# CHAPTER IV

# RESULTS

The results of this thesis are presented in order as shown in the following flow chart.

**4.1 Screening, isolation and identification of organic solvent-tolerant bacteria**

4.1.1 Primary test of the isolates for organic solvent utilization and tolerance (36 isolates)

4.1.2 Secondary test of the isolates for organic solvent utilization and tolerance (12 isolates)

4.1.3 Identification of organic solvent-tolerant bacteria

4.1.3.1 The morphological characteristics

4.1.3.2 The biochemical test

4.1.3.3 16S ribosomal DNA gene

→ **Four bacterial isolates were chosen for further studies.**

**4.2 Characterization of *Deinococcus geothermalis* T27**

**4.3 Characterization of *Bacillus cereus* stain 4/1**

**4.4 Characterization of *Bacillus subtilis* strain 45,**

**4.5 Characterization of *Brevibacillus agri* strain 13**

↓

| Topic number | | | | Topic title |
|---|---|---|---|---|
| *D. geothermalis T27* | *B. cereus* strain 4/1 | *B. subtilis* strain 45 | *Brevibacillus agri* strain 13 | |
| **4.2.1** | **4.3.1** | **4.4.1** | **4.5.1** | **Characterization of organic-solvent tolerant bacteria** |
| 4.2.1.1 | 4.3.1.1 | 4.4.1.1 | 4.5.1.1 | Effect of type and concentration of organic solvent on growth and tolerance |
| 4.2.1.2 | 4.3.1.2 | 4.4.1.2 | 4.5.1.2 | Effect of organic solvent on cell morphology |
| 4.2.1.3 | 4.3.1.3 | 4.4.1.3 | 4.5.1.3 | Effect of organic solvent on fatty acid composition |
| 4.2.1.4 | 4.3.1.4 | 4.4.1.4 | 4.5.1.4 | Organic solvent utilization |
| 4.2.1.5 | 4.3.1.5 | 4.4.1.5 | 4.5.1.5 | Enzymatic activity involving organic solvent utilization |
| | | | | |
| **4.2.2** | **4.3.2** | **4.4.2** | **4.5.2** | **Factors involving of organic-solvent tolerance** |
| 4.2.2.1 | 4.3.2.1 | 4.4.2.1 | 4.5.2.1 | Effect of ions on growth and tolerance |
| 4.2.2.2 | 4.3.2.2 | 4.4.2.2 | 4.5.2.2 | Effect of nutrient on growth and tolerance |

## 4.1 Screening, isolation and identification of organic solvent-tolerant bacteria

Organic solvent-tolerant bacteria can be isolated from soil. Therefore, soil samples were collected from the oil and hydrocarbon contaminated area, hot spring area, agricultural area and natural area (Table 4.1). Enrichment technique was applied to increase quantity of organic solvent-tolerant bacteria. Bacterial screening was then performed on mineral salt basal (MSB) medium and mineral salt basal medium supplemented with 1% yeast extract (MSBY) agar plate incubated at 45°C with toluene or cyclohexane vapor phase. Thirty-six bacterial isolates that grew on toluene or cyclohexane as a sole carbon and energy source were isolated.

### 4.1.1 Primary test of the isolates for organic solvent utilization and tolerance

Thirty-six isolates (Table 4.1) were cultured in mineral salt basal medium and mineral salt basal medium supplemented with 1% yeast extract slant directly overlaid with various types of organic solvent at 45°C for 3 day. The growth of bacteria was observed by colony formation. The bacterial isolates could be divided into 5 groups according to mainly their tolerance towards organic solvents with different log $P_{ow}$ values. (Table 4.2): Group I bacteria tolerate to organic solvent with a broad rang of log $P_{ow}$ value (log $P_{ow}$ 0.7-5.6); Group II bacteria tolerate to organic solvent with log $P_{ow} >$ 3.0; Group III bacteria tolerate to organic solvent with log $P_{ow}$ 2.0-3.0; Group IV bacteria tolerate to organic solvent with log $P_{ow} \leq 2.0$; and Group V bacteria exhibited low tolerance to organic solvent. Within these five groups of organic solvent-tolerant bacteria, twelve bacteria isolates having comparatively good growth in the presence of organic solvent from each group were chosen for further study (Table 4.3). For group III and group V could not choose for further study because bacteria low tolerate to organic solvent.

### 4.1.2 Secondary test of the isolates for organic solvent utilization and tolerance

Twelve bacterial isolates chosen from previous study (primary test) were grown at 45°C for 3 day on mineral salt basal liquid medium, mineral salt basal liquid medium supplemented with 1% yeast extract and mineral salt basal liquid medium supplemented with 16mM glucose. Various types of organic solvent were added as vapor phase or directly into cell suspension (20 % v/v) and number of organic solvent in Table 4.2. The growth of bacteria was observed by measuring the optical density at 560 nm using a spectrophotometer. Four bacteria isolates could be selected as representative(s) of each group because growth of these bacterial greater than others (Fig 4.1-4.3).

**Table 4.1** Source of soil sample used for screening and bacterial isolates obtained

| Enrichment solvent | Source of soil sample | | Bacterial isolate number* |
|---|---|---|---|
| Toluene | Contaminated area | Engine oil, Burirum province | **4/1**, 4/2, 6, 6/2, 6/4 |
| | | Petroleum oil, Krabi province | - |
| | | Machine oil, Krabi province | - |
| | | Machine oil, Bangkok province | 8 |
| | Hot spring area | Mud, Naeklong district,Krabi province | 20, 25/1, 25/2, **T27**, 27/1 |
| | | Mud, Klongtom district, Krabi province | 12, **13**, 22, 36 |
| | | Mud, Jaezon, Lumpang province | - |
| | | Mud, Pnogdoad, Mahongzon province | - |
| | | Mud, Pai, Mahongzon province | - |
| | | Mud, Sunkumpang, Changmai province | **45** |
| | Agricultural area | Casava plantation, Rayong province | - |
| | | Rampatan plantation, Krabi province | - |
| | | Longan plantation, Chanburi province | - |
| | Natural area | Soil source, Chulalongkorn University | - |
| Cyclohexane | Contaminated area | Engine oil, Burirum province | 1, 5/1 |
| | | Petroleum oil, Krabi province | - |
| | | Machine oil, Krabi province | 33 |
| | | Machine oil, Bangkok province | - |
| | Hot spring area | Mud, Naeklong district,Krabi province | 10,23,24,24/1 |
| | | Mud, Klongtom district, Krabi province | 21 |
| | | Mud, Jaezon, Lumpang province | |
| | | Mud, Pnogdoad, Mahongzon province | 3C1,3C3,3C4, 3C5,4C1,4C4, 4C6 |
| | | Mud, Pai, Mahongzon province | |
| | | Mud, Sunkumpang, Changmai province | 44, 46 |
| | Agricultural area | Casava plantation, Rayong province | - |
| | | Rampatan plantation, Krabi province | - |
| | | Longan plantation, Chanburi province | - |
| | Natural area | Soil source, Chulalongkorn University | - |

* The bolded numbers are the bacterial isolate chosen for further studies

**Table 4.2** Primary test for solvent tolerance and solvent utilization on agar overlaid with solvent

| Organic solvents & Log *Pow* | 1. *n*-Decane | 2. *n*-Heptane | 3. *n*-Octane | 4. *n*-Hexane | 5. Diethylphthalate | 6. Cyclohexane | 7. Ethylbenzene | 8. *o*-Xylene | 9. *m*-Xylene | 10. *p*-Xylene | 11. Styrene | 12. Toluene | 13. 1-Heptanol | 14. Benzene | 15. Chloroform | 16. Butyl acetate | 17. *n*-Butanol | 18. Ethyl acetate | Organic solvent used for screening |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5.6 | 4.7 | 4.5 | 3.5 | 3.3 | 3.2 | 3.1 | 3.1 | 3.1 | 3.1 | 3.0 | 2.5 | 2.3 | 2.0 | 2.0 | 1.8 | 0.8 | 0.7 | |
| **Bacterial isolated** | **Group I Tolerance** | | | | | | | | | | | | | | | | | | |
| 3C5 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | + | Cyclohexane |
| 24 | +++ | ++ | +++ | +++ | +++ | ++ | +++ | +++ | ++ | +++ | +++ | ++ | + | - | +++ | +++ | + | - | Cyclohexane |
| **4/1** | +++ | +++ | ++ | + | +++ | ++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | + | +++ | - | + | Cyclohexane |
| 3C4 | ++ | ++ | +++ | + | +++ | +++ | ++ | ++ | ++ | ++ | ++ | +++ | + | + | ++ | ++ | + | + | Cyclohexane |
| 12 | ++ | + | + | +++ | +++ | + | ++ | ++ | + | ++ | +++ | +++ | ++ | +++ | +++ | + | + | + | Toluene |
| 4C4 | + | + | + | + | ++ | + | ++ | + | + | + | ++ | + | + | + | + | + | + | + | Toluene |
| **T27** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | Toluene |
| | **Group I Utilization** | | | | | | | | | | | | | | | | | | |
| 3C5 | - | - | - | - | + | - | - | - | - | - | ++++ | - | + | - | + | + | - | - | Cyclohexane |
| 24 | - | - | + | - | ++ | + | + | + | + | + | ++++ | - | + | - | - | + | + | - | Cyclohexane |
| **4/1** | + | + | + | - | ++ | + | ++ | ++ | ++ | ++ | ++++ | +++ | + | + | + | + | - | - | Cyclohexane |
| 3C4 | + | + | + | + | + | ++ | + | + | + | + | +++ | + | - | + | + | + | + | + | Cyclohexane |
| 12 | + | + | + | + | + | + | - | - | - | - | +++ | - | - | + | + | + | + | + | Toluene |
| 4C4 | + | + | + | - | ++ | - | + | + | + | + | +++ | + | + | + | + | + | - | - | Toluene |
| **T27** | + | + | + | - | + | + | + | + | + | + | +++ | ++ | + | + | + | + | - | - | Toluene |

