CHAPTER III

METERIALS & METHODOLOGY

3.1 Laboratory equipments, chemicals

3.1.1 Laboratory equipments

Laboratory equipments	Company	Country
Autoclave NLS-3020	Sanyo Electric Co.,Ltd	Japan
Autoclave HV-110	Hirayama	Japan
Biological safety cabinet, Forma Class II A2	Thermo Electron Corporation	USA
Fast Plasmid Mini, 0032 007b653	Eppendrof	USA
Gel Document, GeneSnap	Syngene	USA
Gene Amp PCR® system 2700	Applied Biosystems	USA
GC-14A (for solvent utilization)	Shimadzu	Japan
GC-17A (for FAME analysis)	Shimadzu	Japan
Incubator shaker, innova 4000	New Brunswick scientific	USA
Incubator shaker, innova 4340	New Brunswick scientific	USA
Micropitte 20, 100, 200, 1000 µl	Gilson	France
Protector Laboratory Hood	Science Technology	USA
Refrigerated Centrifuge, 5804R	Eppendrof	USA
Scanning Electron Microscope	Jeol	Japan

(SEM) model JSM-5410LV		
Spectrophotometer DU650	Beckman	USA
Transmission Electron Microscope (TEM) model JEM-2100	Jeol	Japan
Ultrasonic	Banderlin	Germany

3.1.2 Laboratory chemicals

(1) Chemicals

Chemicals	**Company**	**Country**
Agar Agar	Scharlau Chemic Microbiology	Spain
Albumin bovine (BSA)	Sigma	USA
Bovine serum albumin (BSA)	Sigma	USA
$CaC_{l2}.2H_2O$	Merck	Germany
$C_4H_{10}N_2Na_2O_6.2H_2O$ (EDTA)	BDH	England
$C_{12}H_{25}O_4SNa$ (SDS)	Sigma	USA
$CoCl_2.6H_2O$	Merck	Germany
$COOK(CHOH)_2COONa.4H_2O$	Carlo Erba Reagenti	Italy
Crystal violet	BDH	England
$CuSo_4.5H_2O$	Scharlau Microbiology	Spain
Dimethyl sulfoxide CAS No.D-5879	Sigma	USA
$FeSO_4.7H_2O$	BDH	England
Glucose monohydrate	Specialty chemicals Limited	Australia

Glycerol (99 % purity)	Univar	Australia
Iodine crystal	BDH	England
KH_2PO_4	Carlo Erba Reagenti	Italy
K_2HPO_4	Riedel	Germany
$MgSO_4.7H_2O$	Carlo Erba Reagenti	Italy
$MnSo_4.H_2O$	Merck	Germany
MoO_3	Merck	Germany
NH_4Cl	May&Baker	England
NaCl	BDH	England
Na_2CO_3	BDH	England
NaOH	Merck	Germany
Na_2HPO_4	Fluka	Germany
p-Nitrophenyl acetate	Fluka	Germany
pGEM - T Easy vector	Promega	USA
Rhamnose	Fluka	Germany
Safanin O	Fluka	Germany
(tri) Sodium citrate	Merck	Germany
Succinic acid	Merck	Germany
Sucrose	Fluka	Switzerland
Taq polymerase (500 Unit)	Fermentas	USA
Tryptone	Himedia	India
Yeast extracts	Scharlau Chemic Microbiology	Spain
$ZnSO_4.7H_2O$	Merck	Germany

(2) Organic solvents for used in this study

n-Butanol (99.5 % purity)	Fluka	Germany
Butyl acetate (99.5 % purity)	Fluka	Germany
Benzene (99 % purity)	Fluka	Germany
Chloroform (99.5 % purity)	Merck	Germany
Cyclohexane (99.8 % purity)	Fluka	Germany
n – Decane (98 % purity)	Fluka	Germany
Diethylphthalate (96 % purity)	Fluka	Germany
Ethyl acetate (99.9 % purity)	LAB-SCAN Co. Ltd.	Thailand
Ethanol (99.8 % purity)	Carlo Erba Reagenti	Italy
Ethylbenzene (99 % purity)	Fluka	Germany
n – Heptane (99 % purity)	Fluka	Germany
n –Heptanol (99 % purity)	Fluka	Germany
n – Hexane (98 % purity)	Fisher Scientific	United
n – Octane (99 % purity)	Fluka	Germany
Styrene (99 % purity)	Fluka	Germany
Toluene (99.5 % purity)	Fluka	Germany
m-Xylene (99 % purity)	Fluka	Germany
o-Xylene (98 % purity)	Fluka	Germany
p-Xylene (98 % purity)	Fluka	Germany

