Materials and Methods

3. MATERIALS AND METHODS

3.1 Materials

Fine chemicals, enzymes and kits used in this study are listed **(Table: 5)**. Bacterial strains and plasmids used in this study are listed **(Table: 6)**.

TABLE 5. List of fine chemicals, enzymes and kits used in this study

Fine chemicals / enzymes / kits	Source
Media components and other analytical grade chemicals	Himedia (Mumbai, India)
Acids and solvents	Sisco Research Laboratories (Mumbai, India)
Agarose, acetonitrile, antibiotics, bromophenol blue, BSA, calcium chloride, DEPC, EDTA, ethidium bromide, formamide, glycine, ONPG, PNPG, DNase, glucose, glycerol, glycine, imidazole, IPTG, lysozyme, β-mercaptoethanol, proteinase K, RNaseA, sodium acetate, SDS, Tris-HCl, X-gal, sodium dihydrogen phosphate, disodium hydrogen phosphate, disodium carbonate, copper sulphate, sodium potassium tartarate, folin ciocalteu, potassium chloride, magnesium sulphate, manganous sulphate, sodium carbonate, calcium chloride, zinc sulphate, silver nitrate, mercuric chloride, lithium chloride, lead acetate, EGTA, caesium chloride, TGB, coomassie blue R250, xylene cyanol, yeast extract, MOPS,	Sigma (St.Louis, USA)
Acrylamide, APS, bis-acrylamide, TEMED	Amersham Biosciences (NJ, USA)
DNA molecular weight markers, dNTPs, protein molecular weight markers, Restriction enzymes, Taq-DNA polymerase, T4-DNA ligase, T4-DNA polymerase	MBI Fermentas (Opelstrasse, Germany)
DNeasy mini kit, gel extraction kit, PCR purification kit, one step RT-PCR kit, plasmid midi kit, RNeasy mini kit	Qiagen (Hilden, Germany)

TABLE 6. List of Bacterial strains / plasmids used in this study

Bacterial strains / plasmids	Description	Source
Zymomonas mobilis subsp. mobilis ZM4	ATCC 31821	NRRL, Peoria, Illinois
Zymomonas mobilis subsp. mobilis NCIMB 11163	A British ale-infecting isolate	A Gift from Prof. K.M. Pappas, University of Athens, Athens
Zymomonas mobilis B14023	Wild type	NRRL, Peoria, Illinois
Escherichia coli DH5α	F^- *ΔlacU*169 (Ø80 *lacZΔM*15)*supE*44 *hsd*R17 *recA*1 *gyrA*96 *endA*1 *thi*-1 *rel*A1	Invitrogen, CA, USA
Escherichia coli BL21(DE3)	F^- *ompT gal dcm lon hsdSB* (rB^-, mB^-) (DE3)	Novagen, CA, USA
$PBSSK^+$	Ap^r*lacI*<polylinker>f1(+)pM B1ori	Stratagene, San Diego, USA
pET-30b	Expression vector	Novagen, CA, USA
p*bga*	Expression vector with beta-galactosidase insert	This study
pZA22	Expression vector	NRRL, Peoria, Illinois
pZA22ßg	Expression vector with beta-galactosidase insert	This study
pTZ103	pTZ57R/T carrying ß-lactamase, YP_161838 (4.2 kb, Amp^r)	Rajnish *et al.,* 2011
pZTE103	pET-30b (+) carrying ß-lactamase, YP_161838 (6.8 kb, Kan^r)	Rajnish *et al.,* 2011

Bioinformatics databases, softwares and tools used in this study are listed **(Table: 7)**.

Primers used in this study are listed **(Table: 8)**.