+, growth; -, no growth

**Table 4.2** (continued)

| Organic solvents & Log $P_{ow}$ | 1. *n*-Decane | 2. *n*-Heptane | 3. *n*-Octane | 4. *n*-Hexane | 5. Diethylphthalate | 6. Cyclohexane | 7. Ethylbenzene | 8. *o*-Xylene | 9. *m*-Xylene | 10. *p*-Xylene | 11. Styrene | 12. Toluene | 13. 1-Heptanol | 14. Benzene | 15. Chloroform | 16. Butyl acetate | 17. *n*-Butanol | 18. Ethyl acetate | Organic solvent used for screening |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5.6 | 4.7 | 4.5 | 3.5 | 3.3 | 3.2 | 3.1 | 3.1 | 3.1 | 3.1 | 3.0 | 2.5 | 2.3 | 2.0 | 2.0 | 1.8 | 0.8 | 0.7 | |
| **Bacterial isolated** | **Group II Tolerance** | | | | | | | | | | | | | | | | | | |
| 24/1 | +++ | ++ | ++ | - | +++ | + | ++ | ++ | ++ | ++ | +++ | + | + | + | + | + | ++ | + | Cyclohexane |
| 23 | +++ | +++ | +++ | +++ | +++ | +++ | - | - | - | + | + | ++ | + | +++ | +++ | - | - | - | Cyclohexane |
| 21 | +++ | ++ | +++ | - | +++ | + | ++ | ++ | + | ++ | +++ | ++ | ++ | +++ | ++ | + | + | + | Cyclohexane |
| 25/1 | +++ | +++ | +++ | ++ | +++ | + | + | ++ | ++ | + | ++ | ++ | + | +++ | + | + | + | - | Toluene |
| 6 | ++ | +++ | +++ | +++ | +++ | +++ | + | + | + | ++ | + | ++ | + | +++ | +++ | +++ | - | + | Toluene |
| **45** | +++ | + | +++ | ++++ | ++++ | + | + | + | + | + | + | + | + | ++++ | ++++ | + | - | + | Toluene |
| | **Group II Utilization** | | | | | | | | | | | | | | | | | | |
| 24/1 | - | - | + | - | + | - | ++ | ++ | + | + | ++++ | ++ | - | - | ++ | + | + | + | Cyclohexane |
| 23 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Cyclohexane |
| 21 | ++ | + | ++ | - | + | + | + | ++ | ++ | ++ | ++ | ++ | + | + | - | + | - | + | Cyclohexane |
| 25/1 | ++ | + | + | + | ++ | ++ | + | + | + | ++ | +++ | ++ | ++ | - | + | + | + | - | Toluene |
| 6 | - | - | - | + | + | + | + | + | + | + | +++ | + | + | + | + | + | - | + | Toluene |
| **45** | ++ | ++ | ++ | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | ++ | + | + | - | + | Toluene |

+, growth; -, no growth

**Table 4.2** (continued)

| Organic solvents & Log $P_{ow}$ | 1. *n*-Decane | 2. *n*-Heptane | 3. *n*-Octane | 4. *n*-Hexane | 5. Diethylphthalate | 6. Cyclohexane | 7. Ethylbenzene | 8. *o*-Xylene | 9. *m*-Xylene | 10. *p*-Xylene | 11. Styrene | 12. Toluene | 13. 1-Heptanol | 14. Benzene | 15. Chloroform | 16. Butyl acetate | 17. *n*-Butanol | 18. Ethyl acetate | Organic solvent used for screening |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5.6 | 4.7 | 4.5 | 3.5 | 3.3 | 3.2 | 3.1 | 3.1 | 3.1 | 3.1 | 3.0 | 2.5 | 2.3 | 2.0 | 2.0 | 1.8 | 0.8 | 0.7 | |
| **Bacterial isolated** | **Group III Tolerance** | | | | | | | | | | | | | | | | | | |
| 3C3 | +++ | + | +++ | ++ | +++ | + | ++ | + | + | +++ | + | + | ++ | + | + | + | - | - | Cyclohexane |
| 33 | +++ | - | + | - | +++ | +++ | - | - | - | + | + | - | + | +++ | - | + | - | - | Cyclohexane |
| 46 | +++ | + | + | ++++ | ++++ | + | + | + | + | + | ++ | + | + | + | ++++ | + | - | - | Cyclohexane |
| 36 | +++ | + | + | +++ | +++ | +++ | + | + | + | + | + | + | + | + | + | + | - | + | Toluene |
| 22 | +++ | - | - | +++ | +++ | - | + | + | + | ++ | + | - | - | +++ | + | ++ | - | - | Toluene |
| 20 | ++ | +++ | +++ | +++ | +++ | - | + | + | + | + | + | ++ | + | ++ | ++ | + | + | + | Toluene |
| 8 | +++ | +++ | +++ | + | +++ | - | +++ | + | + | + | + | + | + | + | + | + | - | + | Toluene |
| 6/2 | + | ++++ | + | - | ++++ | + | + | + | + | + | ++ | + | ++ | + | + | + | - | - | Toluene |
| | **Group III Utilization** | | | | | | | | | | | | | | | | | | |
| 3C3 | ++ | - | ++ | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + | + | - | - | Cyclohexane |
| 33 | +++ | - | + | - | +++ | +++ | - | - | - | + | + | - | + | +++ | - | + | - | - | Cyclohexane |
| 46 | ++ | ++ | ++ | + | ++ | + | ++ | ++ | ++ | ++ | ++++ | ++ | ++ | ++ | - | ++ | - | - | Cyclohexane |
| 36 | + | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | ++ | + | + | - | + | Toluene |
| 22 | +++ | - | - | +++ | +++ | - | + | + | + | ++ | + | - | - | +++ | + | ++ | - | - | Toluene |
| 20 | - | - | - | - | + | - | + | + | + | + | + | + | + | + | + | + | + | + | Toluene |
| 8 | - | - | + | - | +++ | - | ++ | ++ | ++ | ++ | ++++ | ++ | ++ | - | - | - | - | - | Toluene |
| 6/2 | + | + | + | - | + | + | + | + | + | + | +++ | ++ | + | + | + | + | - | - | Toluene |

+, growth; -, no growth

**Table 4.2** (continued)

| Organic solvents & Log $P_{ow}$ | 1. *n*-Decane | 2. *n*-Heptane | 3. *n*-Octane | 4. *n*-Hexane | 5. Diethylphthalate | 6. Cyclohexane | 7. Ethylbenzene | 8. *o*-Xylene | 9. *m*-Xylene | 10. *p*-Xylene | 11. Styrene | 12. Toluene | 13. 1-Heptanol | 14. Benzene | 15. Chloroform | 16. Butyl acetate | 17. *n*-Butanol | 18. Ethyl acetate | Organic solvent used for screening |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5.6 | 4.7 | 4.5 | 3.5 | 3.3 | 3.2 | 3.1 | 3.1 | 3.1 | 3.1 | 3.0 | 2.5 | 2.3 | 2.0 | 2.0 | 1.8 | 0.8 | 0.7 | |
| **Bacterial isolated** | **Group IV Tolerance** | | | | | | | | | | | | | | | | | | |
| 34 | + | - | - | - | +++ | + | + | + | + | ++ | +++ | ++ | ++ | ++ | ++ | +++ | +++ | - | Cyclohexane |
| 10 | - | +++ | - | +++ | - | +++ | + | + | + | + | ++ | ++ | ++ | - | ++ | ++ | ++ | ++ | Cyclohexane |
| 4C1 | + | + | + | + | +++ | + | ++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | +++ | +++ | +++ | + | Cyclohexane |
| 5/1 | + | + | +++ | + | +++ | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++++ | + | + | + | + | Cyclohexane |
| 4/2 | + | + | - | +++ | +++ | + | ++ | + | + | ++ | ++ | + | + | + | + | ++ | - | - | Cyclohexane |
| 4C6/1 | - | + | + | + | + | + | + | + | + | +++ | +++ | +++ | + | + | + | - | + | ++ | Cyclohexane |
| **13** | + | + | + | + | +++ | +++ | +++ | ++ | ++ | +++ | +++ | ++ | ++ | - | +++ | ++ | ++ | ++ | Toluene |
| 27/1 | +++ | + | + | + | +++ | + | + | + | + | + | ++ | + | ++ | ++ | + | + | + | - | Toluene |
| 6/4 | - | + | ++ | - | ++ | - | + | - | - | + | + | - | + | ++++ | + | + | - | - | Toluene |
| 25/2 | - | - | - | - | ++ | + | - | ++ | + | + | ++ | ++ | ++ | - | +++ | - | - | - | Toluene |
| | **Group IV Utilization** | | | | | | | | | | | | | | | | | | |
| 34 | + | - | - | - | +++ | + | + | + | + | ++ | +++ | ++ | ++ | ++ | ++ | +++ | +++ | - | Cyclohexane |
| 10 | - | +++ | - | +++ | - | +++ | + | + | + | + | ++ | ++ | ++ | - | ++ | ++ | ++ | ++ | Cyclohexane |
| 4C1 | + | + | + | + | ++ | + | ++ | ++ | ++ | ++ | ++++ | +++ | - | + | + | + | ++ | + | Cyclohexane |
| 5/1 | + | + | + | + | + | + | + | - | - | + | ++ | + | + | + | + | + | + | + | Cyclohexane |
| 4/2 | - | - | - | + | - | + | - | + | - | + | ++++ | - | - | - | + | - | - | - | Cyclohexane |
| 4C6/1 | - | - | - | - | - | + | + | + | + | - | +++ | + | - | + | + | + | + | - | Cyclohexane |
| **13** | + | - | + | - | + | + | + | - | - | + | ++ | - | + | - | + | - | + | - | Toluene |
| 27/1 | + | ++ | ++ | + | ++ | ++ | ++ | + | + | ++ | + | ++ | + | + | + | ++ | + | - | Toluene |
| 6/4 | - | - | + | - | - | - | + | - | - | - | ++++ | - | - | - | + | + | - | - | Toluene |
| 25/2 | - | - | - | - | ++ | + | - | ++ | + | + | ++ | ++ | ++ | - | +++ | - | - | - | Toluene |

