-1}, 76.9 g l^{-1}, and 66.5 g l^{-1} of ethanol at glucose, fructose, and sucrose concentrations of 200 g l^{-1}, respectively by *Z. mobilis* B-4286. Nellaiah *et al.,* (1988) has reported the production of 80.0 g l^{-1} of ethanol from enzymatically hydrolysed cassava starch at glucose concentrations of 171 g l^{-1}, by *Z. mobilis* B-4286. Nellaiah and Gunasekaran (1992) showed that highest ethanol concentration of 59 g l^{-1} and productivity of 3.57 g l^{-1} could be obtained from cassava starch hydrolysate by immobilized *Z. mobilis*. Amutha and Gunasekaran (1994) showed that concurrent saccharification and fermentation of cassava starch using *Z. mobilis* produced 57 g l^{-1} of ethanol. Baratti *et al.,* (1991) has demonstrated that *Z. mobilis* possess an extracellular sucrase which is mainly responsible for sucrose hydrolysis during fermentation. Kannan *et al.,* (1998) has shown that improved ethanol production of 73.5 g l^{-1} from 150 g l^{-1} sucrose by a mutant of *Z. mobilis* than of its parent strain, B-806 (65.2 g l^{-1}).

2.2 Pre-genomic studies of lactose metabolism in *Z. mobilis*

The only limitation to *Z. mobilis* compared to other ethanol producing microorganism such as *Saccharomyces cerevisiae* is that its utilizable substrate range is restricted to glucose, fructose, and sucrose. Advancements in recombinant DNA technology could be exploited to construct microbial strains to produce ethanol from lactose, maltose and cellobiose. To obtain ethanologenic strains utilizing the above-mentioned substrates, genes from other organisms were transformed into *Z. mobilis*. Our focus is the absence of lactose metabolism in *Z. mobilis*.

Throughout the pre-genomic years, in the nonexistence of well-built computational tools, an extensive series of experiments were carried out for the lactose metabolism in *Z. mobilis*. Carey *et al.,* (1983) transferred the lactose operon into *Z. mobilis*. The beta-galactosidase was expressed in *Z. mobilis* (Goodman *et al.,* .1984). Strzelecki *et al.,* (1986) demonstrated the potency of the lactose operon in *Z. mobilis*.

Various wide-host-range cloning vectors with the aim of allowing the functional display of the lactose operon of *E. coli* and the assembly of the fused beta-galactosidase and other genes were developed. These plasmids have been transformed into *Z. mobilis* and their ability on the lactose and galactose consumption and production of ethanol have been studied (Lodge *et al.,* 1992). *Z. mobilis* could utilize solitary glucose fraction in lactose and produced 1 mole ATP/mole glucose utilized; as a result, there is no enhancement of energy for growth of the cell because the equivalent ATP is utilized for every molecule of the lactose transported diagonally the membrane during proton transfer (Romano, 1986). In a following successive developments, the galactose operon has been cloned into the wide-host-range plasmid

carrying galactose and lactose utilizing genes of *E. coli*. Buchholz *et al.,* (1989) reported the transforming of beta-galactosidase gene into a lactose utilizing strain of *Zymomonas mobilis*, if probable with the gal operon, as a result that the genes encoding the lactose permease and beta-galactosidase would be probable. A galactose metabolizing *Z. mobilis* was developed as follows: a galactose positive recombinant vector, was developed by the cloning of the *E. coli* 's *galETK* genes straight away downstream of the promoter gene of *Z. mobilis* in the vector pZA22 and established into one of the *Zymomonas* strain (Yanase *et al.,* 1991). The recombinant strain was able to produce a minute quantity of ethanol from galactose source. Still though, there was production of beta-galactosidase and the lactose was metabolized to produce galactose and glucose, there was absence of growth of the recombinant *Z. mobilis*.

In additional, to progress the lactose operon expression in *Z. mobilis,* the *Z. mobilis*'s promoter segment was located instantaneously upstream of a reduced beta-galactosidase gene on the vector Pzl1 consequently that collectively led to expression of recombinant lactose permease and beta-galactosidase (Yanase *et al.,* 1991). Nonetheless the recombinant plasmid pZS5, had elevated beta-galactosidase activity although the activity of lac permease was smaller amount. pRUT102, an extensive host choice plasmid with the lac operon and gal operon below the direction of the *Z. mobilis* promoter was brought together by Buchholz *et al.,* (1989). Prut102 containing *Z. mobilis* CP4.45 was proceeded to mutagenesis by NTG together with continuous choice pressure for growth on medium containing lactose. *Z. mobilis* SB6, developed a thick culture and formed ethanol from lactose.

2.3 Pre-genomic studies of beta-lactam resistance in *Z. mobilis*

There have been no previous comprehensive and detailed studies on the ß-lactam resistance-determinants in this non-pathogenic bacterium. There has been earlier report of the cloning and expression of a ß-lactamase gene from *Z. mobilis* B406 in *Escherichia coli* JM 109 (Mukundan *et al.,* 1994).

2.4 Genome sequence of *Z. mobilis*

The genome sequence of *Z. mobilis* ZM4 was reported by Seo *et al.,* (2005). The whole genome of *Z. mobilis* ZM4 [NC_006526] consists of a chromosome of 2,056,416 bp with a typical G+C content of 46.33%. The calculated open reading frames (ORFs, 1,998) occupies 87% of the genome, in which 1,346 ORFs (67.4%) are reported with their functions, 258 ORFs (12.9%) are predicted putative coding sequences for general functions and 394 ORFs (19.7%) are unknown hypothetical genes. The genome sequence shows the presence of enzymes that metabolize sucrose, fructose, glucose, mannose, raffinose and sorbitol. The key enzymes (glucose-6-phosphate dehydrogenase, lactonase, 6-phosphoglucanate dehydratase) necessary for the conversion of glucose to yield ethanol have been reported in this study. More than 54 genes were found to be more expressed when ethanol production is vigorous. NAD(P)H: quinone oxidoreductase and oxidoreductase were found to be highly expressed during ethanol production. Kouvelis *et al.,* (2009) reported the first complete genome sequence of ethanologenic *Z. mobilis* subsp. *mobilis* strain NCIMB 11163.

2.5 Metabolic pathway reconstruction studies in *Z. mobilis*

The metabolic pathway network of *Z. mobilis* was reconstructed by Tsantili *et al.,* (2007). Linear programming analysis was used to study the ethanol production on

the context of anaerobic respiration. The major conclusions of this study were: 1) the maximum ethanol production is in context with the catabolism of the substrates through the Entner-Doudoroff, Pentose-Phosphate and Embden-Meyerhof-Parnass pathways; 2) the lack of system to consume ATP surplus; 3) the increase in ethanol yield due to accessibility of NAD(P)H; 3) the removal of the key enzymes of the above-said pathways affected the ethanol yield; 4) the presence/absence of the genes involved in glycerol metabolism affected the ethanol production; 5) the trans gene and double deletions led to stoppage of ethanol production; 6) The highest ethanol yield was 1.42 mole / mole glucose, 1.08 mole / mole xylose and 1.25 mole / 0.5 mole glucose and 0.5 mole xylose is due to the catabolism of these substrates through the Entner-Doudoroff, Pentose-Phosphate and Embden-Meyerhof-Parnass pathways 7) the deletion of the genes involved in the above-said pathways affected the ethanol yield.

In another instance, Pinto *et al.,* (2009) studied the various methods for the genome-level reconstruction of the metabolism of *Z. mobilis*. The quality of the data and its integration methods were broadly discussed in this study. The various steps involved in the genome-level reconstruction of the ethanol metabolism network were studied by utilizing pathway databases such as BRaunschweig ENzyme DAtabase and Kyoto Encyclopedia of Genes and Genomes.

2.6 Transcript and metabolic studies in *Z. mobilis*

Transcript and metabolic level profiles for *Z. mobilis* under aerobic and anaerobic fermentation conditions were studied by Yang *et al.,* (2009). Studies carried out using Microarray and various chromatographic techniques like HPLC, GC-MS and GC indicated that *Z. mobilis* showed 1.7 % of the ethanol under aerobic

conditions compared to anaerobic conditions. The transcripts of the majority of the enzymes in the ethanol production such as *glk, zwf, pgl, pgk, eno, pdc* were found to be greater under anaerobic fermentation conditions. Under aerobic conditions, acetaldehyde, acetate, lactate and acetoin production was greater thus leading to much lesser ethanol production. Expression of the *pdc* and *adhB* genes concerned with *Z. mobilis* ethanol production was improved at least double in anaerobic fermentation and was constant with fermentation product and growth facts. RNAGENiE interface developed by Carter *et al.,* (2001) can be used for studying the functional transcripts coding for ethanologenic enzymes. This interface combines both neural network (NN) and support vector machine (SVM). This approach has 80-90 % accuracy. By using this interface, ethanologenic enzyme transcripts can be studied. Parkinson *et al.,* (2009) developed ArrayExpress. It is comprised of three constituents: the ArrayExpress Repository—a unrestricted collection of experimental data like that of ethanologenic *Z. mobilis*, the ArrayExpress Warehouse—a catalogue of gene expression profiles and biological data that includes *Z. mobilis* transcriptomics data and the ArrayExpress Atlas—a recent précis database of transcriptomics data of organisms like *Z. mobilis*. In recent times, especially sequencing and transcriptomics data of organisms like ethanologenic *Z. mobilis* are included. By using this interface, one can study all aspects of ethanol production process in *Z. mobilis*.