3.2 Culture medium

3.2.1 The mineral salt basal medium (MSB)

The mineral medium was used for screening, isolation, cultivation and substrate utilization experiments. The mineral medium was comprised of media and trace element (Na *et al.*,2005)

I. Media

4.3 g of K_2HPO_4

3.4 g of KH_2PO_4

2.0 g of $(NH_4)_2SO_4$,

The component was dissolved in 1 liter of distilled water and adjusted pH to pH 7.0 by 1 N NaOH. The mineral medium was autoclaved at 121 °C for 15 minutes.

II. Trace element

0.01 mg of $ZnCl_2.7H_2O$,	0.16 g of $MgCl_2.6H_2O$,
0.01 mg of $CoCl_2.6H_2O$,	0.001 gof $MnCl_2.4H_2O$,
0.001 mg of $CuSO_4$,	0.006 g of $FeSO_4.7H_2O$,
0.001 mg of $NiSO_4.6H_2O$,	0.026 g of $CaCl_2.2H_2O$,
0.001 mg of Na_2SeO_4,	$Na_2MoO_4.2H_2O$ 0.02 mg

Trace element solution was separately prepared from media as a stock solution. They were dissolved in 100 ml of distilled water and it was autoclaved at 121 °C for 15 minutes. Before using, 0.1% (v/v) sterile trace element was supplemented in mineral medium. For solid media, 1.7% agar was added to the MSB media.

3.2.2 Luria Bertani medium (LB)

Trytone	10 g
Yeast extracts	5 g
NaCl	10 g

LB medium was dissolved in 1 liter of distilled water, adjusted to pH 7.0 and autoclaved at 121 °C for 15 minutes. For solid media, 1.7% agar was added to the LB media.

3.2.3 Half luria bertani medium (HLB)

Trytone	5 g
Yeast extracts	2.5 g
NaCl	5 g

HLB medium was dissolved in 1 liter of distilled water, adjusted to pH 7.0 and autoclaved at 121 °C for 15 minutes. For solid media, 1.7% agar was added to the HLB media.

3.3 Methodologies

The experimental methodologies are described in order of the experiments performed. All of the methods used in this research can be simplified and shown as a flow chat below:

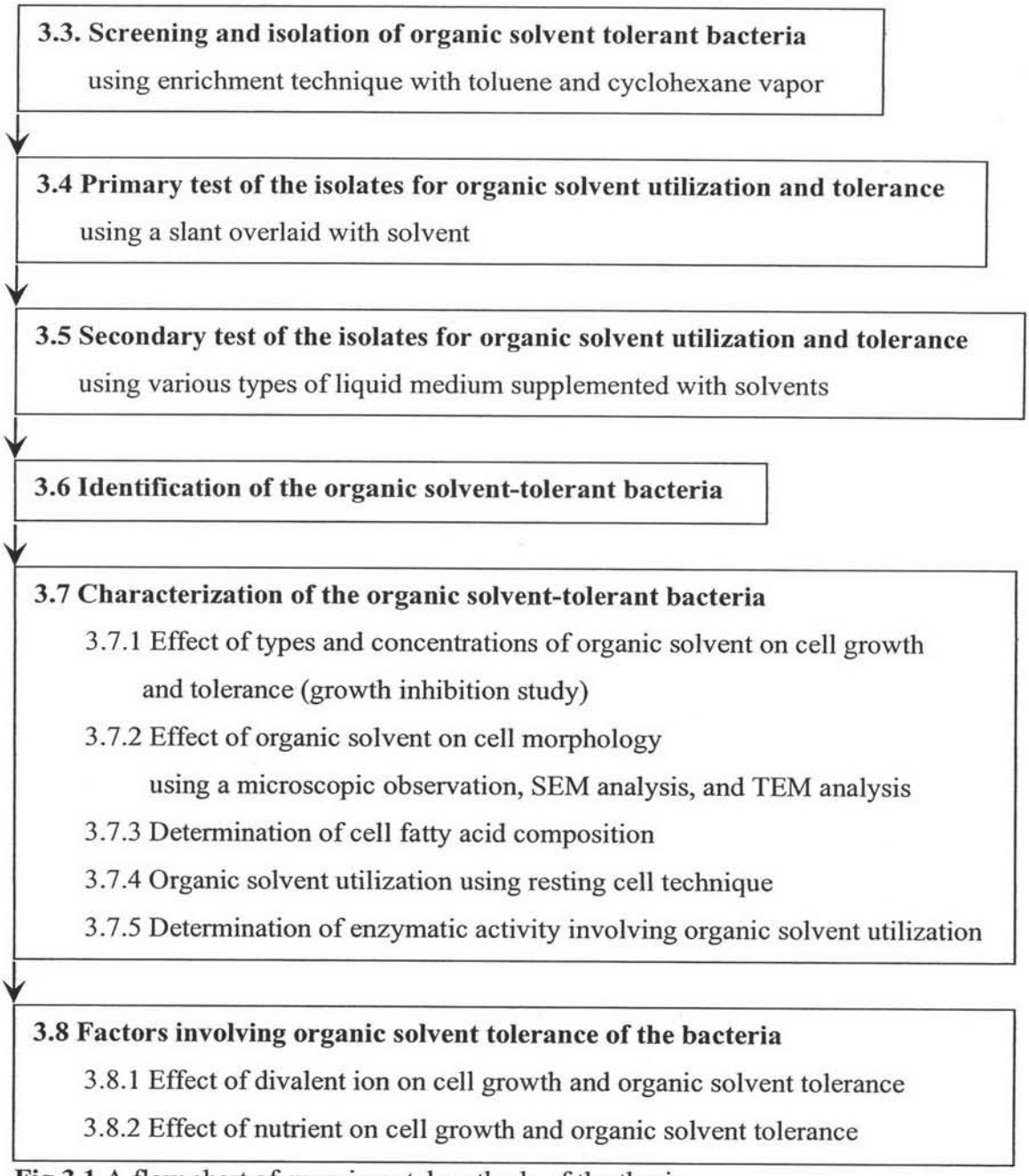

Fig 3.1 A flow chart of experimental methods of the thesis

3.3.1 Screening and isolation of organic solvent tolerant bacteria using enrichment technique with toluene and cyclohexane vapor

Soil samples were taken from contaminated area, hot spring area, agricultural area and natural area in various provinces, Thailand. Approximately 5 g of soil was acclimated in a 50-ml screw-capped vial in the presence of toluene or cyclohexane vapor for 1 week at 45°C. After the acclimatization, 1 g of soil was dispersed in 4 ml of sterile water and allowed to stand for 30 min. One milliliter of this soil suspension was added to 9 ml of MSB medium. Then, 0.5 ml of toluene or cyclohexane was added to the small tube inside the screw-capped vial. The vial was incubated with shaking (120 rpm) at 45°C for 5 days. Subsequently, 1 ml of the culture was transferred to 9 ml of fresh MSB medium and incubated for 2 days. Serial dilutions of the second culture were plated out on MSB agar plates and incubated at 45°C in a closed container with a beaker containing liquid toluene or cyclohexane. Colonies obtained were reinoculated into the liquid medium to confirm the utilization of toluene or cyclohexane. Pure cultures utilizing toluene and cyclohexane as a sole carbon source were kept on HLB agar plates.