+, growth; -, no growth

**Table 4.2** (continued)

| Organic solvents & Log $P_{ow}$ | 1. *n*-Decane | 2. *n*-Heptane | 3. *n*-Octane | 4. *n*-Hexane | 5. Diethylphthalate | 6. Cyclohexane | 7. Ethylbenzene | 8. *o*-Xylene | 9. *m*-Xylene | 10. *p*-Xylene | 11. Styrene | 12. Toluene | 13. 1-Heptanol | 14. Benzene | 15. Chloroform | 16. Butyl acetate | 17. *n*-Butanol | 18. Ethyl acetate | Organic solvent used for screening |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5.6 | 4.7 | 4.5 | 3.5 | 3.3 | 3.2 | 3.1 | 3.1 | 3.1 | 3.1 | 3.0 | 2.5 | 2.3 | 2.0 | 2.0 | 1.8 | 0.8 | 0.7 | |
| **Bacterial isolated** | **Group V Tolerance** | | | | | | | | | | | | | | | | | | |
| 3C1 | ++ | + | + | + | +++ | + | + | + | + | + | + | + | + | + | + | + | + | + | Cyclohexane |
| 44 | + | + | +++ | + | +++ | + | + | + | + | + | + | + | + | + | + | + | + | - | Cyclohexane |
| 16/2 | - | - | - | - | ++ | - | - | - | + | - | - | - | - | - | - | - | - | - | Toluene |
| 14 | +++ | + | + | - | +++ | + | + | + | + | + | + | + | + | + | + | + | + | - | Toluene |
| 1 | +++ | - | - | - | +++ | - | - | - | - | - | - | - | - | - | - | + | - | - | Toluene |
| | **Group V Utilization** | | | | | | | | | | | | | | | | | | |
| 3C1 | + | + | + | + | + | + | + | + | + | + | + | ++ | + | + | + | + | + | + | Cyclohexane |
| 44 | + | + | + | + | ++ | + | ++ | ++ | + | + | ++ | ++ | + | + | + | + | + | - | Cyclohexane |
| 16/2 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Toluene |
| 14 | - | - | - | - | + | + | + | + | + | + | ++++ | + | + | + | + | + | + | - | Toluene |
| 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Toluene |

+, growth; -, no growth

**Table 4.3** Organic solvent tolerant bacterial isolates chosen for further study

| Group of bacterial isolates | Bacterial isolates chosen for further study |
|---|---|
| **I**<br>**Organic solvent with a broad rang log $P_{ow}$ value** | 12 3C4 **4/1** **T27** |
| **II**<br>**Organic solvent with log $P_{ow}$ > 3.0 value** | 25/1 21 6 **45** |
| **III**<br>**Organic solvent with log $P_{ow}$ 2.0-3.0 value** | - |
| **IV**<br>**Organic solvent with log $P_{ow}$ < 2.0 value** | **13** 4C6/1 4C1 5/1 |
| **V**<br>**Low tolerate to organic solvent** | - |

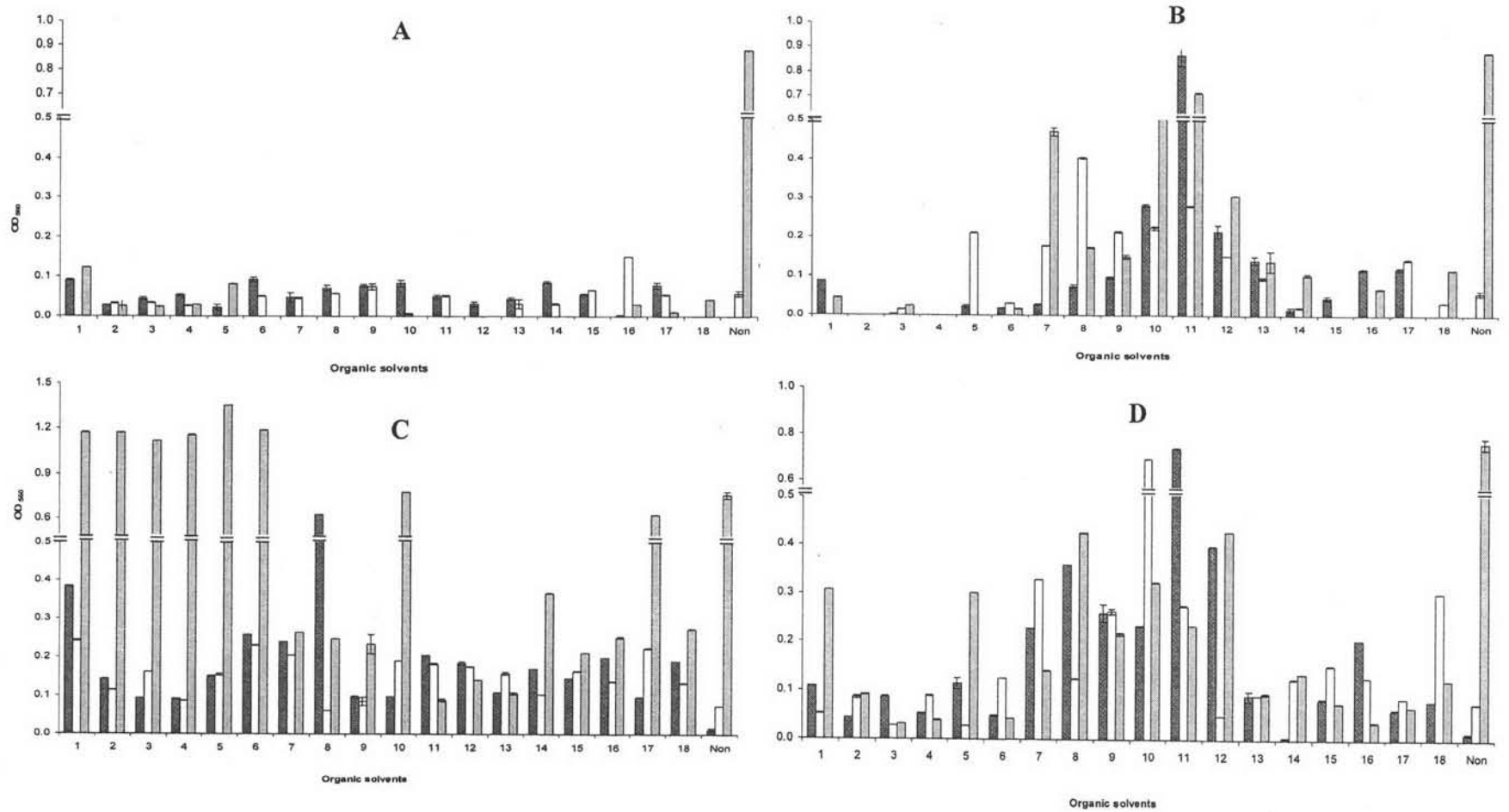


**Fig 4.1** Secondary test of bacterial isolates in group I (according to Table 4.3) for their tolerance and utilization of solvent a broad rang of log $P_{ow}$ value. Bacterial isolate 12 (A, B) and isolate 3C4 (C, D) were grown at 45 °C for 3 days in MSB (■), MSBG (□), MSBY (▨) medium in presence of solvent vapor (A, C) and directly added (5%, v/v) (B, D). Data are means of the results from at least three individual experiments. The numbers on x-axis represent type of organic solvent shown in Table 4.4.