Medina *et al.,* (2010) developed a integrative web interface that includes a comprehensive group of processes for the examination of gene expression data like that of ethanol production. Each and every one of the genomic data examination services are included and associated to numerous alternatives for the purposeful interpretation of the experiments like that of ethanol production. Diverse methods can be used to appreciate the functional base of the experiments like ethanol production.

Numerous resources like Gene Ontology, Kyoto Encyclopedia of Genes and Genomes and STRING can be used for studying ethanologenic pathways.

Barrett *et al.,* (2008) reported the the biggest open repository, The Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI). It is meant for gene expression data like that for ethanol-production mediated changes. As well, GEO has erstwhile groups of functional genomic data like that of *Z. mobilis*. These data (microarrays and next-generation sequencing) are deposited by the researchers involved in the genome sequencing of *Z. mobilis*. The database has a lithe system that can study completely annotated, unprocessed and processed data like that of *Z. mobilis* genome sequencing projects. It has various tools that can be used to study the gene-expression data during ethanol production by *Z. mobilis*.

Recently, Widiastuti *et al.,* (2011) reconstructed and later validated the genome-level metabolism network of *Z. mobilis*. The major findings of this study were 1) the Pentose-Phosphate pathway and tricarboxylic pathways lacked some enzymes. The genes coding for phosphogluconate dehydrogenase, transaldolase, 2-oxoglutarate dehydrogenase complex and malate dehydrogenase were absent in *Z. mobilis*. This would be helpful for competent and homofermentative ethanol production; 2) *pdc* and *adh* genes were found to be synchronized in the ethanol production and amino acid synthesis; 3) On the basis of the model calculations and experimental interpretation, ethanol yield on xylose was lesser when compared to glucose , even as there is no variation between xylose and glucose consumptions in the biomass yield; 4) ethanol production was two-fold in *Z. mobilis* than *S. cerevisiae* and *E. coli*; 5) Among the 102 vital reactions for production of ethanol, 35 reactions are partly united and 67 are completely/metabolically attached.

Recently, Bochner *et al.,* (2010) reported for the first time the Phenotype Microarray profiles of *Z. mobilis*. The results of this study concluded that *Z. mobilis* showed differences from the majority of bacteria and was similar to yeast in the ethanol production, restricted utilization of carbon supply, and elevated sugar tolerance other than that its method of producing a redox potential.

2.7 *in silico* and systems biology studies in *Z. mobilis*

Lee *et al.,* (2010) demonstrated that utmost *in silico* yields of ethanol in the *Z. mobilis* metabolic model was 1.85 mol ethanol/mol glucose. The observations of the model concluded that the capability for ethanol manufacture reduced when pyruvate decarboxylase was deleted from the metabolic simulation. Further, the simulations were carried out by the researchers to examine the maximum *in silico* yield of ethanol for carbon sources like glucose, xylose, arabinose. Glucose showed a elevated highest *in silico* yield of ethanol than xylose and arabinose. The calculated growth and ethanol manufacture rate were found to reduce steadily as the flux rate of NADH oxidase improved. Highest *in silico* yields of ethanol in the *Z. mobilis* metabolic model was 2 mol ethanol/ mol glucose. Alcohol dehydrogenase was the lone enzyme which is essential for the ethanol production not considering of the carbon source.

More recently, Shrestha *et al.,* (2010) generated the putative three-dimensional structure of Pyruvate decarboxylase (PDC) dimer, a key ethanologenic enzyme of *Z. mobilis*. A comparison was made in this study for the structures of the PDC with respect to ethanologenic enzyme-substrate interactions that would be related to the diverse biochemical features related to ethanol production. PDC is a major enzyme in homoethanol fermentation process. The models gave information necessary for upgrading of PDC enzyme for industrial manufacture of ethanol.

Recently, Yang *et al.,* (2010) used systems biology approach to characterize a *Z. mobilis* mutant showing sodium acetate tolerance that has prospective significance in ethanol production. The genomic modifications linked with sodium acetate tolerance and ethanol production were studied by the researchers using microarray relative genome sequencing and pyrosequencing.

More recently, Moon *et al.,* (2011) studied structural features of Iron-dependent alcohol dehydrogenase 2 (ZMADH2) enzyme from *Z. mobilis* ZM4 in the presence/absence of NAD^+ Cofactor. This enzyme has the capability of shifting the equilibrium towards the production of ethanol. The catalytic iron attaches in close proximity to the nicotinamide ring of NAD+, that confines and establishes the ethanol to the active site of the enzyme. Researchers studied the structures of the ZMADH2 with and without NAD^+. Later, they modelled ethanol in the active site and concluded that there is a characteristic metal-dependent enzymatic system. The activity of the ZMADHs for ethanol production is extremely better than that for ethanol oxidation. In contrast, the oxidation rate of ethanol by ZMADH2 is significantly enhanced in aerobic environment, consequently supplying the NADH necessary for the respiratory chain. For the duration of the oxidation of ethanol in aerobic system, NAD^+ attaches to the active site of ZMADH2. Thus, this study give in-sights to the importance of alcohol dehydrogenase enzyme in ethanol production in *Z. mobilis*.

2.8 Bioinformatics approach in Post-genomic studies

2.8.1 Protein sequence and structure databases

Protein Sequence Databases such as GenBank (Benson *et al.,* 2002), Swiss-prot (Bairoch and Apweiler, 2000), TrEMBL (O'Donovan *et al.,* 1999), Protein Identification Resource (Barker *et al.,* 1999), nr databases could be of usual resources for computational retrieval of the sequences. GenBank, a national institute of health genetic sequence database, an annotated compilation of the entire freely accessible DNA sequences. The Swiss-Prot is part of the Swiss Institute of Bioinformatics in charge of research and development of protein databases. TrEMBL, an *in silico*-annotated complement to SWISS-PROT. Protein Identification Resource provides the scientific community with an highly efficient on-line computational system designed for the identification and analysis of protein and their coding sequences. The nr database is the largest database of nucleotides available in NCBI BLAST. Protein Structure Databases such as PDB (Westbrook *et al.,* 2000), DALI (Holm and Sander, 1996) could help to understand the various structural features of the enzymes of interest in *Z. mobilis*. PDB contains information of the experimentally-determined structures of proteins. DALI is a server for comparing the 3D-Protein structures. This would helpful to understand the features such as active-site and functional domains that might be lacking in the non-functional enzymes.

2.8.2 Protein family databases

Protein Family Databases such as Pfam (Bateman *et al.,* 1999, 2002), PRODOM (Corpet *et al.,* 2000), SCOP database (Murzin *et al.,* 1995), CATH superfamily (Orengo *et al.,* 1998), ENZYME (Bairoch, 1993) could be used to study the function aspects of the putative enzymes of *Z. mobilis*. Pfam is a database of large

collection of protein families, represented by multiple sequence alignments and hidden Markov models. ProDom is used for analysis of multiple sequence alignments of large numbers of protein sequences. SCOP database is a large classification of structural domains of the proteins on the basis of amino acid sequence similarity and 3-D structures. CATH is a manually curated protein domain structure classification. ENZYME is an information repository in relation to the enzyme nomenclature.

2.8.3 Similarity search algorithms and programs

Smith–Waterman algorithm (Smith and Waterman, 1982), BLAST program (Altschul *et al.,* 1990), FASTA program (Pearson, 1990) and PSI-BLAST (Altschul *et al.,* 1997) could be used to know the homologous partners of the enzymes of interest in *Z. mobilis*, there by would help to understand the similarity and identity of the previously unstudied enzymes prior to the pre-genomic era. Smith–Waterman is an algorithm used for determining regions between two protein sequences. BLAST compares protein sequences to database and finds local similarity regions. FASTA is a program that provides sequence similarity searching against protein databases.