3.3.2 Primary test of the isolates for solvent utilization and tolerance

Bacterial isolates isolated from toluene and cyclohexane enriched condition were tested their organic solvent tolerance and organic solvent utilization. Cells were streaked on MSB and MSB slant supplemented with 1% yeast extract. The streaks were then allowed to dry for 20 to 30 min at room temperature and, then organic solvent was directly poured on top of the slant surface to a depth of 5 mm and incubated at 45°C for 5 days. Cell growth was visually determined.

3.3.3 Secondary test of the isolates for solvent utilization and tolerance

Cells were grown in three types of medium: 1) MSB; 2) MSB supplement with 16mM glucose (MSBG) and 3) MSB supplement with 1% yeast extract broth (MSBY). Organic solvent was provided as either a vapor phase of a direct addition (20% v/v). Cells were grown at 45°C for 5 days with shaking condition at 250 rpm and growth was spectrophotometrically determined at the absorbance 560 nm.

3.3.4 Identification of the organic solvent-tolerant bacteria

3.3.4.1 The morphological characteristics of the organic solvent-tolerant bacteria

Morphology of bacteria

Bacterial staining was used to study shape, size of bacteria and to classify bacteria into Gram positive or Gram negative. Bacteria were grown in LB agar medium for 24 hours, stained and visualized though a microscopy (solutions for bacterial staining and a protocol shown in APPENDIX A)

Characteristics of bacterial colonies

Bacterial were grown in HLB medium agar for 24 hours, and then the physiological feature on an agar plate was observed; for example, color, from, diameter, surface and edge.

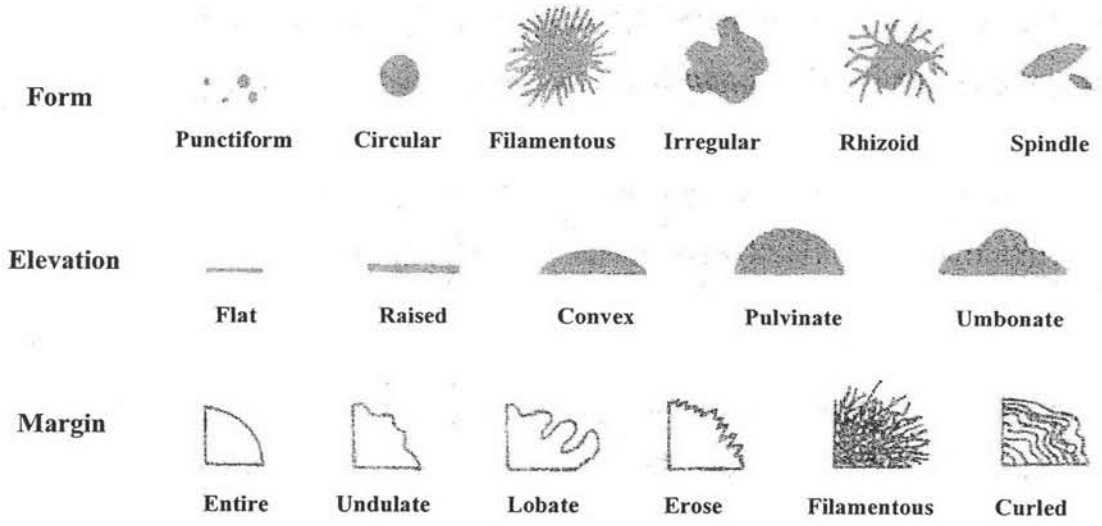

Fig 3.2 Characteristics of colonies

3.3.4.2 The biochemical test of the organic solvent-tolerant bacteria

Biochemical characterizations of bacteria were performed by the laboratory of Institution for Scientific Research, Department of Medical Sciences, and Ministry of Pubic Health in Thailand. Biochemical test methods are briefly shown in APPENDIX A.