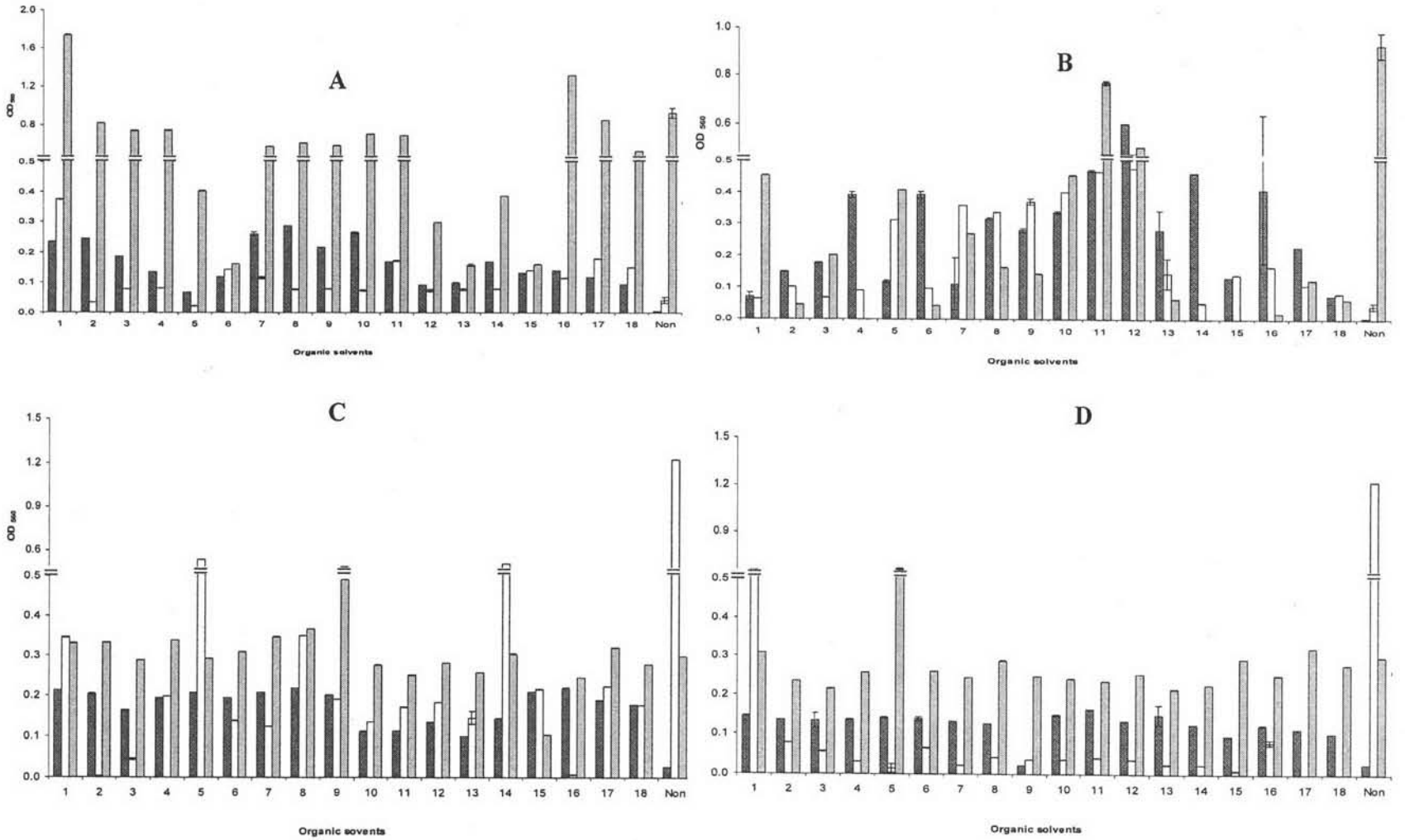


**Fig 4.1** (continued) Bacterial isolate 4/1 (A, B) and T27 isolate (C, D) were grown at 45 °C for 3 days in MSB (■), MSBG (□), MSBY (▨) medium in presence of solvent vapor (A, C) and directly added (5%, v/v) (B, D). Data are means of the results from at least three individual experiments. The numbers on x-axis represent type of organic solvent shown in Table 4.4.

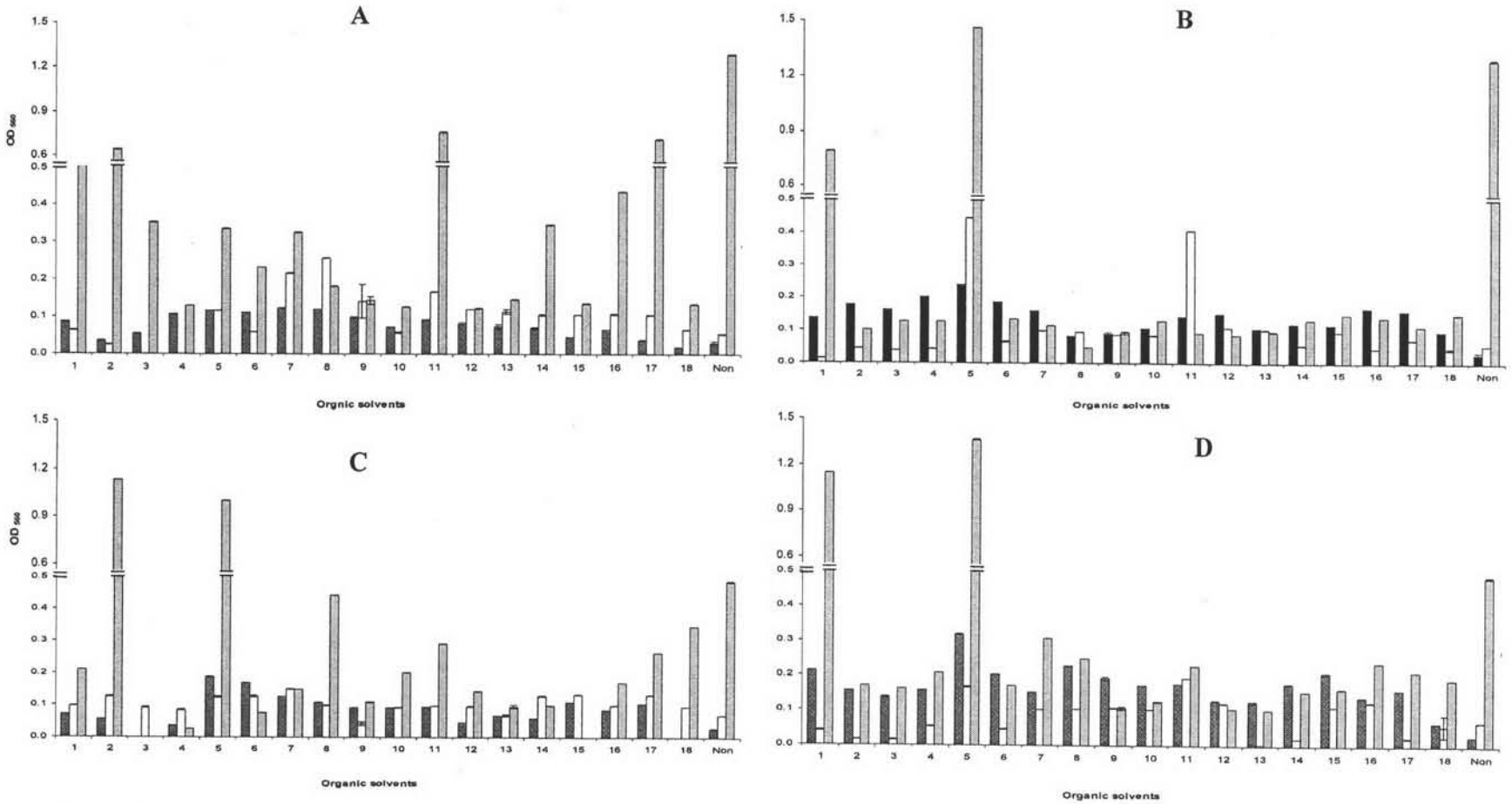


**Fig 4.2** Secondary tests of bacterial isolates in group II (according to Table 4.3) for their tolerance and utilization of organic solvent with log $P_{ow} > 3.0$ value. Bacterial isolate 25/1 (A, B) and isolate 21 (C, D) were grown at 45 °C for 3 days in MSB (■), MSBG (□), MSBY (▨) medium in presence of solvent vapor (A, C) and directly added (5%, v/v) (B, D). Data are means of the results from at least three individual experiments. The numbers on x-axis represent type of organic solvent shown in Table 4.4.