2.8.4 Functional motif search compendium and servers

Functional motifs of the previously unstudied enzymes could be studied using PRINTS (Attwood *et al.,* 2002), PROSITE (Bucher and Bairoch, 1994; Falquet *et al.,* 2002), and BLOCKS (Henikoff *et al.,* 1999, 2000). PRINTS is a compendium of protein conserved motifs. PROSITE comprises of documentation entries explaining protein domains, families and functional sites as well as their related patterns and profiles to recognize them. BLOCKS is a server used to annotate protein sequences.

2.8.5 Protein family and scheme databases

Protein Family and scheme databases such as Pfam (Bateman *et al.,* 2002), PROSITE (Falquet *et al.,* 2002), PRINTS (Attwood *et al.,* 2002), and BLOCKS (Henikoff *et al.,* 1999, 2000) could be accessed to know the possible active-sites, motifs, patterns that are present/absent in the enzymes in *Z. mobilis*. This could be useful understand the characteristics of the enzymes.

2.8.6 Enzyme databases

The SCOP (Murzin *et al.,* 1995), CATH (Orengo *et al.,* 1998) and FSSP (Holm and Sander, 1996) databases could be used to study the structure and function relationship between the previously characterized enzymes and the uncharacterized enzymes in *Z. mobilis*. FSSP is a protein structure database of families of structurally similar proteins. The ENZYME database (Bairoch, 1993) and enzyme active site database PROCAT (Wallace *et al.,* 1996) could be used to study the active site residues responsible for the functioning of the enzymes in *Z. mobilis*. PROCAT is a database of 3D enzyme active site templates.

2.8.7 Multiple sequence alignment algorithms, programs and databases

Mutation probability (Gonnet *et al.,* 1992), PAM (Gonnet *et al.,*1992), BLOSUM (Henikoff and Henikoff, 1992) matrices, Smith–Waterman algorithm (Smith and Waterman, 1982), Needleman–Wunsch algorithm (Needleman–Wunsch, 1970), BLAST (Altschul *et al.,* 1990) tools including BLASTp, BLASTx, tBLASTn, megaBLAST, and PSI-BLAST, FASTA (Pearson and Lipman, 1988; Pearson, 1990) databases and computational programs/servers for multiple sequence alignments like Clustal W (Higgins *et al.,* 1994), PILEUP (Genetics Computer Group, 1994), MALI

(Vingron and Argos, 1989), and PIMA (Smith and Smith, 1992) can be used for assess the possible functional role of uncharacterized enzymes and their homologous partners in the available databases. BLOSUM is a substitution matrix used for protein sequence alignment. The Needleman-Wunsch algorithm is used to perform global alignment of protein sequences. ClustalW2 is a general purpose multiple sequence alignment program for proteins. PileUp generates a multiple sequence alignment from a group of interrelated protein sequences using progressive, pairwise alignments. PIMA is a tool that allows to search library of functional diagnostic profiles with a protein sequence.

2.8.8 Functional domain search databases

PRODOM (Corpet *et al.,* 2000), DALI (Holm and Sander, 1996), SCOP (Murzin *et al.,* 1995), and DomainParser (Xu *et al.,* 2000) databases could be used to study the functional domains of the enzymes in *Z. mobilis*. DomainParser is a algorithm for the decomposition of a multi-domain protein into individual structural domains. SITE database (Zhang *et al.,* 1999) is a structure based function prediction database that would be helpful to study the uncharacterized enzymes by their structure prediction.

2.8.9 Physico-chemical, bio-chemical and bio-physical prediction tools and servers

The protein sequence of the putative enzymes could be analysed using Protparam, Protscale (Gasteiger *et al.,* 2005) and SAPS (Brendel *et al.,* 1992). ProtParam calculates physicochemical biophysical and biochemical properties like instability index, molecular weight, grand average of hydropathicity (GRAVY), theoretical isoelectric point, total number of negatively and positively charged amino-

acid residues, aliphatic index, estimated half-life (*Escherichia coli*, *in vivo*, in hours) and extinction coefficient. ProtScale predicts the profile of the protein on the basis of its amino acid composition. SAPS predicts major features of protein sequence like charge-clusters, compositional domains, hydrophobic segments, repetitive structures and multiplets. Meta servers like MetaPP (Rost *et al.,* 2004), ProSAL and SCRATCH could be used for predicting structural and functional features of the putative enzymes of *Z. mobilis*.

2.8.10 Sub-cellular localization and functional categorization tools

The sub-cellular localization and the functional categorization of enzymes could be predicted using PRED-CLASS (Pasquier *et al.,* 2001), ProtCompB, ProtFun 2.2 (Jensen *et al.,* 2003), PSORT (Nakai and Horton, 1999), and SVMProt (Cai *et al.,* 2003). PRED-CLASS is a system of cascading neural networks that classifies any protein, given its amino acid sequence alone, into membrane, globular, fibrous or mixed (fibrous and globular) protein class. ProtCompB V-3 uses a combination of several methods like linear discriminant function-based predictions, direct comparison with homologous proteins of known localization, prediction of sequences of functional peptides to classify the sub-cellular localization of proteins. ProtFun generates *ab initio* predictions of function of a protein from its sequence. PSORT examines the input sequence by applying the accumulated regulations for a variety of sequences of recognized protein signals and reports the opportunity for the protein input sequence to be. SVMProt classification system is trained from representative proteins of a number of functional families and Pfam curated protein families.

2.8.11 Signal peptide and cleavage site prediction tools

Signal peptide and cleavage site of the enzymes could be predicted using iPSORT (Bannai *et al.,* 2002), Phobius (Kall *et al.,* 2004), PrediSi (Hiller *et al.,* 2004), PSORT (Nakai and Horton, 1999), sigcleave (von Heijne, 1986), SignaIP (Bendsten *et al.,* 2004), SIG-Pred and SOSUI signal (Gomi *et al.,* 2004). iPSORT is N-terminal signals subcellular localization site predictor. Phobius is a collective transmembrane topology and signal peptide analyser. Predisi is a software tool for predicting signal peptide sequences in real time with a high accuracy and is based on a position weight matrix approach by a frequency correction that takes the amino acid bias present in proteins in consideration. Sigcleave calculates the position of cleavage flanked by a signal sequence and the exported protein. SIG-Pred predicts cleavage sites of signal peptides on the weight matrices basis. SignalP 3.0. integrates a forecast of cleavage sites and a signal peptide/non-signal peptides on the basis of a amalgamation of a number of artificial neural networks and hidden Markov models. SOSUIsignal calculates 3-domain structure of signal peptide.

2.9 Antibiotic Resistance in non-pathogenic bacteria

Antibiotic resistance is observed in both pathogenic and non-pathogenic microorganisms. Non-pathogenic bacteria that are resistant to antibiotics may then become a source for the dissemination of antibiotic resistance genes to any newly invading harmful bacteria. It is known that human pathogens are predisposed to antibiotics. Even before the use of these drugs for the treatment of infections, antibiotic resistance determinants appeared due to horizontal gene transfer across the non-pathogenic microorganisms. Even human commensals are reported to have antibiotic resistance (Sibold *et al.,* 1994).

The extensive use of antibiotics is reported to be linked with the development of antibiotic resistance in pathogenic and non-pathogenic strains of *E. coli* (Livermore, 1997; Olukoya and Olasupo, 1997). There are reports of antibiotic-resistant non-pathogenic *E. coli* contributing to the spread of antibiotic-resistance determinants through meat, milk and gut (Kruse and Sorum, 1994). Therefore, there is potential risk of transfer of antibiotic-resistance transfer from non-pathogenic bacteria to pathogenic bacteria (Phillips *et al.,* 2004; Wassenaar, 2005). Environment plays an important role in the transfer of genetic pool of antibiotic-resistance between pathogenic and non-pathogenic bacteria (Furuya and Lowy, 2006).

Antibiotic resistance in pathogens is contributed by antibiotic resistance genes like beta-lactamases and penicillin-binding proteins. Antibiotic-resistance genes in non-pathogenic bacteria are not studied extensively as characterized in pathogenic bacteria. Genome-sequencing projects have opened the new approaches for the identification of antibiotic-resistance in bacteria (Fisher *et al.,* 2005). Until now there have been few investigations on the genetic and biochemical basis of the development and spread of antibiotic-resistance amongst the non-pathogenic bacteria.