3.3.4.3 Determination of ribosomal DNA gene of the organic solvent-tolerant bacteria identification

Genomic DNA from individual bacteria stains was extracted by a standard method (Sambrook et al., 1989). The 16S rDNA gene fragment was amplified from the genomic DNA of each bacteria by polymerase chain reaction (PCR) using the bacterium-specific primer : forward primer (63f) (5' CAGGCCTAACACATGCAAGTC 3') and a reverse primer 1387r (5' GGGCGGWGTGTACAAGGC 3') (Marchesi *et al.*,1998) A reaction mixture (total volume of 25 μl) contained 1X Taq buffer with $(NH_4)_2SO_4$, 0.2 mM

dNTP, 2 mM $MgCl_2$, 0.4 μM of each primer, 1μl DNA sample (500 ng DNA), Taq polymerase 5 units/μl (Fermentas, USA). The reaction mixture was subjected to pre-denaturation at 94°C for 3 min, 30 cycles consisting of denaturation at 95°C for 1 min, 55°C and 1 min for annealing, chain extension at 72°C for 1.3 min, with an additional extension time of 72°C for 5 min, The fragment was then cloned into pGEM-T Easy Vactor (Promega, USA) and transformed into competent cell of *Escherichia coli* DH5α (APPENDEX A). The recombinant plasmid from the was isolated using the QIAprep Spin Miniprep kit (Qiagen, Germany). The insert was then sequenced by the Macrogen Inc. (Seoul, Korea) and compared to the most similar sequences with Blastn program in the NCBI database (http://www.ncbi.nlm.nih.gov).

3.3.5 Characterization of the organic solvent-tolerant bacteria

3.3.5.1 Effect of types and concentrations of organic solvent on cell growth and tolerance

Four bacterial strains were grown on HLB liquid medium incubated on a rotary shaker at 250 rpm, at 45°C. After late log phase, they were treated directly with 5% and 20% (v/v) of various types of organic solvents. Cell survival was determined as colony forming units (CFUs) and spectrophotometrically at the absorbance 560 nm.

3.3.5.2 Effect of organic solvent on cell morphology

Macroscopic observation after gram strain

Cells were grown in HLB medium at 45°C for 6 h ethyl acetate, *n*-decane, and chloroform was added to medium 20% (v/v) for *Deinococcus geothermalis* T27 and *Breviacillus agri* strain 13, *Bacillus subtilis* strain 45, and *Bacillus cereus* strain 4/1 respectively, then incubated for 6 h. The control was cells grown in the absence of organic solvent. The morphology of the solvent tolerant bacteria was observed after gram staining under a microscope.

Scanning Electron Microscope (SEM)

Four bacterial strains were grown on HLB liquid medium (100 ml) incubated on a rotary shaker at 250 rpm, at 45°C. After late log phase, they were treated with 20% (v/v) organic solvent, i.e. ethyl acetate for *Deinococcus geothermalis* strain T27 and *Brevibacillus agri* strain 13; *n*-decane for *Bacillus subtilis* strain 45 and chloroform for *Bacillus cereus* strain 4/1, and continue shaking at 250 rpm, at 45°C for another 6 h. The cells were harvested by centrifugation at 5000 rpm for 15 min. Cells were prepared for the ultra-structure analysis at Scientific and technological research equipment center, Chulalongkorn University, Thailand. Cells were fixed with glutaraldehyde (2.5% in 0.1M phosphate buffer, pH 7.2) for 2 h, washed twice with phosphate buffer, and filtered with 0.45 um Millipore filter (Millipore Corporation, USA). The sample was then dehydrated in series of increasing concentrations of ethanol (30%, 50%, 70%, 90% and absolute ethanol), supjected to critical-point drying with carbon dioxide (Balzers CPD020, Balzers AG, Liechtenstein), and mounted on metal stubs with gold-palladium. Each sample was examined

using a JEOL scanning electron microscope; model JSM-5410LV (Jeol, Tokyo, Japan) at 15 kV.