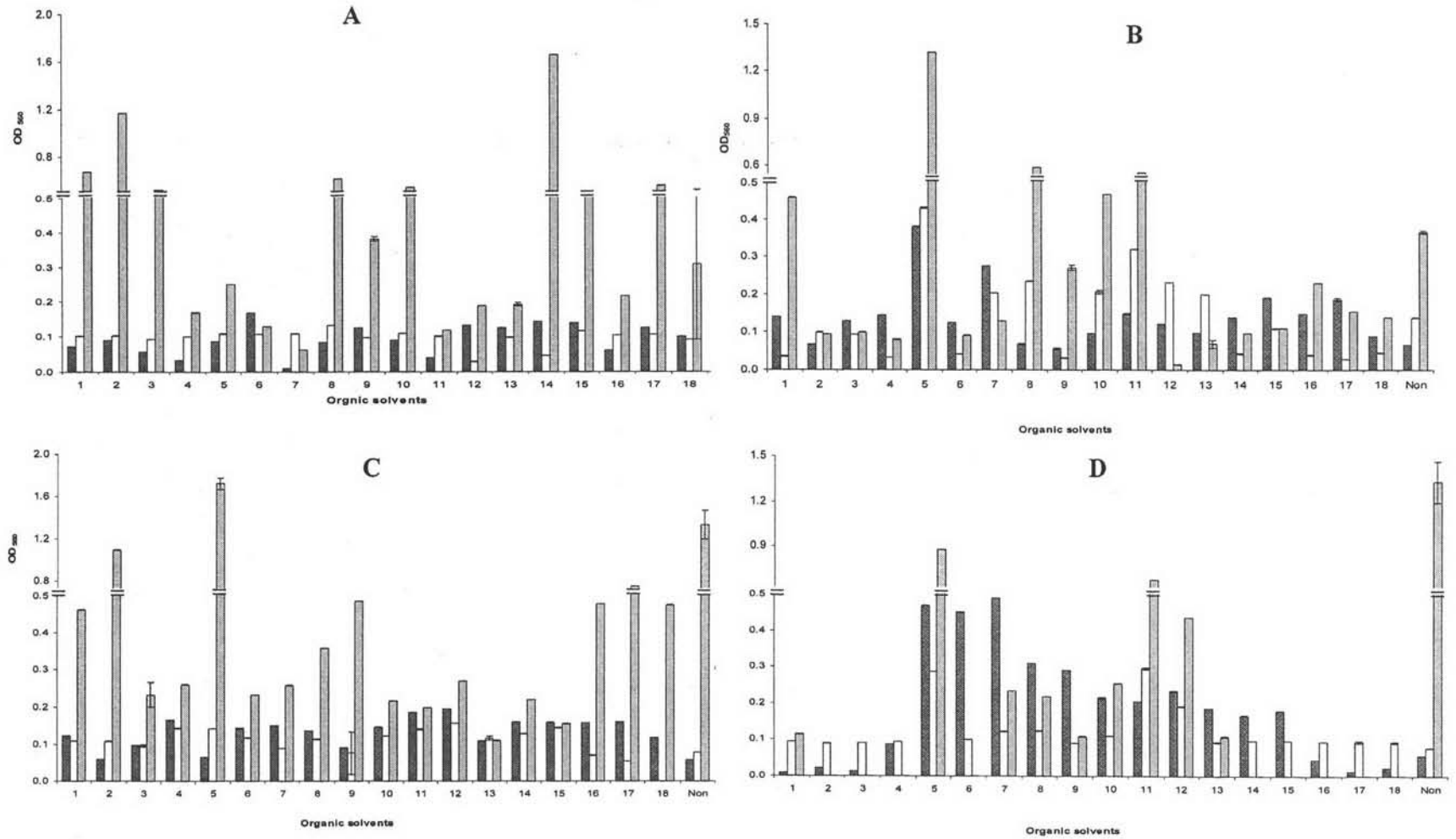


**Fig 4.2** (continued) Bacterial isolate 6 (A, B) and isolate 45 (C, D) were grown at 45 °C for 3 days in MSB (■), MSBG (□), MSBY (▨) medium in presence of solvent vapor (A, C) and directly added (5%, v/v) (B, D). Data are means of the results from at least three individual experiments. The numbers on x-axis represent type of organic solvent shown in Table 4.4.

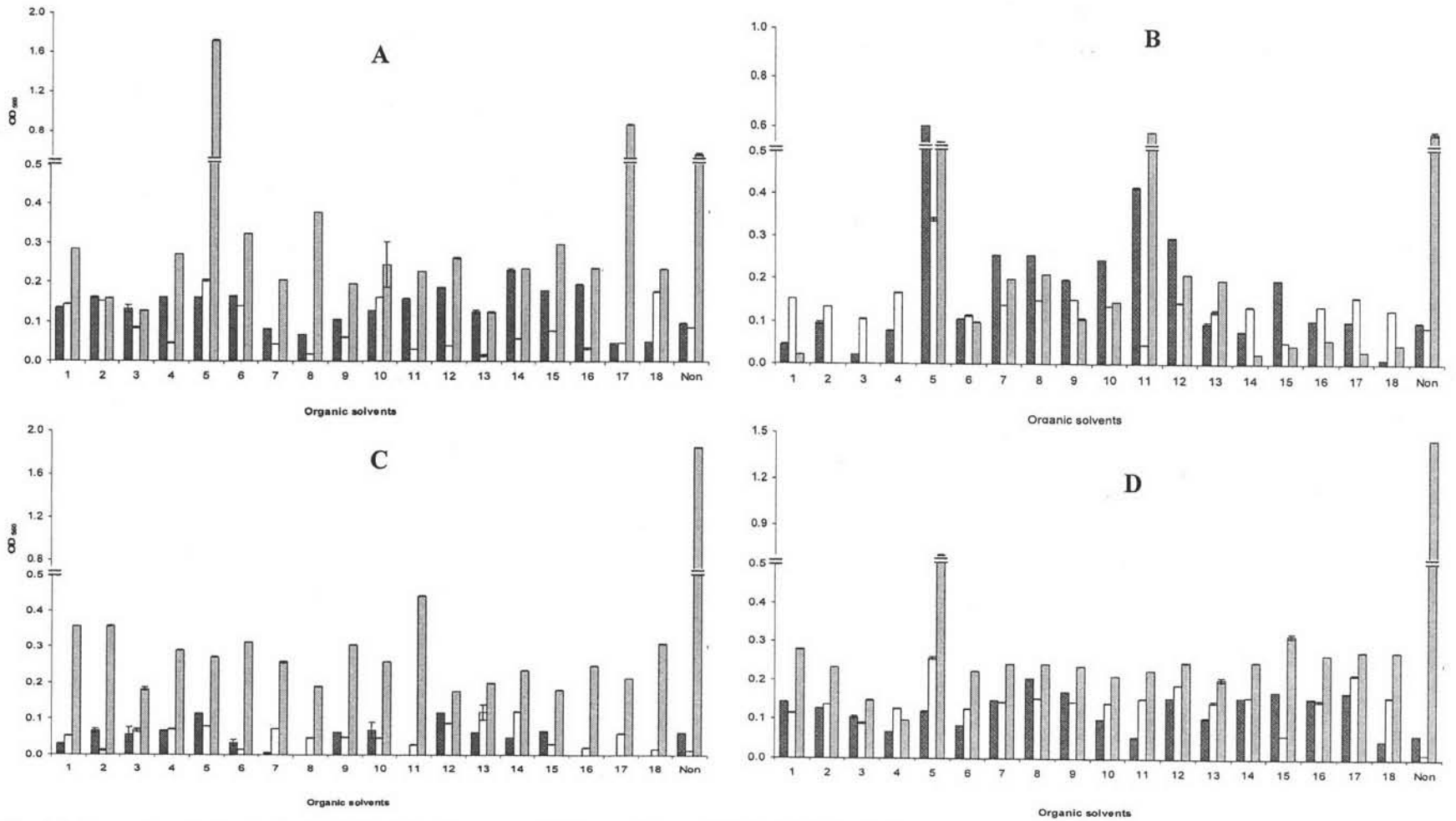


**Fig 4.3** Secondary tests of bacterial isolates in group IV (according to Table 4.3) for their tolerance and utilization of organic solvent with log $P_{ow} < 2$. Bacterial isolate 13 (A, B) and isolate 4C6/1 (C, D) were grown at 45 °C for 3 days in MSB (■), MSBG (□), MSBY (▨) medium in presence of solvent vapor (A, C) and directly added (5%, v/v) (B, D). Data are means of the results from at least three individual experiments. The numbers on x-axis represent type of organic solvent shown in Table 4.4.