TABLE 7. List of Bioinformatics databases / softwares / tools used in this study

Databases/ softwares / tools	URL
NCBI Genome	http://www.ncbi.nlm.nih.gov/sites/genome
TreeView	http://taxonomy.zoology.gla.ac.uk/rod/treeview.html
blastp	http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins
ProtParam	http://web.expasy.org/protparam/
SignaIP 4.0	http://www.cbs.dtu.dk/services/SignaIPS
PSORTb v3.0.2	http://www.psort.org/psortb/
SIG-Pred	http://www.bioinformatics.leeds.ac.uk/prot_analysis/Signal.html
PRED-CLASS v1..0	http://athina.biol.uoa.gr/PRED-CLASS/input.html
Conserved Domain Database	http://www.ncbi.nlm.nih.gov/cdd/
NMPDR	http://www.nmpdr.org/FIG/wiki/view.cgi
STRING v9.0	http://string-db.org/
KEGG PATHWAY	http://www.genome.jp/kegg/pathway.html
ClustalW2	http://www.ebi.ac.uk/Tools/msa/clustalw2/
RCSB	http://www.pdb.org/pdb/home/home.do
SWISS-MODEL	http://swissmodel.expasy.org
ESyPred3D v3.0	http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/
Swiss-PdbViewer v4.04	http://spdbv.vital-it.ch/
PyMOL	http://www.pymol.org
Cn3D	http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml
WHAT_CHECK	http://swift.cmbi.ru.nl/gv/whatcheck
PROCHECK	http://www.ebi.ac.uk/thornton-srv/software/PROCHECK
SAVES	http://nihserver.mbi.ucla.edu/SAVES/
PMDB	http://mi.caspur.it/PMDB/
CASTp	http://sts.bioengr.uic.edu/castp/
DockingServer	http://www.dockingserver.com/web
iPSORT	http://ipsort.hgc.jp/
SOSUIsignal	http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal_submit.html
ConFunc	http://www.sbg.bio.ic.ac.uk/~confunc/
3d2GO	http://www.sbg.bio.ic.ac.uk/pyre/pfd/

NCBI Genome is a resource centre for information on genomes' chromosomes, assemblies, annotations and maps. TreeView is a tree drawing software. blastp is a program to find out protein sequences similar to the query. ProtParam is a tool to calculate number of amino acids, theoretical isoelectric point, instability index (II) value, aliphatic index value, grand average of hydropathicity (GRAVY) value,

estimated half-life (in h *Escherichia coli*, *in vivo*). SignaIP 4.0 server incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks and hidden Markov models. PSORTb v3.0.2 is a bacterial protein localization prediction tool. SIG-Pred predicts signal peptides and their cleavage sites on the basis of weight matrices. PRED-CLASS v1.0 is a system of NN that classifies proteins into four classes namely membrane, globular, fibrous and mixed (fibrous and globular). Conserved Domain Database is a resource for functional annotated units in proteins. NMPDR database is a curated genome annotation database. STRING v9.0 is a database of known and predicted protein interactions. KEGG PATHWAY is a collection of manually drawn pathway maps. ClustalW2 is a general purpose multiple sequence alignment program. RCSB is a repository of 3D structures of proteins. SWISS-MODEL is a protein structure homology-modeling server. ESyPred3D v3.0 is a automated homology modeling program. Swiss-PdbViewer v4.04 is an application to analyze protein structures. PyMOL is a molecular visualization system. Cn3D is an application to view 3D structures. WHAT_CHECK is a molecular modelling software suite. PROCHECK is used to check stereochemical quality of a protein structure. SAVES is a Structural analysis and verification server. PMDB is a Protein 3D model collection database. CASTp is a server to find out the binding sites and active sites of proteins. DockingServer is a web-based interface of molecular docking of protein and ligand. iPSORT is a subcellular localization site predictor for N-terminal sorting signals. SOSUIsignal is a signal peptide prediction tool. ConFunc is an automated protein function prediction method. 3d2GO is an automated protein function prediction from 3D structure.