Transmission Electron Microscope (TEM)

Four bacterial strains were grown on HLB liquid medium (100 ml) incubation in rotary shaker at 250 rpm, at 45°C. After late log phase, they were treated with 20% (v/v) organic solvent, i.e. ethyl acetate for *Deinococcus geothermalis* strain T27 and *Brevibacillus agri* strain 13; *n*-decane for *Bacillus subtilis* strain 45 and chloroform for *Bacillus cereus* strain 4/1, and continue shaking at 250 rpm, at 45°C for another 6 h. The cells were harvested by centrifugation at 5000 rpm for 15 min. Cells were then harvested, washed twice with 0.1 M phosphate buffer prior to cell preparation for TEM analysis according to Bozzola, 2007 (Bozzola, 2007). Briefly, cells were fixed using 2.5% glutaraldehyde in 0.d1 M phosphate vuffer at 4°C overnight followed by osmium tetroxide post-fixation (1% in 0.1 M phosphate buffer) for 2 h. Then, cells were mixed, solidified with 1.5% agar, cut in to a 0.5-mm^3 cubic, and dehydrated with 35%, 50%, 70%, 90%, and absolute ethanol, respectively. Then, the cell sample was embedded in low-viscosity Spurr resin in absolute ethanol and polymerized with the resin in a stable oven at 70°C for 8h. The sample was thin setioned (60-90 nm) using an ultramicrotome, post-stained with uranyl acetate and lead citrate, and examined with a JEM-2100 JEOL transmission electron microscope (Jeol, Tokyo, Japan) at an accelerating voltage of 120 kV, at Scientific and technological research equipment center, Chulalongkorn University, Thailand. Bacterial cell dimensions wore measured from the electron micrograph images (Neumann et al., 2005) using SemAfore 4.01 program (JEOL Skandinaviska AB, Sweden). Average cell size of

gram-negative cocci was calculated according to Tumber et al., 1993 (Tumber *et al.*,1993) and gram-positive bacilli was calculated according to Neumann et al., 20055 (Neumann *et al.*,2005) by using 20 individually bacterial cells per population from three individual experiments (APPENDIX C).

3.3.5.3 Determination of cell fatty acid composition

Four bacterial strains were grown on HLB liquid medium (100 ml) incubated on a rotary shaker at 250 rpm, at 45°C. After late log phase, they were treated with 20% (v/v) organic solvent, i.e. ethyl acetate for *Deinococcus geothermalis* strain T27 and *Brevibacillus agri* strain 13; *n*-decane for *Bacillus subtilis* strain 45 and chloroform for *Bacillus cereus* strain 4/1, and continue shaking at 250 rpm, at 45°C for another 6 h. The cells harvested and quickly by centrifugation at 5000 rpm for 5 min and washed with 0.85% NaCl. Cells were dried using lyophilization.

Fatty acid extraction

Weigh the cell sample of bacteria with and without organic solvent that contains 26.0 and 25.3 mg for *Deinococcus geothermalis* strain T27; 25.7 and 25.8 mg for *Bacillus subtilis* strain 45; 25.3 and 25.4 mg for *Bacillus cereus* strain 4/1; 25.3 and 25.4 mg for *Brevibacillus agri* strain 13 respectively. 4 % (v/v) conc. sulfuric acid in methanol with 0.01% Butylhydroxytoluene (antioxidant) (2 ml) was added to the vial. Then 100 µl of 10 mg/ml C19:0 was added (as an internal standard). The mixture was incubated at 95°C for 2 h. Then, 1ml of distilled water was added to every sample, followed by 1ml of hexane. The samples were mixed by vortexing for 30 sec. The sample was centrifuged at 1000 rpm for 5

min. The upper phase is collected in a new vial containing a little amount of sodium sulfate for absorbing the residual water. The sample from the vial containing sodium sulfate was transferred to micro-tube and the fatty acid composition of each sample was analyzed using gas chromatography (GC) (Unagul *et al.*,2007).

Analysis of fatty acid composition by GC-FID

Analysis of FAME in hexane was performed using a quadruple GC system with a flame ionization detector (FID) equipped with a split/splitless injector. A CP-Sil 88 capillary column (Chrompack, Middelburg, the Netherland; 50 m; inner diameter 0.25 mm; 0.25 μm film thickness) was used for the separation of the FAME. GC conditions were as followed: the injector temperature was maintained at 240°C and the detector temperature was maintained at 270°C. The injection was splitless; the carrier gas He was use at a flow rate of 2 ml min^{-1}; injection used 1 μl. (Unagul *et al.*,2007).