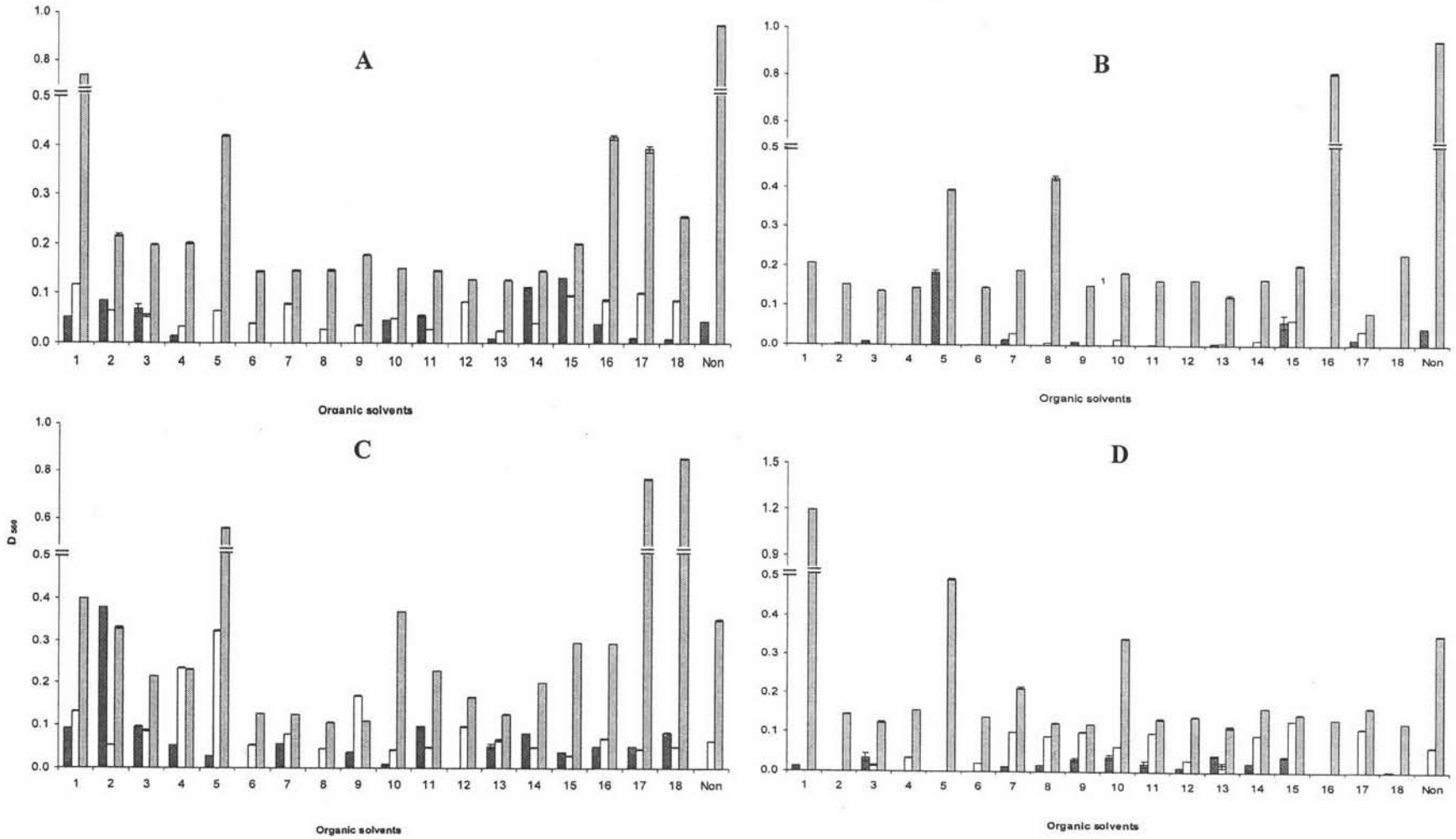


**Fig 4.3** (continued) Bacterial isolate 4C1 (A, B) and isolate 5/1 (C, D) were grown at 45 °C for 3 days in MSB (■), MSBG (□), MSBY (▨) medium in presence of solvent vapor (A, C) and directly added (5%, v/v) (B, D). Data are means of the results from at least three individual experiments. The numbers on x-axis represent type of organic solvent shown in Table 4.4.

### 4.1.3 Identification of organic solvent-tolerance bacteria

#### 4.1.3.1 The morphological characteristics of organic-solvent tolerance bacteria

Thirty-six bacterial strains were identified for their morphological characteristic by bacterial gram staining (Appendix A). Furthermore, the characteristic of cell colonis on Luria Bertani (as described in Method 3.3.4.1) agar were observed. The characteristics of the four bacterial isolates (isolate T27, 4/1, 45, and 13) are shown in Table 4.4.

#### 4.1.3.2 The biochemical test of organic-solvent tolerance bacteria

The biochemical tests were used to identify the species of bacteria (Appendix A). The biochemical characterization results (Table 4.5) were compared to the Manual of Clinical Microbiology supporting by the laboratory of Institution for Scientific Research, Department of Medical Sciences, and Ministry of Public Health in Thailand, and used to identify type of the bacteria.

#### 4.1.3.3 16S ribosomal DNA gene for organic-solvent tolerance bacterial identification

The determination of 16S ribosomal DNA gene sequence was used to identify organic solvent tolerant bacteria. The specific primers (63f and 1387r) (Marchesi *et al.*,1998) were used to amplify the target sequence as described in Materials and Methods 3.3.3.3. These primers were found to be useful for 16S rDNA gene identification of bacterial species from environmental samples than other PCR primers. After PCR amplification, PCR products were analyzed by using 0.8% agarose gel electrophoresis. The length of the amplified 16S rDNA fragment product was

approximately 1.3 kbps. Subsequently, the blastN program was used to compare and analyze the 16S rDNA sequence against NCBI database (www.ncbi.nlm.gov). The result of alignment of 16S rDNA sequences of 4 bacterial isolates in this study are shown in Table 4.6.

**Table 4.4** Morphological characteristic of bacteria

| Groups | Bacterial Isolated | Morphology | | Colony characteristics | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Gram stain | Cell shape | Color | From | Elevation | Margin | Surface |
| I | **4/1** | **Positive** | **Bacilli** | **Cream** | **Circular** | **Convex** | **Entire** | **Smooth** |
| | 12 | Positive | Bacilli | Cream | Circular | Convex | Entire | Smooth |
| | 24 | Positive | Rod | Yellow-Cream | Circular | Flat | Entire | Rough |
| | **T27** | **negative** | **Cocci** | **Pink** | **Circular** | **Convex** | **Entire** | **Smooth** |
| | 3C5 | Positive | Bacilli | Transparent | Circular | Convex | Undulate | Smooth |
| | 3C4 | Positive | Bacilli | Cream | Circular | Convex | Undulate | Smooth |
| | 4C4 | Positive | Bacilli | Cream | Circular | Convex | Entire | Smooth |
| II | 6 | Positive | Rod | Yellow | Circular | Flat | Undulate | Rough |
| | 23 | Positive | Coccobacilli | Transparent | Circular | Convex | Entire | Smooth |
| | 25/1 | Positive | Coccobacilli | Yellow | Circular | Convex | Entire | Smooth |
| | 21 | Positive | Rod | Yellow-Cream | Circular | Flat | Entire | Smooth |
| | 24/1 | Positive | Bacilli | Yellow-Cream | Circular | Flat | Entire | Rough |
| | **45** | **Positive** | **Bacilli** | **Yellow-Cream** | **Circular** | **Convex** | **Entire** | **Smooth** |
| III | 6/2 | Positive | Bacilli | White be turbid | Circular | Convex | Entire | Smooth |
| | 8 | Positive | Rod | Yellow-Cream | Circular | Convex | Entire | Smooth |
| | 20 | Positive | Coccobacilli | Yellow-Cream | Circular | Flat | Undulate | Rough |
| | 22 | Positive | Rod | Cream | Circular | Flat | Entire | Rough |
| | 33 | Positive | Cocci | Transparent | Circular | Convex | Entire | Smooth |
| | 36 | Positive | Coccobacilli | Transparent | Circular | Convex | Entire | Smooth |
| | 46 | Positive | Bacilli | Cream | Circula | Flat | Entire | Smooth |
| | 3C3 | Positive | Bacilli | Transparent | Circular | Convex | Entire | Smooth |

PG; poor growth, - ; non detect, NR; not reported

**Table 4.4** (continue) Morphological characteristic of bacteria

| Groups | Bacterial Isolated | Morphology | | Colony characteristics | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Gram stain | Cell shape | Color | From | Elevation | Margin | Surface |
| IV | 4/2 | Positive | Bacilli | Yellow | Circular | Flat | Entire | Rough |
| | 5/1 | Positive | Bacilli | White be turbid | Circular | Convex | Entire | Rough |
| | 6/4 | Positive | Bacilli | White | Circular | Convex | Entire | Smooth |
| | 10 | Positive | Rod | Cream | Circular | Convex | Entire | Smooth |
| | **13** | **Positive** | **Bacilli** | **Cream** | **Circular** | **Flat** | **Entire** | **Smooth** |
| | 25/2 | Positive | Rod | Transparent | spindle | Convex | Entire | Smooth |
| | 27/1 | Positive | Rod | White | Circular | Convex | Entire | Smooth |
| | 34 | Positive | Bacilli | Cream | Circular | Convex | Entire | Smooth |
| | 4C1 | Positive | Coccobacilli | White be turbid | Circular | Convex | Entire | Smooth |
| | 4C6 | Positive | Coccobacilli | Cream | Circular | Convex | Entire | Smooth |
| V | 1 | Positive | Bacilli | Pink | Circular | Convex | Entire | Smooth |
| | 14 | Positive | Bacilli | Yellow-Cream | Circular | Flat | Undulate | Rough |
| | 16/2 | Positive | Cocci | Cream be turbid | Circular | Convex | Undulate | Smooth |
| | 44 | Positive | Bacilli | Cream | Circular | Convex | Entire | Smooth |
| | 3C1 | Positive | Rod | White | Circular | Convex | Undulate | Smooth |