Antibiotic resistance appears through any one of the four general mechanisms: modification of the drug target site, efflux of the antibiotic from the cell, reduced influx of antibiotics and destruction or modification of the antibiotics. Destruction or modification of the antibiotic is mediated by hydrolysis, group transfer and redox mechanisms. β-lactamase catalyzes the hydrolysis of beta-lactam antibiotics like penicillins leading to antibiotic resistance in bacteria. β-lactamases are categorized into four classes on the basis of their isoelectric point, three-dimensional structure, and substrate specificity. They are class A β-lactamases (penicillinases), class B β-lactamases (metallo β-lactamases), class C β-lactamases (AmpC-type β-lactamases)

and class D β-lactamases (oxacillanases) (Bush *et al.,* 1995). A number of sequenced genomes have β-lactamase gene sequences that have not been functionally confirmed (Audic *et al.,* 2007; Nampoothiri *et al.,* 2008). In addition to chromosomal-encoded β-lactamases, plasmid-driven β-lactamase resistance has been reported (Alexander and Jollick, 1977; Bradford *et al.,* 1997). β-lactamase genes are extended across bacterial genomes by means of horizontal-gene transfer and/or by mobile transposon-like elements called integrons (Lauretti *et al.,* 1999).

There are several genes coding for β-lactamases (BLA), β-lactamase domain-containing proteins (BDP) and penicillin-binding proteins (PBP) that could contribute to the ß-lactam antibiotic resistance in non-pathogenic bacteria. Such studies are of significant value for understanding the evolution of resistance genes and plasmids in the non-pathogenic bacteria that are considered to be the progenitors of those in clinical isolates. Several non-pathogenic bacteria such as *Branhamella catarrhalis*, *Lysobacter enzymogenes* exhibit the ß-lactam resistance (Ninane *et al.,* 1978; Tigerstrom and Boras, 1990). The antibiotic resistance genes are located in the chromosome of both pathogens and non-pathogens and are responsible for ß-lactam resistance. Chromosomally located antibiotic resistance genes are well regulated compared to plasmid-mediated resistance (Wright, 2007).

Post-genomic studies have enabled to initiate the projects such as ROAR (Reservoirs of Antibiotic Resistance) network to study the spread of resistance-determinants by non-pathogenic bacteria (Levy and Salyers, 2002).

2.10 Bacterial cellulose synthesis

Bacterial cellulose production gained importance because of the pioneering work carried out in the bacterium *Gluconacetobacter xylinus*. There have been reports

of cloning of the cellulose synthase operon and production of cellulose in *G. xylinus* (Ross *et al.,* 1987; Wong *et al.,* 1990). There have been reports of cellulose production in *Agrobacterium tumefaciens*, *Escherichia coli, Klebsiella pneumoniae, Rhizobium leguminosarum* bv. *trifolii*, *Sarcina ventriculi* and *Salmonella* spp. (Ausmees *et al.,* 1999; Deinema and Zevenhuizen, 1971; Matthysse *et al.,* 1995; Napoli *et al.,* 1975; Nobles *et al.,* 2001; Ross *et al.,* 1991; Zogaj *et al.,* 2001).

2.10.1 Biochemical detection of bacterial cellulose synthesis

Calcofluor and Congo Red binding properties of bacterial cellulose have been reported earlier (Ausmees *et al.,* 1999; Deinema and Zevenhuizen, 1971; Matthysse *et al.,* 1995). There have been reports of detection of bacterial cellulose aggregrates by the use of cellulase enzymes (Deinema and Zevenhuizen, 1971; Matthysse *et al.,* 1981; Zogaj *et al.,* 2001).

2.10.2 Cellulose synthase operon genes

Bacterial cellulose synthesis machinery consists of structural genes, of which cellulose synthase is a vital one. In some bacterium such as *G. xylinus* strains, more than one cellulose synthase operon is present (Saxena and Brown, 1995). The two essential genes of bacterial cellulose synthase operon are cellulose synthase (*bcsA*) and c-di-GMP binding protein (*bcsB*) that have been reported to be fused in *G. xylinus* (Kimura *et al.,* 2001). The other essential gene cellulose (*bcsZ*) has been reported in *A. tumefaciens*, *G. xylinus*, and *R. leguminosarum* bv. *trifolii* (Standal *et al.,* 1994).

2.10.3 Cellulose synthase catalytic subunit

Cellulose synthase catalytic subunit (BcsA) is the first gene of the cellulose synthase operon and it has the conserved motifs D, D, D35Q(R, Q) XRW, PVDPYE,

HAKAGN (L, I) N, QTP, FFCGS and RFLPL. It is located in cytoplasmic membrane (Lin *et al.,* 1990).

2.10.4 Cellulose synthase regulator protein

Cellulose synthase regulator protein (BcsB) has been reported in *A. tumefaciens* and *R. leguminosarum* bv. *Trifolii*. It has as proline/alanine enriched domain at the N-terminus and a transmembrane domain at the C-terminus. Cellulose synthase catalytic subunit (BcsA) and Cellulose synthase regulator protein (BcsB) have been reported in *G. xylinus* to be present in the cytoplasmic membrane (Kimura *et al.,* 2001).

2.11 Beta-galactosidase

Beta-galactosidases (EC 3.2.1.23) are present in a wide variety of organisms including plants, animals and microorganisms, and are known to catalyze both hydrolytic and transglycosylation reactions. The thermostable beta-galactosidase from *Aspergillus niger*, *Bacillus stearothermophilus*, *Pyrococcus woesei*, *Thermus sp* are relatively stable from 35–80 °C. Cold-active and cold-adapted beta-galactosidase from psychrophilic microorganisms like *Arthrobacter psychrolactophilus*, *Pseudoalteromonas haloplanktis* are in general quite efficient in compensating the reduction of reaction rates by induced low temperatures through improvement of the turnover number (k_{cat}) or of the physiological efficiency (k_{cat}/K_m) and are relatively stable from 0–25 °C. Whey has been utilised for the production of ethanol, exopolysaccharide and single cell protein by employing beta-galactosidase from microorganisms like *Aspergillus oryzae*, *Kluveromyces lactis*, *Kluveromyces marxianus*, *Lactobacillus delbrueckii subsp. bulgaricus, Saccharomyces cerevisiae*. Transglycosylation and transgalactosylation properties of beta-galactosidase from *A.*

niger, *Bacillus megaterium*, *Beijerinckia indica, Bifidobacterium infantis*, *Bifidobacterium longum*, *Enterobacter cloacae*, *Geobacillus stearothermophilus*, *K. marxianus*, *Lactobacillus sp*, *Lactobacillus reuteri*, *Penicillium expansum* have been utilized for production of glucose, galactose, heteropolysaccharide, galacto-oligosaccharides. Beta-galactosidase based medical and industrial applications include cleavage of blood group A and B glycotopes, biosensor for specific lactose determination in milk and disease diagnosis, treatment of lactose malsorption, production of lactose hydrolysed milk. Immobilization of beta-galactosidase on anion exchange resin, cellulose-gelatin carrier system, DEAE agarose, glyoxyl / epoxy / BrCN groups, glutaraldehyde, polyelectrolyte surfaces, silicon surface, sepabeads-epoxy supports partially modified with boronate, iminodiacetic, metal chelates, and ethylenediamine improves its stability and reusage. Co-production of beta-galactosidase with other enzymes like amylase, beta-glucosidase has been demonstrated in *G. stearothermophilus*.

2.11.1 Cold-active and thermostable beta-galactosidases

The production of cold-stable β-D-galactosidases and microorganisms that resourcefully ferment lactose is of high biotechnological interest, particularly for removal of lactose in milk and dairy products at low temperatures, cheese whey bioremediation and bio-ethanol production **(Table: 1)**. Recently, a gene encoding β-D-galactosidase was isolated from the genomic library of Antarctic bacterium *Arthrobacter* sp. 32c. Although, the highest activity of this purified enzyme was found at 50 °C, 60 % of the highest activity of this enzyme was determined at 25 °C and 15 % of the highest activity was detected at 0 °C. The cold-stable properties of *Arthrobacter* sp. 32c β-D-galactosidase could be useful for commercial, industrial

conversion of lactose into galactose and glucose in milk products and could be an exciting substitute for the production of bioethanol from lactose-based feedstock (Hildebrandt *et al.,* 2009).

Nakagawa *et al.,* (2007) have overexpressed a cold-stable beta-galactosidase from *A. psychrolactophilus* strain F2 in *E. coli* using the cold expression system. The purified recombinant enzyme, rBglAp exhibited similar enzymatic properties to the native enzyme, it had high activity at 0 ºC, its most favourable temperature was 10 ºC, and it was achievable to swiftly inactivate the rBglAp at 50 ºC in 5 min. rBglAp was capable to hydrolyze both ONPG and lactose with K_m values of 2.7 and 42.1 mM, respectively, at 10 ºC. rBglAp is a cold-active and extremely heat labile enzyme and has major possible application to the food industry (Nakagawa *et al.,* 2007). Hoyoux *et al.,* (2001) have purified beta-galactosidase from the Antarctic Gram-negative bacterium *P. haloplanktis* TAE 79. The purified enzyme is characterized for optimum activity at low temperature. Heat-induced unfolding examined by intrinsic fluorescence spectroscopy shown lower melting point values for both *P. haloplanktis* wild-type and recombinant beta-galactosidase compared to the mesophilic enzymes. Assays of hydrolysis of lactose in milk showed that *P. haloplanktis* beta-galactosidase is comparatively better than current commercial beta-galactosidase, suggesting that the cold-active beta-galactosidase could be used in lactose hydrolysis in dairy products processed in refrigerated plants (Hoyoux *et al.,* 2001).