3.3.5.4 Organic solvent utilization using resting cell technique

To measure the potential of microbial isolates for substrate attenuation in the medium, the test organisms were first grown in MSBY supplemented with ethyl acetate vapor phase for *Deinococcus geothermalis* T27; *n*-decnae or cyclohexane vapor for *Bacillus subtilis* strain 45; toluene vapor for *Bacillus cereus* strain 4/1; *n*-butanol or ethyl acetate vapor for *Brevibacillus agri* strain 13. After 24 h incubation on a rotary shaker at 250 rpm and 45°C, the cells were harvested by centrifugation at 5000 rpm for 10 min. The cells were washed twice with 0.85% NaCl, and then resuspended in the MSB medium to a desired cell density containing each of the individual substrate ethyl acetate for *Deinococcus*

geothermalis T27; *n*-decane or cyclohexane for *Bacillus subtilis* strain 45; toluene for *Bacillus cereus* strain 4/1; *n*-butanol or ethyl acetate for *Brevibacillus agri* strain 13) (Dashti *et al.*,2008).

Batch experiments were conducted in duplicate using 5 ml of culture volume in 15-ml serum bottles sealed with Teflon-coated silicone septa and aluminum crimp cap. The concentration of organic solvent vapor was measured during incubation in a rotary shaker 250 rpm at 45°C by GC.

Organic solvent degradation was measured by performed using a GC system with a flame ionization detector (FID) equipped with a split/splitless injector gas chromatograph equipped with Zebron ZB-WAX column (60m by 0.25 mm, 0.25 μm thickness). The injector, column and detector were maintained at 250, 180 and 255°C, respectively. The injection was split less mode; the carrier gas He was used at a flow rate of 2 ml min^{-1}.

3.3.5.5 Determination of enzymatic activity involving organic solvent utilization

Preparation of bacterium inoculums for enzymatic assay

Cells were grown on HLB liquid medium treated with and without 5% (v/v) ethyl acetate for *Deinococcus geothermalis* T27 (for esterase activities) and *n*-butanol for *Brevibacillus agri* strain 13 (for alcohol dehydrogenase (ADH) activities). Cells were incubated on a rotary shaker at 250 rpm, 45°C for 24 h.

Preparation of cell-free extract for enzyme assays

The cells were collected by centrifugation at 5,000 rpm, 4°C for 15 minutes and washed with cold normal saline. The cells were then resuspended in 3.0 ml of cold 50mM Tris-HCl buffer pH 8.0 and passed though French Pressure Cell Press (Thermo Electron Corporation,USA) two times at 16,000 lb/in^2. The cell derris was then removed by centrifugation at 12,000 rpm, 4°C for 15 minutes. The supernatant obtained was designated as intracellular cell-free extracts which kept at 4°C for esterase and ADH activity assay. All procedures were performed at 4°C.

Determination of protein

The protein concentration was estimated by the method of modified Lowry method (Dulley and Grieve,1975; Lowry *et al.*,1951) with bovine serum albumin as a protein standard.

Reagents

A solution: 2% Na_2CO3 in 0.1 M NaOH containing 0.5% Sodium dodecyl sulfate (SDS)

B solution: 0.5% $CuSO_4.5H_2O$ in 1% Potassium Sodium tartrate

C solution: Phenol reagent

The enzyme solution (0.4 ml) prepared as described in 3.4.5.3 was mixed with 2 ml of a mixed solution A+B (A: B, 50:1) were added. Then, the mixture was incubated for 10 minute at 30°C. Subsequently, 0.2 ml of C solution was added, rapidly mixed and then, incubated for 30 min at 30°C. Finally, to determine the quantity of protein, the absorbance of the sample was measured at 750 nm.

Enzymatic assays

Enzymatic activity was measured spectrophotometrically by monitoring the change in absorbance. The reaction was started by adding the protein followed the reaction for 40 minutes at 25°C.