PG; poor growth, - ; non detect, NR; not reported

**Table 4.5** The biochemical test result of four bacterial isolates were chosen

| **Biochemical Tests** | **Bacterial isolates** | | | |
|---|---|---|---|---|
| | **No.45** | **No.4/1** | **No.13** | **No.27** |
| Gram stain | + bacilli | + bacilli | + bacilli | + cocci |
| Hemolysis | β | β | β | |
| Anaerobic growth | | + | | |
| TSI | K/A | K/A | K/K | N/N |
| $H_2S$ | | | | - |
| Catalase | + | + | + | + |
| Oxidase | + | + | + | + |
| Motility | + | + | + | - |
| Indole | | | | |
| Citrate | + | + | - | - |
| Urease | + | + | - | - |
| Nitrate | + | + | - | - |
| $N_2$ Gas | | | | - |
| Esculin | | | | - |
| 42°C(Growth) | | | | |
| Acetate | | | | |
| VP | + | + | - | + |
| Gelatinase | + | + | + | +w |
| Egg Yolk | - | + | - | |
| Glucose/Gas | + | + | + | |
| Lactose | | | | - |
| Maltose | | | | |
| Mannitol | + | - | + | - |
| D-Xylose | + | - | - | - |
| Sucrose | | | | |
| L-Arabinose | + | - | - | |
| Fructose | | | | - |
| Trehalose | + | + | + | |
| Starch hydrolysis | + | + | + | + |
| LDA | | - | | |
| Lysine | | | | |
| Arginine | - | | - | - |
| Ornithine | | - | | |
| Alkaline phos. | | | | |
| Camp test | | | | |
| Flagella | | | | |
| Bacterial identification | *Bacillus subtilis* | *Bacillus cereus* | *Bacillus circulans* | *Dienococcus sp.* |

**Table 4.6** Comparison of 16S ribosomal DNA gene for bacterial identification

| Bacterial Isolated | Blast | | | Results |
|---|---|---|---|---|
| | % Similarity | Accession No. | Organism | |
| 4/1 | 98% | AB244465.1 | *Bacillus cereus* strain: C10-1 | *Bacillus cereus* |
| | | CP000001.1 | *Bacillus cereus* E33L | |
| | | AY138279.1 | *Bacillus cereus* strain 2000031513 | |
| T27 | 99% | AJ864721.1 | *Deinococcus geothermalis* strain E50051 | *Deinococcus geothermalis* |
| | | CP000359.1 | *Deinococcus geothermalis* DSM 11300 | |
| | | AJ000002.1 | *Deinococcus geothermalis* strain E50053 | |
| 45 | 100% | EU221342.1 | *Bacillus subtilis* strain PAB1C4 | *Bacillus subtilis* |
| | | EU221340.1 | *Bacillus subtilis* strain YM1C11 | |
| | | AB305020.1 | *Bacillus subtilis* strain: BS1 | |
| 13 | 99% | AJ586388.1 | *Brevibacillus agri* strain R-20121 | *Brevibacillus agri* |
| | | AB039334.1 | *Brevibacillus agri* strain:M1-5 | |
| | | AB112716.1 | *Brevibacillus agri* strain:DSM 6348T | |

## 4.2 *Deinococcus geothermalis* T27

### 4.2.1 Characterization of organic-solvent toleran

#### 4.2.1.1 Effect of types and concentrations of organic solvent on growth and tolerance

Growths of *D. geothemalis* T27 were monitored after solvent shock with various types and quantities of organic solvents. Cells were grown to late log phase (about 6 h) before the solvent was added (Method 3.3.5.1). Cell growth and cell survival was assessed by viable cell number ($CFU.ml^{-1}$). Cell survival after solvent shock are displayed in Fig 4.4, showing a relationship between cell number and the amount of solvent added (5% (v/v) and 20% (v/v)) to the media compared to that in the absenc of organic solvent. Organic solvents were re-classified into three groups:

1) organic solvent with log $P_{ow} \geq 3.0$; (*n*-decane (5.6), diethylphthalate (3.3), cyclohexane (3.2), *o*-xylene (3.1), *m*-xylene (3.1), *p*-xylene (3.1)

2) organic solvent with log $P_{ow}$ 2-3; (styrene (3.0), toluene (2.5), 1-hepanol (2.3), benzene (2.0)

3) organic solvent with log $P_{ow} \leq 2$ (butyl acetate (1.8), *n*-butanol (0.8), ethyl acetate (0.7).

The results demonstrated that *D. geothemalis* T27 exhibited tolerance to a broad rage of organic solvent. When exposed to solvent with log $P_{ow}$ greater than 3, i.e. *n*-decane (5.6), and diethyl phthalate (3.3), the number of viable cells only slightly decreased at both solvent concentrations tested suggesting a relative high tolerance of cells. Although the log $P_{ow}$ value of cyclohexane (3.2) is close to that of diethylphathalate, cells were adversely affected by the solvent exposure in that cell viability was decreased by ten times 3-h of solvent addition. The exposure of isomers of

xylene (3.1) showed comparatively similar results in that they exhibited toxicity to cells causing cell viability to significantly decrease (Fig 4.4 A, B). The investigation was also carried out with solvents with a log $P_{ow}$ value between 2-3. Styrene (3.0) and *n*-heptanol (2.3) were clearly toxic to cells at both solvent concentrations. The viable cell numbers were rapidly decreased when the solvent was added. While toluene and benzene showed a modest toxicity effect to cells. A gradual increase of viable cell numbers within 1 h of benzene addition suggested that cells could adapt to the presence of high concentration of benzene (Fig 4.4 C, D).

Further investigation on solvent tolerance of *D. geothemalis* T27 was extended using solvent with a log $P_{ow}$ value lower 2.0. *D. geothemalis* T27 demonstrated a sensitivity to *n*-butanol, which it showed and high tolerance towards butyl acetate and ethyl acetate when exposed to butyl acetate and ethyl acetate; Cells were able to tolerate the solvent toxicity, although slight decrease of the numbers of viable cells was observed immediately after solvent addition (Fig 4.4 E, F).

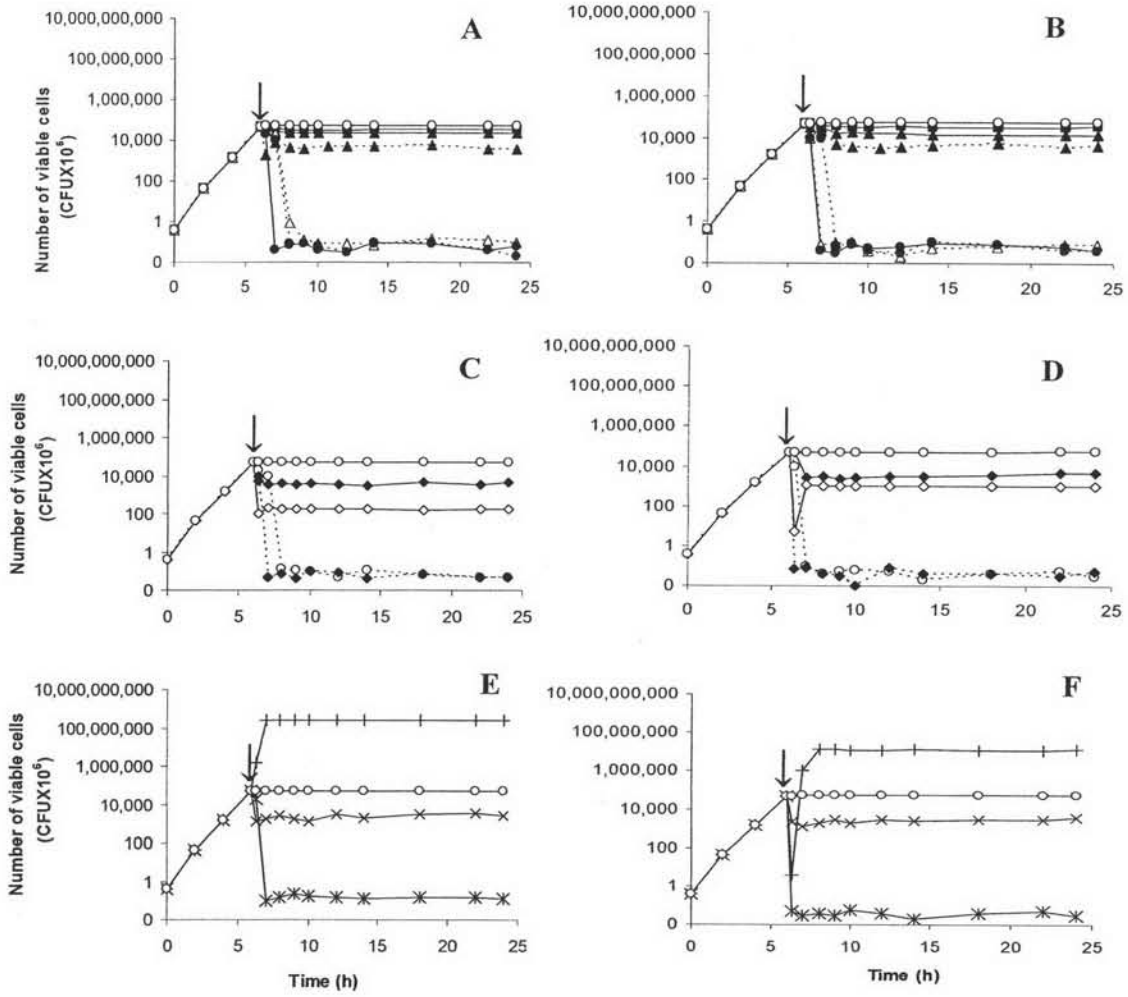


**Fig 4.4** Growth inhibitions of *D. geothemalis* T27 when exposed to organic solvent with log $P_{ow} \geq 3$ (A, B), log $P_{ow}$ 2-3 (C, D) and log $P_{ow} \leq 2$ (E, F). Organic solvent was added (↓) to the final volume of 5% (A, C, E) and 20% (B, D, F). Cell growth in absence to organic solvent (○) was carried out as a positive control.