Chen *et al.,* (2008) have cloned and expressed a thermostable beta-galactosidase gene *bga*B from *B. stearothermophilus* in *B. subtilis* WB600. The optimum temperature for this beta-galactosidase activity was 70 °C. Kinetics of

thermal inactivation and half-life times for this thermostable enzyme at 65 and 70 °C were 50 and 9 h, respectively. This enzyme exhibited a high level of transgalactosylation activity in hydrolysis of milk lactose. The results suggest that this recombinant thermostable enzyme may be suitable for the processes such as hydrolysis of lactose and production of galacto-oligosaccharides (Chen *et al.,* 2008).

Lauro *et al.,* (2008) have cloned, expressed, purified and characterized a beta-galactosidase (Aaβ-gal) from thermoacidophilic bacterium *Alicyclobacillus acidocaldarius*. The recombinant Aaβ-gal is optimally active and stable at 65 °C (Lauro *et al.,* 2008).

TABLE 1. Cold-active, thermostable beta-galactosidases and their properties

Beta-galactosidase producers	Thermostability (°C)	Reference
Arthrobacter sp. 32c	0-60	(Hildebrandt *et al.,* 2009)
A. psychrolactophilus F2	0-50	(Nakagawa *et al.,* 2007)
P. haloplanktis TAE 79	0-40	(Hoyoux *et al.,* 2001)
B. stearothermophilus	45-80	(Chen *et al.,* 2008)
A. acidocaldarius	40-90	(Lauro *et al.,* 2008)
T. ethanolicus	40-80	(Volkov *et al.,* 2005)

Volkov *et al.,* (2005) have determined the nucleotide sequence of a 4936-bp genomic DNA fragment from the thermophilic bacterium *Thermoanaerobacter ethanolicus*. The fragment contained three open reading frames (ORFs). One of the ORF corresponded to the *Lac* A gene for a thermostable ß-galactosidase. Native recombinant LacA showed the highest activity at 75–80 °C. Immobilized on aldehyde silochrome, LacA was even more thermostable and retained its high activity (Volkov *et al.,* 2005). Thus, the cold-active and thermostable beta-galactosidases play a vital role in hydrolysis of lactose, bioethanol and galacto-oligosaccharide production because of their thermostable property.

2.11.2 Immobilization of beta-galactosidase

Immobilization has shown to improve beta-galactosidase's stability and reusage **(Table: 2)**.The immobilization of the ß-galactosidase of *Thermus* sp. T2 was performed using ionic adsorption onto two different supports: a new anionic exchanger resin, based on the coating of Sepabeads internal surfaces with polyethylenimine (PEI) polymers, and conventional DEAE-agarose. Immobilization proceeded unusually rapid in both cases, but the adsorption strength was much greater in the case of PEI-Sepabeads than in DEAE-supports at both pH 5 and 7. Interestingly, the PEI-derivatives remained wholly active at pH 5 and 7 after several weeks of incubation at 50 ºC, conditions that permit the lactose hydrolysis in milk (Pessela *et al.,* 2003).

Pessela *et al.,* (2004) have used a battery of new heterofunctional epoxy supports to immobilize beta-galactosidase. The capability of a standard Sepabeads-epoxy support to immobilize beta-galactosidase from *Thermus* sp. strain T2 can be equal with other Sepabeads-epoxy supports partially modified with boronate, iminodiacetic, metal chelates, and ethylenediamine. Immobilization yields depended on the support, ranging from 95 % using Sepabeadsepoxy-chelate, Sepabeads-epoxy-amino, or Sepabeads-epoxy-boronic to 5 % using Sepabeads-epoxy-IDA. In count, rate of immobilization differed when using different supports. Amazingly, the immobilized beta-galactosidase derivatives showed outstandingly improved but different stabilities after favoring multipoint covalent attachment by long-term alkaline incubation. The enzyme immobilized on Sepabeadsepoxy-boronic was found to be the steadiest. The crosslinking with aldehyde-dextran allowed the stabilization of the quaternary structure of the enzyme. The optimal derivative was extremely

active in lactose hydrolysis even at 70 °C (over 1000 IU/g), maintaining its activity after extended incubation times under these conditions and with no risk of product contamination with enzyme subunits (Pessela *et al.,* 2004).

An immobilized preparation of the beta-galactosidase of *E. coli* using diverse supports and immobilization strategies (bearing glyoxyl, epoxy, BrCN groups or by glutaraldehyde crosslinking on matrices containing primary amino groups) have been obtained. In each and every one cases, the immobilization yield was 100 % with activity recoveries between 50 % and 100 % (using *o*-NPG as substrate). The enzyme immobilized on Eupergit 250 L exhibited an increase in the enzyme activity by a factor of 2. Synthetic activity / hydrolytic activity ratio (*Vs*/*Vh*) was lower than 0.1 with the enzyme immobilized on BrCN at 4 ºC and pH 7, while the soluble enzyme gave a ratio of 0.46 and the immobilized enzyme on Eupergit 250 L gave a ratio of 0.8. Eupergit C immobilized enzyme and soluble enzyme showed enhanced *Vs*/*Vh* ratio when temperature was decreased (Pessela *et al.,* 2007).

Immobilization of ß-galactosidase-producing permeabilized dead cells of *K. lactis* ATCC 8583 into gelatin using glutaraldehyde as cross-linker has been performed. Thirty percent activity was obtained by immobilized cells relative to free disrupted cells (Numano˘glu and Sungur, 2004).

Hamlin *et al.,* (2007) have constructed an electrostatic self-assembly (ESA) for the immobilization of beta-galactosidase onto polyelectrolyte multilayer assemblies of the polyanion poly [1-[4-(3-carboxy-4 hydroxyphenylazo)

benzenesulfonamido]-1, 2-ethanediyl, sodium salt] (PAZO) and the polycation poly (ethylenimine) (PEI) (Hamlin *et al.,* 2007).

TABLE 2. Immobilization agents of beta-galactosidases

Beta-galactosidase producers	Immobilization agents	Reference
Thermus sp. T2	Sepabeads-epoxy supports, Sepabeads-epoxy-chelate, Sepabeads-epoxy-amino, Sepabeads-epoxy-boronic, Sepabeads-epoxy-IDA, PEI-sepabeads	(Pessela *et al.,* 2003, Pessela *et al.,* 2004)
E. coli	Glyoxyl, epoxy, BrCN groups	(Pessela *et al.,* 2007)
K. lactis ATCC 8583	Gelatine	(Numanoˇglu and Sungur, 2004)
A. oryzae	Silica	(Mariotti *et al.,* 2008)
Streptococcus thermophilus	Calcium alginate (CA), K-carrageenan, gellan-xanthan (GX)	(Goel *et al.,* 2006)
K. lactis , *A. oryzae*	Poly (vinylalcohol) hydrogel	(Grosová *et al.,* 2009)

Mariotti *et al.,* (2008) have considered the hydrolysis of whey lactose by immobilized beta-galactosidase of *A. oryzae* on silica. The most excellent immobilization results were attained by using glutaraldehyde as support activator and enzyme stabilizer. The optimized enzyme concentration for immobilization was 15-20 mg g^{-1} of support (Mariotti *et al.,* 2008). The usage of calcium alginate (CA), K-carrageenan and gellan-xanthan (GX) gel beads for entrapment of cells of *Streptococcus thermophilus* containing ß-galactosidase enhanced the stability of enzyme at higher temperatures (>55 ºC) (Goel *et al.,* 2006). Grosová *et al.,* (2009) have immobilized ß-galactosidases of *K. lactis* and *A. oryzae* and yeasts in poly vinylalcohol hydrogel lens-shaped capsules. In the process of SSF with co-immobilized enzyme of *K. lactis* and *S. cerevisiae*, the galactose output increased from 3 g l^{-1} h^{-1} to 4.1 g l^{-1} h^{-1}, thus condensed the time of preparation of D-galactose

(Grosová *et al.,* 2009). Thus, immobilization has shown to improve the stability of beta-galactosidase and reduces the processing time in food and other industries.