The esterase activity in *Deinococcus geothermalis* T27 grown with and without ethyl acetate exposure was determined photometrically in a 50 mM potassium phosphate buffer (pH 8.0) solution by using *p*-nitrophenyl acetate solution (100 mM dissolved in dimethyl sulfoxide) at 25°C and 420 nm. One unit of esterase activity was one unit will hydrolyze 1.0 µmole of *o*-nitrophenyl acetate to acetic acid and *p*-nitrophenol per minute at pH 8 at 25°C (Stoops *et al.*,1969). The principle and calculations for esterase enzymatic assay in APPENDIX B.

ADH activity assay (PMS-DCIP reductase activity assay). The ADH activity in *Brevibacillus agri* strain 13 grown with and without ethyl acetate exposure. Phenazine methosulfate (PMS) reductase activity of cell-free extracts were measured spectrophotometrically by coupling with 2, 6-Dichlorophenol indophenol (DCIP) at 25°C. The reduction of oxidized DCIP was measured as the reduction of absorbance at 600 nm. The reaction mixture consisting of: 50 mM Tris-HCl (pH 8.0 or 9.0), 0.4 mM PMS, 0.22 mM DCIP, 10 mM butanol (substrate) and 0 - 50 mM ethylamine (activator) in a total volume of 1 ml. The enzyme activity observed with substrate was subtracted from that obtained without substrate. One unit of enzyme activity was defined as the amount of enzyme catalyzing 1 µmole of substrate per minute under this assay condition. The millimolar extinction coefficients of DCIP at different pH are shown below.

Millimolar extinction coefficients of DCIP at different pHs

pH	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
ε_{600}	1.69	2.23	3.43	11.13	14.72	15.36	15.61	15.83

Enzymatic Assay of NAD^+-dependent ADH. Enzyme assay was performed at 25°C as follows. The enzyme activity of NAD-dependent ADH was measured by the increase in A_{340} of NADH in a reaction mixture (total, 1 ml) consisting of 50 mM Tris-HCl (pH 8.0), 5 mM NAD^+, and enzyme solution. The reaction was started by adding1-butanol at a final concentration of 10 mM. The enzyme activity observed with substrate was subtracted from that obtained without substrate (Toyama *et al.*,1995).

Native-polyacrylamide gel electrophoresis (PAGE) and activity staining. Cell-free extracts obtained from different growth or induction conditions were loaded onto a native-PAGE. After electrophoresis, the gel was stained for quinoprotein ADH activity in a reaction mixture containing phenazine, methosulfate, nitroblue, nitrobluetetrazolium and a mixture of alcohol substrates (1mM ethanol, 10mM 1-butanol and 10mM 1,2-propanediol) and final volume 5mM of NAD^+ (Toyama *et al.*,1995).

3.3.6 Factors involving organic solvent tolerance of the bacteria

3.3.6.1 Effect of divalent ion on cell growth and organic solvent tolerant

Four bacterial strains were grown on HLB medium supplement with Fe^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}, Na^{+} at 2, 10, and 20 mM and incubated on a rotary shaker at 250 rpm, at 45°C for 6 h. At late log phase, an organic solvent was added to the final volume of 5% and 20% (v/v) and continue shaking at 250 rpm, at 45°C for further 6 h. Cell survival was determined as number of viable cells (CFUs) and spectrophotometrically at the absorbance 560 nm.

3.3.6.2 Effect of nutrient on cell growth and organic solvent tolerance

Four bacterial strains were grown in LB, HLB and MSB containing addition al ingredient: 1) (16 mM) glucose, fructose, galactose, sucrose, xylose, rhamnose and mannitol; 2) (16 mM) citrate and succinate; 3) 1% of yeast extract, 1% tryptone and incubated on a rotary shaker at 250 rpm, at 45°C for 6 h. At late log phase, an organic solvent was added to the final volume of 5% and 20% (v/v) and continued shaking at 250 rpm, at 45°C for another 6 h. Cell survival was determined as number of viable cells (CFUs) and spectrophotometrically at the absorbance 560 nm.