TABLE 8. List of primers used in this study

Primer	Sequence (5' to 3')	Purpose
ZMO0103F	ATGAAGTGTCGGACGATCATAACGG	SQ-PCR of ORF ZMO0103
ZMO0103R	TTATTTCTTATGGGATGCCTTGTCT	SQ-PCR of ORF ZMO0103
ZMO1650F	GTGGCCTCGTCTCTTTCCCATAGAC	SQ-PCR of ORF ZMO1650
ZMO1650R	TTACTCTGCTGCGATATTTTCATAA	SQ-PCR of ORF ZMO1650
ZMO1967F	ATGATGAGGAGGCGGGATGTTTTGG	SQ-PCR of ORF ZMO1967
ZMO1967R	CTAACTTTCCGATTGGAGGATGTCG	SQ-PCR of ORF ZMO1967
ZMO0893F	ATGGCATTTTCGGAAAGACTGGGAA	SQ-PCR of ORF ZMO0893
ZMO0893R	TTATGAAGAAAGAGCGCTGTCCCAT	SQ-PCR of ORF ZMO0893
ZMO1866F	GTGAAAAGACCAGGTAAAGAACTCC	SQ-PCR of ORF ZMO1866
ZMO1866R	TCAGACATGCACAGCGACGACATCG	SQ-PCR of ORF ZMO1866
ZMO0777F	ATGCCAACGACCTCCCATTCAGGCA	SQ-PCR of ORF ZMO0777
ZMO0777R	TCAGGGCATAAGACCTAGCACCGAA	SQ-PCR of ORF ZMO0777
ZMO1094F	ATGAAACTCCGTCTTCTCGGTTCGG	SQ-PCR of ORF ZMO1094
ZMO1094R	CTAGATTTCTGTTGTCCAGCCATCA	SQ-PCR of ORF ZMO1094
ZMO0904F	CCGCCATGACGGCATT	Q-PCR of ORF ZMO0904
ZMO0904R	CCAACGGGTCCAGTTTCATT	Q-PCR of ORF ZMO0904
adhBF	CGCAGAAGCCACCATTCAG	Q-PCR of *adhB*
adhBR	GCTGGAATACCAATGGAAGCA	Q-PCR of *adhB*
bgaSQ-F	ATGGACAAGATGGGGAAAATAGCGAAAAG	SQ-PCR of ORF ZMO0904
bgaSQ-R	TTATTGCTGCTCTTGGGCTTCCTTTATAT	SQ-PCR of ORF ZMO0904
bgaF	TATAGGATCCATGGACAAGATGGGGAAAATAGCGAAAG	Cloning of ORF ZMO0904
bga R	ACTAGTCGACTTATTGCTGCTCTTGGGCTTCCTTTATAT	Cloning of ORF ZMO0904
ZMO1083 F	GCATTGCCTCGGGTTATCTTTCTGA	SQ-PCR of intervening region of ORFs ZMO1083 and ZMO1084
ZMO1084 R	CACCATTTCGTCCCATGACAAC	SQ-PCR of intervening region of ORFs ZMO1083 and ZMO1084
ZMO1084 F	GGATAGATAGATTATTAGGACGGGATGGAG	SQ-PCR of intervening region of ORFs ZMO1084 and ZMO1085
ZM01085 R	GCTTTGATGCTGTTTTCTTGGCT	SQ-PCR of intervening region of ORFs ZMO1084 and ZMO1085
ZMO1083F1	ATGGGATTGACGCTGCTGATTGA	SQ-PCR of ORF ZMO1083
ZMO 1083R1	TCATTTCTTTACTGCCCCCTTCTC	SQ-PCR of ORF ZMO1083
ZMO1084 F1	ATGACAAAAGCCAAAAAAGCAACCG	SQ-PCR of ORF ZMO1084
ZMO1084R1	TCATTCTTCGGACTCATCGTTTTCTG	SQ-PCR of ORF ZMO1084
ZMO1085F1	ATGAAACGCATAAACAGGCATTACTCTC	SQ-PCR of ORF ZMO1085
ZMO1085R1	TTAATGTTTATCCGTCTTATCAGCATC	SQ-PCR of ORF ZMO1085

3.2 Bacterial culture conditions

Z. mobilis was grown in Rich Medium (glucose, 20 g l^{-1}; KH_2PO_4, 20 g l^{-1} and yeast extract 10 g l^{-1}) under static condition at 30 ºC. Whenever required, media were supplemented with antibiotics. *E. coli* strains were grown in LB medium at 37°C

under agitation. LB medium consisted of (g/l) tryptone 10, yeast extract 5, sodium chloride 10 and pH adjusted to 7.2. For solid medium, agar 20 g/l was added.

3.3 Recombinant DNA techniques

Genomic DNA isolation, plasmid isolation, restriction digestions, ligations, bacterial transformation, agarose gel electrophoresis and PAGE analysis were performed as described by Sambrook *et al.,* (1989). Alternatively, Plasmid midi kit and Dneasy mini kit (Qiagen, Hilden, Germany) were used for the isolation of plasmids and genomic DNA respectively.

3.4 Protein estimation

The protein estimation was performed with Lowry's assay using Bovine Serum Albumin as the standard.

3.5 Estimation of minimum inhibitory concentration of ß-lactam antibiotics

The minimal inhibitory concentrations (MIC) of antibiotics were calculated by microtitre broth dilution technique using 96-well microtitre plates. The MIC assay was performed as described in the earlier study (Andrews, 2001).

3.6 Disk diffusion assay of ß-lactam antibiotics

Z. mobilis ZM4 (NC_006526) and *Z. mobilis* NCIMB 11163 were subjected to disk diffusion assay with various ß-lactam antibiotics by Kirby-Bauer disk diffusion susceptibility assay.