2.11.3 Notable industrial applications of beta-galactosidase

Microbial beta-galactosidases have a prominent position in terms of their role in production of various industrially-relevant products like biosensor, lactose hydrolyzed milk, ethanol. Marrakchi *et al.,* (2008) have developed a biosensor associating two distinct enzymatic activities, that of the beta-galactosidase and that of the glucose oxidase, in order to apply it for the quantitative detection of lactose in commercial milk samples. To eliminate interferences with glucose, a differential mode of measurement was used in this biosensor (Marrakchi *et al.,* 2008). A putative beta-galactosidase from *Streptococcus mitis* with a choline-binding domain was recently identified. This peculiar property makes it useful for biotechnological applications (Campuzano *et al.,* 2009).

Panesar *et al.,* (2007) have carried out trialling to overcome the problem of enzyme extraction and poor permeability of cell membrane to lactose. Permeabilized *K. marxianus* NCIM 3465 cells were used for the production of lactose-hydrolyzed milk. The ethanol-permeabilized yeast cells gave 89 % hydrolysis of milk lactose under optimized conditions (Panesar *et al.,* 2007).

Domingues *et al.,* (2005) have investigated the constant production of extracellular heterologous beta-galactosidase and ethanol by a recombinant flocculating *S. cerevisiae*. Jointly with extracellular beta-galactosidase production, an ethanol productivity of 9 g l^{-1} h^{-1} was obtained for the bioreactor fed with 50 g l^{-1}

initial lactose concentration at 0.45 h^{-1} dilution rate. In adding together to beta-galactosidase and ethanol production, this system allowed for complete lactose metabolism (Domingues *et al.,* 2005).

In 2005, a kinetic analysis of alcoholic fermentation of lactose using strain NCYC869-A3/T1, a recombinant *S. cerevisiae* flocculent strain expressing both the LAC4 (coding for ß-galactosidase) and LAC12 (lactose permease) genes of *K. Lactis* was carried out. The lactose was wholly utilized in all the fermentations. The increase in ethanol production improved linearly when the initial lactose concentration was increased between 5 and 200 g l^{-1}. Ethanol productivity improved with increasing initial lactose concentration up to 150 gl^{-1} (1.23 g l^{-1} h^{-1}) (Guimaraes *et al.,* 2005).

Rodríguez *et al.,* (2006) have constructed and analyzed two hybrid proteins from the beta-galactosidase of *K. lactis*, intracellular, and its *A. niger* homologue that is extracellular. One of the hybrid proteins obtained has interesting properties for its biotechnological utilization that increases the yield of the protein released to the growth medium. Changes introduced in the construction, besides to improve secretion, conferred to the protein biochemical characteristics of biotechnological interest (Rodríguez *et al.,* 2006). Thus, beta-galactosidase plays a key role in food and other allied industries.

2.11.4 Galacto-oligosaccharides production by beta-galactosidase

In recent years, much investigation has been carried out in the field of pro- and prebiotics as functional foods. Galacto-oligosaccharides (GOS) are used as nondigestible, carbohydrate-based food ingredients in human and animal nutrition.

Much of the research is focussed upon microorganisms that produce beta-galactosidases with improved quality for production of galacto-oligosaccharides **(Table: 3)**. The synthesis of GOS with a high yield of 55 % from 275 g l^{-1} lactose at 50 °C for 12 h was performed using transglycosylating beta-galactosidase producing *E. cloacae*. The enzyme showed a extensive range of acceptor specificity for transglycosylation and catalyzed glycosyl transfer from ONPGal to various chemicals resulting in novel saccharide yields from 0.8% to 23.5 % (Lu *et al.,* 2009).

Wu *et al.,* (2006) have screened a mutant strain of *B. indica* L3 for the production of heteropolysaccharide-7 (PS-7). The highest amount of PS-7 formed by the mutant was 2.88 g/L with a viscosity of 4530 cP in lactose-based MSM medium. The PS-7 manufacture was enhanced by the addition of 4 g/L glucose into lactose-based MSM medium, reaching 5.52 g l^{-1} with a viscosity of 39531 cP. PS-7 of 6.18 g/L with a viscosity of 45772 cP was produced from the mutant grown in whey medium. The PS-7 production from the mutant reached 7.04 g l^{-1} when 4 g l^{-1} glucose was added to the whey medium (Wu *et al.,* 2006).

Li *et al.,* (2009) have cloned a novel gene encoding transglycosylating beta-galactosidase (BGase) from *P. expansum* F3 and subsequently expressed on the cell surface of *S. cerevisiae* EBY-100 by galactose induction. The BGase-anchored yeast could directly utilize lactose to produce GOS, as well as the by-products glucose and a small quantity of galactose. The glucose was consumed by the yeast, and the galactose was used for enzyme expression, thus to a great extent facilitating GOS synthesis. The GOS yield reached 43.64 % when the recombinant yeast was cultivated

in yeast nitrogen base-Casamino Acids medium containing 100 g/liter initial lactose at 25 °C for 5 days (Li *et al.,* 2009).

In 2007, the process by which GOS formed from lactose was optimized using beta-galactosidase from *Lactobacillus* sp. It proved to be beneficial to directly apply the crude cell-free enzyme extract for the conversion, since similar GOS yields and composition were obtained as when using the pure enzyme preparation, but expensive purification could be avoided (Splechtna *et al.,* 2007). Hsu *et al.,* (2007) have studied the production of GOSs by transgalactosylation using ß-galactosidase of *B. longum* BCRC 15708. Two types of GOSs, tri- and tetrasaccharides, were formed after ß-galactosidase action on 40 % lactose. Trisaccharides were the major type of GOS formed. In general, an increase of the initial lactose concentration in the reaction mixture resulted in a higher GOS production. A highest yield of 32.5 % (w/w) GOSs could be achieved from 40 % lactose solution at 45 °C, pH 6.8, when the lactose conversion was 59.4 %. The corresponding productivity of GOSs was 13.0 g l^{-1} h^{-1} (Hsu *et al.,* 2007).

A mutagenesis advance was applied to the beta-galactosidase (BgaB) of *G. stearothermophilus* KVE39 to improve its enzymatic transglycosylation of lactose into oligosaccharides. A straightforward screening strategy based on the reduction of the hydrolysis of a potential transglycosylation product (lactosucrose), provided mutant enzymes possessing enhanced synthetic properties for the autocondensation product from nitrophenyl-galactoside and GOS from lactose. Alter of one arginine residue to lysine (R109K) augmented the oligosaccharide yield compared to that of the wild-type BgaB. Consequently, Site-saturation mutagenesis at this position

demonstrated that valine and tryptophan further enlarged the transglycosylation performance of BgaB. During the transglycosylation reaction with lactose of the evolved ß-galactosidases, a key trisaccharide ß-D-galactopyranosyl-(1→3)-ß-D-galactopyranosyl-(1→4)-D-glucopyranoside (3'-galactosyl-lactose)] was formed (Placier *et al.,* 2009).

Spletchna *et al.,* (2007) have investigated GOS formation from lactose in discontinuous and constant modes of conversion using beta-galactosidase (ß-gal) from *L. reuteri*. In the continuous stirred tank reactor, ß-gal from *L. reuteri* showed up to 2-fold higher specificity toward the formation of ß-(1→6)-linked GOS, with ß-D-Galp-(1→6)-D-Glc and ß-D-Galp-(1→6)-D-Gal being the main GOS components formed under these conditions (Spletchna *et al.,* 2007).

Cheng *et al.,* (2006) have used *Bacillus sp* for the production of low-content GOS from lactose that resulted in the highest yield of trisaccharides and tetrasaccharides. GOS production was improved by mixing beta-galactosidase with glucose oxidase. The low content GOS syrups, produced by beta-galactosidase was subjected to the fermentation by *K. marxianus*, whereby glucose, galactose, lactose and other disaccharides were at a low level, resulting in up to 97 % and 98 % on a dry weight basis of high-content GOS with the yields of 31 % and 32 %, respectively (Cheng *et al.,* 2006).

In 2008, a procedure was proposed for producing non-monosaccharide and high-purity GOS from lactose by *P. expansum* F3 β-galactosidase immobilized in calcium alginate. A purity of 28.7 % (w/w) GOS was obtained from 380 g l^{-1} lactose

solution at pH 5.4 and 50 °C. The immobilized enzyme was used for repeated GOS synthesis and showed good working stability. Digestible sugars in the GOS were dwindling after fermentation with *S. cerevisiae* L_1 or *K. lactis* L_3 entrapped in the calcium alginate. Purity greater than 37 % with yields greater than 27 % of non-monosaccharide GOS were obtained by *S. cerevisiae* L_1 for 19 batches and purity greater than 97 % with a yield greater than 20 % of high-purity GOS was produced using *K. lactis* L_3 for two batches (Li *et al.,* 2008).

Layer and Fischer (2006) have performed *in vitro* glycosylation of peptides and proteins by trans-galactosylation of protected serine and threonine by β-D-galactosidase. The trans-mono-galactosylation of serine with a surplus of lactose produced 28 % of *N*-*tert*-butoxycarbonyl-1-*O*-β-D-galactopyranosyl-L-serinemethyl ester. The same transformational conditions, when applied to threonine, produced *N*-*tert*-butoxycarbonyl-1-*O*-β-D-galactopyranosyl-L-threonine-methylester in lower quantities. Mono-galactosylated serine and threonine are further galactosylated in the examined experimental setup to yield bi-galactosylated products also, especially at 50 °C with completely dissolved lactose (Layer and Fischer, 2006).

In 2009, oligosaccharides in bovine cheese whey permeate was characterized by a combination of nanoelectrospray Fourier transform ion cyclotron resonance mass spectrometry and matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry. In adding together to sialyllactose (the most abundant oligosaccharide in bovine colostrum), 14 other oligosaccharides were identified, half of which have the same composition of human

milk oligosaccharides. These oligosaccharides could potentially be used as additives in infant formula and products for the pharmaceutical industry (Barile *et al.,* .2009).

TABLE 3. Galactooligosaccharides production by beta-galactosidase

Beta-galactosidase producers	Galactooligosaccharides (GOS) and by-products	Reference
E. cloacae	GOS, glucose, galactose	(Lu *et al.,* 2009)
B. indica L3	Heteropolysaccharide-7	(Wu *et al.,* 2006)
P. expansum F3	GOS, glucose, galactose	(Li *et al.,* 2009, Li *et al.,* 2008)
Lactobacillus sp	ß-D-Galp-(1→6)-D-Glc, ß-D-Galp-(1→6)-D-Lac, ß-D-Galp-(1→6)-D-Gal, β-D-Galp-(1→3)-D-Lac, β -D-Galp-(1→3)-D-Gal	(Spletchna *et al.,* 2007)
B. longum BCRC 15708	tri-, tetrasaccharides, lactose, galactose, glucose	(Hsu *et al.,* 2007)
G. stearothermophilus KVE39	Lactosucrose, ß-D galactopyranosyl-(1→3)-ß-D-galactopyranosyl-(1→4)-D-glucopyranoside (3'-galactosyl-lactose)]	(Placier *et al.,* 2009)
L. reuteri	ß-D-Galp-(1→6)-D-Glc, ß-D-Galp-(1→6)-D-Gal, β -D-Galp-(1→3)-D-Gal β-D-Galp-(1→6)-D-Lac β-D-Galp-(1→3)-D-Lac	(Spletchna *et al.,* 2007)
L. bulgaricus	Sialyllactose, 14 other oligosaccharides	(Barile *et al.,* 2009)
L. delbrueckii subsp. bulgaricus	Galactose, lactic acid , acetic acid, ethanol	(Shene and Bravo, 2007)
B. infantis	GOS, lactose, monosaccharides	(Jung and Lee, 2008)
Lactobacillus plantarum	ß-D-Galp-(1→6)-D-Lac, ß-D-Galp-(1→6)-D-Glc	(Iqbal *et al.,* 2010)
Bacillus circulans	*N*-acetylactosamine, N-acetylglucosamine	(Kaftzik *et al.,* 2002)

Spletchna *et al.,* (2006) have formed prebiotic GOS from lactose using the β-D-galactosidases (β -Gals) of *L. reuteri* L103 and L461. Greatest GOS yields were 38 % when using an initial lactose concentration of 205 g l^{-1} and at 80 % lactose conversion. Disaccharides other than lactose and trisaccharides made up the enormous majority of GOS formed. The main products were identified as β-D-Galp-(1→6)-D-

Glc (allolactose), β-D-Galp-(1→6)-D-Gal, β-D-Galp-(1→3)-D-Gal, β-D-Galp-(1→6)-Lac, and β-D-Galp-(1→3)-Lac. There were no key products with β1→4 linkages. Both intermolecular and intramolecular transgalactosylation were observed. D-Galactose proved to be a very competent galactosyl acceptor; thus, a relatively large amount of galactobioses was formed (Spletchna *et al.,* 2006).

Li *et al.,* (2008) have analyzed a novel beta-galactosidase BgaBM from *B. megaterium* that displayed wide acceptor specificity for transglycosylation with a series of acceptors, including pentose, hexose, hydroxyl, and alkyl alcohol using o-nitrophenyl-β-D-galactoside (ONPG) as a donor (Li *et al.,* 2008). Kwon *et al.,* . (2007) have established that β-D-galactosidase displayed on *Bacillus* spores by fusion to the spore coat proteins may be used as a whole-cell immobilized biocatalyst for transgalactosylation in water-solvent biphasic reaction systems resulting in the synthesis of octyl-β-D-galactopyranoside at concentrations up to 27.7 mM (8.1 g/liter) with a conversion yield of 27.7 % (wt/wt) after 24 h from 100 mM lactose and 100 mM octanol dissolved in phosphate buffer and ethyl ether, respectively (Kwon *et al.,* 2007). In 2007, an exopolysaccharide producing strain of *L. delbrueckii subsp. bulgaricus* was isolated from yogurts grown at dilution rates between 0.06 and 0.8 h^{-1}. A major fraction of the galactose moiety from lactose from deproteinized whey was metabolized; molar yield of galactose from lactose varied between 0.173 and 0.791 with increasing dilution rates. The process was heterofermentative with maximum concentrations of lactic acid (30.7 g l^{-1}), acetic acid (11.7 g l^{-1}) and ethanol (0.96 g l^{-1}) obtained at dilution rates of 0.12, 0.36 and 0.12 h^{-1}, respectively. Greatest EPS concentration (830 mg l^{-1}) and maximum specific EPS production rate [188 mg (g biomass h)$^{-1}$] were obtained at a dilution rates of 0.36 h^{-1} and 0.67 h^{-1} respectively

(Shene and Bravo, 2007). Nguyen *et al.,* (2007) have established the synthesis of prebiotic GOS from lactose, with the maximum GOS yield of 38.5 % of total sugars at about 75 % lactose conversion using heterodimeric beta-galactosidase from the probiotic strain *Lactobacillus acidophilus* R22 (Nguyen *et al.,* 2007).

In 2008, synthesis of GOS from 36 % lactose using a recombinant beta-galactosidase of *B. infantis* in *Pichia pastoris* was investigated. The trangalactosylation ratio reached up to 25.2 % with 83.1 % conversion of initial lactose and the highest yield of GOS was 40.6 %. The GOS syrup was possessed of a 13.43 % GOS, 5.06 % lactose, and 8.76 % monosaccharides. The prebiotic effect of GOS promoted the growth of *B. breve* ATCC 15700 and *L. acidophilus* ATCC 33323 (Jung and Lee, 2008). Iqbal *et al.,* (2010) have observed that a recombinant beta-galactosidase from *Lactobacillus plantarum* has a high transgalactosylation activity and was used for the synthesis of prebiotic GOS. The maximal GOS yield was 41 % (w/w) of total sugars at 85 % lactose conversion (600 mM initial lactose concentration). The main individual products formed were ß-D-Galp-(1→6)-D-Lac, accounting for 34 % of total GOS, and ß-D-Galp-(1→6)-D-Glc, totalling up 29 % of total GOS (Iqbal *et al.,* 2010). In 2002, the use of ionic liquids as substitute solvents for enzyme catalysis was investigated. Beta-galactosidase from *Bacillus circulans* catalysed the synthesis of *N*-acetylactosamine starting from lactose and *N*-acetylglucosamine in a transglycoslyation reaction. The adding up of 25 % v/v of 1, 3-di-methyl-imidazolmethyl sulfate as a water-miscible ionic liquid suppresses the secondary hydrolysis of the formed product, resulting in doubling-up the yield to almost 60 %. The enzyme can be reused a number of times after ultrafiltration of the reaction mixture without loss of activity (Kaftzik *et al.,* 2002). Thus, beta-

galactosidase plays a significant role in production of galacto-oligosaccharides that can be used as food and feed for human-beings and animals respectively.

2.11.5 Role of beta-galactosidase in whey utilization

Whey has enormous therapeutic applications due to its composition in terms of proteins, lactose, minerals and valuable milk nutrients. The disposal of whey remains a major problem for the dairy industry especially in developing countries where a relatively insignificant part of whey is used for production of protein concentrates or permeates and a significant part of it is disposed off into the water streams causing severe water pollution resulting in high BOD and 5–6 % dissolved solids. The major options for treatment or bioconversion of whey into commercially important products, ethanol and β- galactosidase **(Table: 4)** which finds an increasing use because of growing lactose intolerant population. Thus, microbial beta-galactosidases production is an important area in whey utilization.