3.7 Atomic Force microscopy of *Z. mobilis* and the extracellular cellulosic material

The morphology and microstructure of *Z. mobilis* and the extracellular cellulosic material were examined using Atomic Force Microscopy (A100, APE

Research, Trieste, Italy). *Z. mobilis* was grown on Rich medium glucose. The *Z. mobilis* cells were peeled off and then transferred to cover slips and further analysed by atomic force microscope (Recouvreux *et al.,* 2008).

3.8 Transcript analysis of beta-lactamases, cellulose synthase operon genes and beta-galactosidase

The total RNA was isolated from mid-log phase cultures (OD_{600} 0.7) of *Z. mobilis* grown in Rich medium as described previously (Conway *et al.,* 1991). The entire RNA samples were subjected to DNase I (MBI Fermentas, Opelstrasse, Germany) treatment to eliminate the genomic DNA. The quality and amount of RNA was calculated using Nanodrop (ND-1000 Spectrophotometer, Wilmington, DE, USA). The RNA integrity was analyzed by formaldehyde agarose gel electrophoresis (Sambrook and Russell, 2001). The Semi-Quantitative PCR (SQ-PCR) was performed for the cDNA synthesized from whole RNA template using gene-specific primers. SQ-PCR primers were designed by NetPrimer software and primers were purchased from Sigma Genosys, Bangalore. The level of expression of the transcripts in *Z. mobilis* ZM4 was estimated. The *adhB* (alcohol dehydrogenase B), a house-keeping gene was employed as endogenous control. SQ-PCR was performed using Qiagen One-Step RT-PCR kit master mix (Qiagen, Hilden, Germany) kit in an Eppendorff PCR system (Eppendorff, USA) according to manufacturer's instruction. Qiagen One-Step RT-PCR master mix (15 µl) consisting of 5x Qiagen One-Step RT-PCR buffer (2.0 µl), 10 mM dNTP mix (0.4 µl), 0.6 µM of each of forward and reverse gene-specific primers for the respective genes, 20 U/µl RNase inhibitor (0.5 µl) and 3 µg RNA template. SQ-PCR thermal cycling conditions were used as follows: 60 °C for 30 min, 95 °C for 15 min, 40 cycles each of 94 °C for 15 sec, annealing temperature for the respective genes (in °C) for 2 min and 72 °C for 2 min, finally 72 °C for 10

min. Qiagen One-Step RT-PCR master mix devoid of template control was used as negative control. The resultant PCR products (15 µl) were electrophoresed on 1.2 % agarose gel in 1 x TAE (40 mM Tris acetate, 1 mM EDTA, pH 8) buffer at steady voltage of 80 V/cm and stained with ethidium bromide (0.5 µg ml^{-1}). Computation of intensity and confirmation of SQ-PCR product was done using the Bio-Rad quantity One software.

3.9 SDS-PAGE and Zymogram analysis of beta-lactamases

SDS-PAGE and ß-lactamase activity staining was performed as described earlier (Liang *et al.,* 2003). Starch (0.1%) was copolymerized in 10% SDS-PAGE gels. A 75 µl sample was mixed with 25 µl of sample loading buffer and loaded in the gel. After completion of the SDS-PAGE, gels were divided into two portions. One portion was treated with 12.5% trichloroacetic acid followed by coomassie brilliant blue R-250 dye staining. The other portion was soaked and shaken for 10 min two times in 25% (v/v) isopropanol in 10 mM phosphate buffer (pH 7.0) to get rid of SDS and at last soaked in 100 mM phosphate buffer (pH 7.0) for 15 min prior to penicillinase staining. The process of penicillinase activity staining was performed as reported earlier (Liang *et al.,* 2003). The gel was saturated in the substrate solution (100 mg penicillin G in 50 ml, 100 mM phosphate buffer, pH 7.0) with moderate shaking for 30 min. Following rinsing, the penicillinase activity was examined with 0.6% iodine in 6% potassium iodide solutions. The transparent region of penicillinase activity was seen. Later, the gel was destained with 5% acetic acid.

3.10 Beta-lactamase assay

Beta-lactamase assay was performed by observing the hydrolysis of the ß-lactam antibiotics at 240 nm after addition of the enzyme (Hitachi U-2900 UV visible Spectrophotometer) with 1 cm path length cuvette. The beta-lactamase assay was

initiated by addition of 10 µl of the beta-lactamase extract to 2 ml of 0.1 mM ß-lactam antibiotics in 10 mM phosphate buffer, pH 7 (preincubated at 37 °C). Absorbance was observed every minute for the period of three min. One enzyme unit was defined as the amount of ß-lactamase needed to hydrolyse 1 µmol of substrate per min under the assay conditions (Waley, 1974).

3.11 Beta-galactosidase assay

Cells were centrifuged and resuspended in Z-buffer (100 mM $NaPO_4$ [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$) and then permeabilized by treatment with sodium dodecyl sulfate and chloroform. Assays were performed in Z-buffer containing 50 mM ß-mercaptoethanol. Activities (change in optical density at 420 nm [OD420] per min) were normalized to actual cell density (OD650) and were compared to those of appropriate controls assayed at the same time. Activities are expressed in Miller units (Miller, 1972).

3.12 Determination of cellulose synthesis in *Z. mobilis*

3.12.1 Congo Red Assay

Z. mobilis was grown on Rich medium glucose agar containing Congo red (Römling *et al.,* 1998) to study the colony morphology. To study the secretion of cellulose, *Z. mobilis* was grown for 72 h at 30 ºC on RMG agar plates (glucose, 20 g l^{-1}; KH_2PO_4, 20 g l^{-1}; yeast extract 10 g l^{-1} and 1.6 % agar) supplemented with Congo red 40 µg.ml^{-1}.

3.12.2 Calcofluor Assay

Z. mobilis was grown on Rich medium glucose containing calcofluor, a fluorochrome to study the fluorescence (Da Re and Ghigo, 2006). Calcofluor binding by *Z. mobilis* was observed on RMG agar plates (glucose, 20 g l^{-1}; KH_2PO_4, 20 g l^{-1};

yeast extract 10 g l^{-1} and 1.6 % agar) supplemented with Calcofluor 200 µg.ml^{-1}. The *Z. mobilis* cells were placed on glass slide along with calcofluor white stain and observed in microscope to study the fluorescence (Gallo *et al.,* 1989).

3.12.3 Acetic-nitric (updegraff) cellulose assay

Determination of cellulose in *Z. mobilis* performed using acetic-nitric (Updegraff) cellulose assay by dissolving the extracellular component of *Z. mobilis* in acetic-nitric reagent and finally with anthrone reagent (Updegraff, 1969).

3.13 Determination of copy number of beta-galactosidase

Real time PCR analyses were performed using SYBR green detection system using an Applied Biosystems 7500 Real Time PCR system (Applied Biosystems, CA, USA). The primers used for Real Time PCR were designed using the Primer Express software, and the primers were ordered from Sigma Genosys, Bangalore. A two step SYBR green PCR assay was performed in a final reaction volume of 25 µl using the Power SYBR green master mix (Applied Biosystems, CA, USA). The primer concentration used for the Real Time PCR assay was 900 nM. The cycling conditions used for the Real Time PCR analysis was as follows: 50 ^{0} C for 2 min, followed by 40 cycles of 95 ^{0} C for 10 min, 95 ^{0} C for 15 sec, and 60 ^{0} C for 1 min. PCR amplifications were performed in triplicates and appropriate no template control was included for every gene studied. The PCR products were subject to melt curve analysis to confirm the specificity of the primers used. Absolute quantitation of copy number was performed by dilution of the linearized plasmid containing gene. The dilutions were prepared and a standard curve was constructed by plotting the C_t values of the dilutions against the logarithm of the copy number using the SDS version 1.4 software (Applied Biosystems, CA, USA). Relative quantification assay was

performed by quantitation of the target gene with respect to the endogenous control. The endogenous control was used to normalize the levels of target gene during the quantification assay. Real Time PCR was performed in duplicates and the data generated during the Real Time PCR run were analyzed using the SDS version 1.4 software (Applied Biosystems, CA, USA).

3.14 Preparation of *Z. mobilis* competent cells

Electrocompetent cells of *Z. mobilis* were prepared by the method of Liang and Lee (1998) with modifications. *Z. mobilis* B14023 cells were grown on RM medium in stationary flasks at 30°C to an absorbance 0.3-0.4 A600. The cells were harvested by centrifugation at 4000 rpm for 5 min at 4°C. The cells from a 100 ml culture were suspended in 10 ml of sterile 10% glycerol (supplemented with 0.85% NaCl), centrifuged and finally resuspended in 2-3 ml of 10% glycerol. This competent cells were stored at –70^{o}C for long-term.