Oberoi *et al.,* (2008) have found that *K. marxianus* NCIM 3465 showed greatest beta-galactosidase activity of 1.62 IU mg^{-1} dry weight using whey and cauliflower waste. Although a minor increase in enzyme production was seen by incorporating 5 % to 10 % cauliflower waste in whey, nearly 15% increase in beta-galactosidase production was observed when cauliflower waste level was increased to 20%. Supplementing whey with 20 % cauliflower waste also lowered the production time. Lactose concentration in whey, mainly responsible for increasing the BOD of the effluent water, decreased from 4.2 % to nearly 0 % at 24 h. Thus, this study established that both these by-products / residues could be effectively used for beta-galactosidase production at commercial scale (Oberoi *et al.,* 2008). In 2004, a strain

of *K. lactis* M2 obtained from whey samples has maximum enzyme activity (up to 8103 EU/ml). This yeast strain could be of valuable application in bioconversion of whey (Moeini *et al.,* 2004). Oda and Nakamura (2009) have found that NBRC 1963 of *K. marxianus* converted lactose [in media containing 20 % (w/v) sugar cheese whey] most economically to ethanol (Oda and Nakamura, 2009).

Several mathematical models have been developed for ethanol production from whey using beta-galactosidase. In 2007, mathematical models for semi-continuous ethanolic fermentation in a whey medium employing co-immobilized *S. cerevisiae* strain and β-D-galactosidase was developed. Kinetic parameters of biomass growth, experimentally determined fluxes of ethanol and water, kinetic constants of ethanol separation, the degree of sugar utilization and ethanol productivity, the time of ethanol separation were predicted using this model (Staniszewski *et al.,*2007). Hatzinikolaou *et al.,* (2005) have meticulously examined kinetics and stability of beta-galactosidase of a wild type strain of *A. niger* in the direction of its potential use for the hydrolysis of acid whey permeate lactose. An incorporated process, concerning the simultaneous hydrolysis–ultrafiltration of whey lactose that included the specific kinetic properties of the beta-galactosidase was developed and modelled (Hatzinikolaou *et al.,* 2005). Ozmihci and Kargi (2007) have used lactose utilizing yeast strain, *K. marxianus* DSMZ-7239 for ethanol production from cheese-whey powder (CWP) solution in batch experiments and developed a kinetic model describing the rate of sugar utilization and substrate inhibition as function of the initial substrate and the biomass concentrations (Ozmihci and Kargi, 2007). In 2009, a mathematical model for ethanol fermentation with yeast *S. cerevisiae* and beta-galactosidase on whey was developed (Staniszewski *et al.,* 2009).

Rech and Ayub (2007) have reported the consequence of simple feeding strategies to obtain high-cell-density cultures of *K. marxianus* maximizing β–galactosidase productivity using cheese whey as basic medium. The best fed-batch strategy was established to be the feeding of three-fold lactose concentration in the cheese whey-medium during 25 h, resulted in beta-galactosidase productivity of 291 U l^{-1} h^{-1}, signifying an increase of more than 50 % compared to batch cultivations (Rech and Ayub, 2007). Bansal *et al.,* (2008) have carried out studies related to beta-galactosidase production using whey containing 4.4 % (w/v) lactose inoculated with *K. marxianus* MTCC 1389 and alleviated water pollution problems caused due to its disposal into the water streams (Bansal *et al.,* 2008). Saad (2004) has demonstrated that submerged culture of *Aspergillus japonicus* produced β-D-galactosidase, with 2.95 U mg^{-1} protein specific activity, when developed on cheese whey permeate fortified with 0.5 % yeast after 4 days incubation at 28 °C. Rates of lactose hydrolysis in whey was about 55 %, after 4 h incubation at 45 °C. This enzyme was found suitable for obtaining fermentable sugars from whey wastes (Saad, 2004).

TABLE 4. Beta-galactosidases used for ethanol production from whey

Beta-galactosidase producers	Reference
K. marxianus NCIM 3465	(Oberoi *et al.,* 2008)
K. lactis M2	(Moeini *et al.,* 2004)
K. marxianus NBRC 1963	(Oda and Nakamura, 2009)
S. cerevisiae	(Staniszewski *et al.,* 2007)
A. niger	(Hatzinikolaou *et al.,* 2005)
K. marxianus DSMZ-7239	(Ozmihci and Kargi, 2007)
S. cerevisiae	(Staniszewski *et al.,* 2009)
K. marxianus	(Rech and Ayub, 2007)
K. marxianus MTCC 1389	(Bansal *et al.,* 2008)
Aspergillus japonicus	(Saad, 2004)
Streptococcus thermophilus 95/2, *Lactobacillus delbrueckii subsp. bulgaricus* 77	(Tari *et al.,* 2009)

In 2009, appropriate conditions for the production of beta-galactosidase from whey permeate has been evaluated. This enzyme is to be used in the production of

lactose-hydrolyzed milk (Jokar and Karbassi, 2009). Tari *et al.,*(2009) have investigated beta-galactosidase production by *Streptococcus thermophilus* 95/2 (St 95/2) and *Lactobacillus delbrueckii* ssp. *bulgaricus* 77 (Lb 77) in a medium containing whey (5%), corn steep liquor (4%), potassium phosphate (2 %) and peptone (2 %) at 43 ºC for 8 h using RSM. The associative growth provided 6.4 % and 39 % more beta-galactosidase activity using pure St 95/2 and Lb 77 strains, respectively (Tari *et al.,* 2009). Ozmihci and Kargi (2008) have studied ethanol fermentation of cheese whey powder solution using the pure culture of *K. marxianus* (DSMZ 7239). Total Sugar of 50 gL^{-1} was fermented to ethanol in a continuously operated packed column bioreactor (PCBR) using olive pits as support particles for cell attachment (Ozmihci and Kargi, 2008). Thus, whey utilization by beta-galactosidase reduces the burden of water pollution and provides beneficial products like ethanol and protein concentrates.

2.11.6 Medical and veterinary applications of beta-galactosidase

Beta-galactosidase with its transgalactosylation property finds prominent medical applications such as treatment of disorders and development of digestive supplements. Anderson *et al.,* (2005) have isolated an endo beta-galactosidase from *Clostridium perfringens* ATCC 10543 capable of liberating both the A trisaccharide and B trisaccharide from glycoconjugates containing blood group A and B glycotopes, respectively. Recombinant EABase damaged the blood group A and B antigenicity of human type A and B erythrocytes and also released A-Tri and B-Tri from blood group A^+- and B^+-containing glycoconjugates. The exceptional specificity of this beta-galactosidase should make it useful for studying the structure and function of blood group A- and B-containing glycoconjugates (Anderson *et al.,* 2005). In 2009,

a recombinant endo-beta-galactosidase (ABase), which releases A/B antigen was developed. It removed 82 % of A antigen and 95 % of B antigen in human A/B red blood cells, and concealed anti-A/B antibody binding and complement activation effectively. It was also found to remain active at 4 °C. *in vivo* infusion into a blood type A demonstrated a marked reduction of A antigen expression in the glomeruli of kidney (85 % at 1 h, 9 % at 4 h and 13 % at 24 h) and the sinusoids of liver (47 % at 1 h, 1 % at 4 h and 3 % at 24 h) without grave adverse effects. This substitute approach might be useful for minimizing antibody removal and anti-B cell immunosuppression as an adjuvant therapy in ABO-incompatible kidney, liver and possibly heart transplantation (Kobayashi *et al.,* 2009). Liu and Roffler (2006) have examined the expression of *E. coli* beta-galactosidase in muscle fibers and concluded that repeated intramuscular injections of beta-galactosidase can encourage strong immune responses in immune-competent animals and cause abolition of transduced muscle fibers by inflammatory cells (Liu and Roffler, 2006).

Recently, a beta-galactosidase from the mesoacidophilic fungus *Bispora sp.* MEY-1 under simulated gastric conditions, has shown greater stability (100%) and hydrolysis ratio (>80 %) toward milk lactose than the commercially available beta-galactosidase from *A. oryzae* ATCC 20423. Thus, this beta-galactosidase may be a superior digestive supplement for alleviating symptoms associated with lactase deficiency (Wang *et al.,* 2009). Sanchez-Aparicio *et al.,* (2009) have developed recombinant β-D-galactosidases accommodating one or two different peptides from the foot-and-mouth disease virus nonstructural protein 3B per enzyme monomer that allowed differentiation between sera of FMDV-infected pigs, cattle, and sheep and those of naive and conventionally vaccinated animals. These FMDV infection-

specific biosensors can provide an effective and versatile alternative for the serological distinction of FMDV-infected animals (Sanchez-Aparicio *et al.,* 2009).