3.15 Electrotransformation of *Z. mobilis*

Gene PulserTM (Bio-Rad Life Science, Hercules, CA) was used for generating exponential pulses. The output of the pulse generator was directed through a Pulse Controller (Bio-Rad) containing a 20 W resistor in series with the sample, and a selection of resistor of 100 to 1000 W in parallel with the sample. A peak voltage of 1.5 kV, corresponded to the field strength of 7.5 kV/cm, and 25 µF capacitance and a parallel resistor of a specified ohm. A 200 µl aliquot of *Z. mobilis* B14023 was mixed with 10 µl of plasmid DNA (~5 µg) to be transformed in a chilled electroporation chamber with an electrode gap of 0.2 cm and held on ice for 5 min. Then the mixture of cells and DNA was pulsed and immediately, the cells were mixed with 1 ml of RM

medium at 30°C. The cells were then diluted with the same media and plated on RM agar with selective antibiotics.

3.16 Purification and characterization of beta-galactosidase

The beta-galactosidase was purified according to the procedure described in The Recombinant Protein Handbook (Amersham Biosciences). The clarified supernatant of *E. coli* BL21 (DE3), harboring the p*bga* obtained after disruption of the cells by sonication was ammonium sulphate precipitated at 37 °C. The pellet obtained at 40-60% ammonium sulphate saturation was suspended in assay buffer and further subjected to anion exchange chromatography.

Active fractions were passed through HiTrap Fast Flow DEAE-sepharose column (Amersham, Uppsala, Sweden) equilibrated with a buffer containing 20 mM bis-trispropane (pH 6.5), 3 mM EDTA, and 0.2 M saccharose. The column was washed with 7 column volumes in the presence of 0.3 M KCl. The column was developed with a salt gradient, and the beta-galactosidase eluted at approximately 240 mM KCl in the buffer system. Gel filtrations (with active fractions obtained after the anion exchange step) were carried out with prepacked Superose column equilibrated with 0.4 M Tris-HCl (pH 7.0), 2 mM EDTA, 0.2 M KCl, and 0.1 M saccharose.

The effect of pH on beta-galactosidase activity was measured by performing assays in 50 mM citrate buffer (pH 2.0-5.0), 50 mM sodium acetate buffer (pH 4.0-7.0), 50 mM sodium phosphate buffer (pH 6.0-9.0) and 50 mM Tris-HCl buffer (pH 8.0-9.0). For stability at different pH levels, the beta-galactosidase was pre-incubated in 50 mM of the buffers at 30 ºC for 30 minutes and the residual activity was measured. The effect of temperature on beta-galactosidase activity was measured by assays set at temperature ranging from 25 ºC to 80 ºC in 50 mM sodium phosphate

buffer (pH 7.0). For determination stability at different temperatures, the beta-galactosidase was pre-incubated in 50 mM of sodium phosphate buffer (pH 7.0) at temperature ranging from 25 ºC to 80 ºC for 30 min and the residual activity was measured. The effect of metal ions and other additives on enzyme activity was studied by incubation with the 10 mM of different metal ions or other additives for 30 minutes and the residual activity was measured. The substrate specificity of the beta-galactosidase was studied using different substrates (0.1 mM each).

3.17 Preparation of cellular fractions of *Z. mobilis*

A overnight starter culture of *Z. mobilis* in RMG media was prepared. This starter was inoculated into 100 ml RMG medium and allowed to grow till late log phase. Sixty ml of culture was centrifugation at 10,000× g for 10 min at 4°C. The remaining 50 ml was set aside for preparation of periplasmic fraction. The supernatant was discarded and the cells were resuspended in 5 ml of 10 mM phosphate buffer, pH 7.4. The cells were sonicated and centrifuged to obtain the soluble fraction and the β-lactamase assay with the soluble fraction was performed.

The remaining 40 ml of cells were centrifuged and the cell pellet was resuspended thoroughly in 30 ml of 30 mM Tris-HCl pH 8, 20% sucrose. 60 µl 0.5 M EDTA, pH 8 (final concentration of 1 mM) was added and stirred slowly at room temperature for 10 min using magnetic stirrer. The cells were collected by centrifugation at 10,000 × g at 4°C for 10 min. The supernatant was removed and discard. The pellet was resuspended thoroughly in 30 ml of ice-cold 5 mM $MgSO_4$ and the cell suspension was stirred slowly for 10 min on ice. The periplasmic proteins were released into the buffer during this step. It was then centrifuged at 4°C for 10 min at 10,000 × g to pellet the shocked cells. A 1 ml sample from the supernatant

(periplasmic fraction) was transferred to a microcentrifuge tube and concentrated using SpeedVac. The β-lactamase assay with the periplasmic fraction was performed.