**A, B:** Cell exposure to solvent with log $P_{ow} \geq 3$

- ■ *n*-decane
- ▲ diethylphthalate
- --▲-- cyclohexane
- --Δ-- *o*-xylene
- ● *m*-xylene
- --●-- *p*-xylene

**C, D:** Cell exposure to solvent with log $P_{ow}$ 2-3

- --○-- styrene
- ♦ toluene
- --♦-- 1-heptanol
- ◊ benzene

**E, F:** Cell exposure to solvent with log $P_{ow} \leq 2$

- \+ butyl acetate
- * *n*-butanol
- × ethyl acetate

#### 4.2.1.2 Effect of organic solvent on cell morphology

*D. geothermalis* T27 demonstrated unique ability to tolerate ethyl acetate. Effect of organic solvent stress on morphology of *D. geothermalis* T27 was determined. Cells were grown in HLB medium at 45°C for 6 h, than ethyl acetate was added to medium (20% v/v) and further incubated for 6 h. The control was cells grown in the absence of organic solvent. The morphology of the bacterium exposed to solvent analyzed by the gram strain, SEM and TEM (Fig 4.5). As compared to the morphology of cells grown with and without solvent exposure, *D. geothermalis* T27 cells in a direct contact to ethyl acetate appears to decreased in size with an approximately 40% size reduction (Table 4.7). Cells dimensions of cylindrical bodies were directly measured from the TEM photographs to calculate cell volume and cell surface area (APPENDIX C).

**Table 4.7** Cell size of *D. geothermalis* T27 exposed and not exposed to ethyl acetate

| Condition | Surface area ($\mu m^2$) | Volume ($\mu m^3$) |
|---|---|---|
| non exposed | 0.115 ± 0.010 | 1.227 ± 0.089 |
| exposed | 0.071 ± 0.038 | 0.947 ± 0.027 |

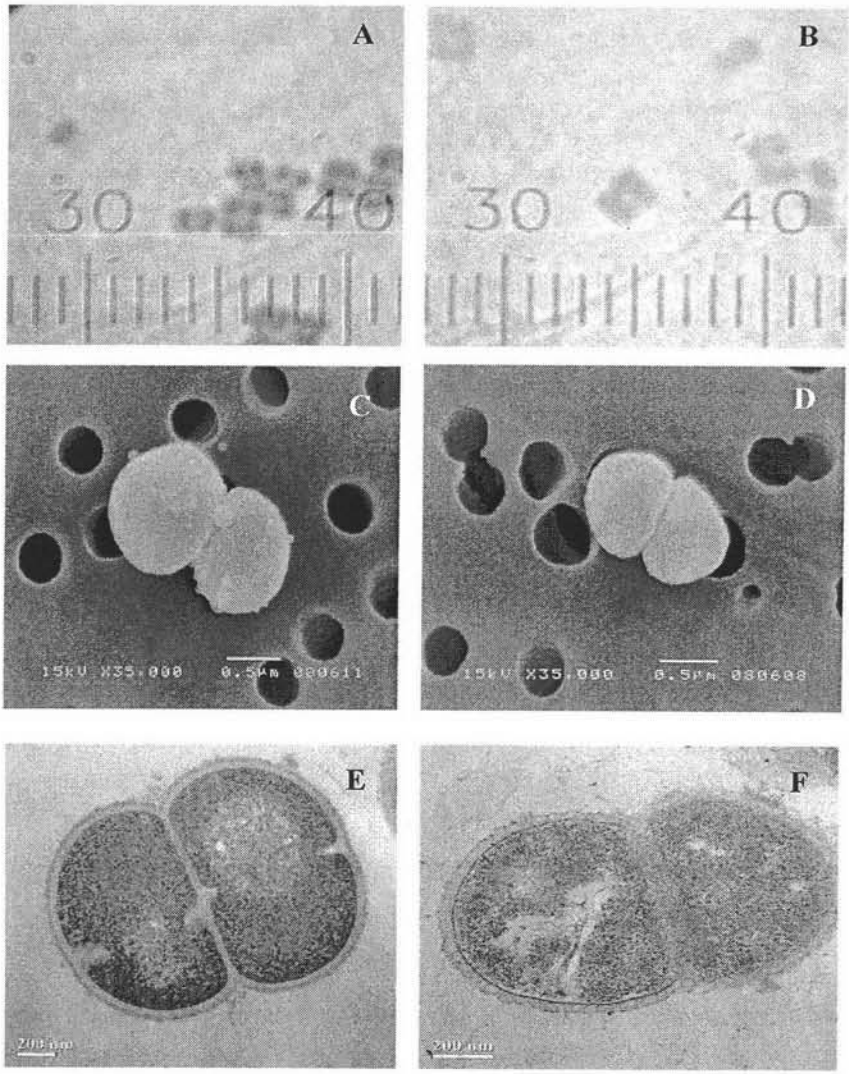


**Fig 4.5** Cell morphology of *D. geothermalis* T27. Gram strain (A, B), scanning electron micrographs (C, D) and transmission electron micrographs (E, F) of cells grown in HLB medium for 12 h without organic solvent at 45°C (A, C, E). Gram strain, scanning electron micrographs and transmission electron micrographs of cell were grown in HLB medium for 6 h followed by 6 h ethyl acetate exposure (B, D, F).

#### 4.2.1.3 Effect of organic solvent on fatty acid composition

Fatty acid composition of cells exposed and non-exposed to ethyl acetate was determined based on a method previously described (Unagul *et al.*,2007). The effect of ethyl acetate exposure on the fatty acid composition of cells exposed to ethyl acetate for 6 h was determined (Table 4.8). As shown in Table 4.8, no increase in the concentration of unsaturated fatty acids was observed in ethyl acetate-adapted cells. When *D. geothermalis* T27 grown in HLB medium, it contains approximately 24% and 30% of C15:0 and C17:0, respectively, in relative to all compositions. Fatty acid composition analysis of cells exposed to ethyl acetate revealed that there was no significant change in the level of fatty acid composition or proportion.

**Table 4.8** Fatty acid composition of *D. geothermalis* T27 non-exposed and exposed to ethyl acetate

| Fatty acid | Bacterial cell fatty acid (% wt) | |
|---|---|---|
| | Non-exposure | Ethyl acetate exposure |
| C12:0 | 1.115 | - |
| C13:0 | 1.143 | - |
| C14:0 | 2.113 | 0.453 |
| C14:1 | 1.565 | - |
| C15:0 | 24.517 | 11.613 |
| C15:1 | 1.713 | - |
| C16:0 | 11.773 | 8.986 |
| C16:1 | 5.792 | - |
| C17:0 | 29.464 | 14.691 |
| C17:1 | 2.637 | 1.338 |
| C20:0 | 1.483 | - |

#### 4.2.1.4 Organic solvent utilization

The ability of *D. geothermalis* T27 to utilize of organic solvent as a carbon source was investigated using resting cell technique in minimal medium containing 2.838 mM and 0.709 mM of ethyl acetate (Dashti *et al.*,2008). *D. geothermalis* T27 could degrade 88% and 70% of 2.838 mM and 0.709 mM ethyl acetate respectively in MSB liquid medium within 24 h (Fig 4.6).

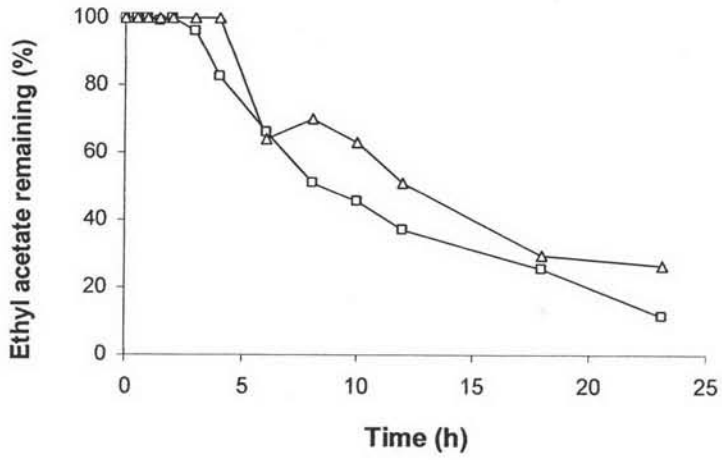


**Fig 4.6** Utilization of ethyl acetate by *D. geothermalis* T27. Cells were grown in minimal medium containing 2.838 mM (□) and 0.709 mM (Δ) of ethyl acetate.

#### 4.2.1.5 Enzymatic activity involving organic solvent utilization

*D. geothermalis* T27 were grown in 2 conditions of liquid medium 1) mineral medium containing 1% yeast extract, 2) mineral medium containing 1% yeast extract and 5% (v/v) of ethyl acetate. Cells were grown at 45 ºC for 24 h. The specific activities of these enzymes were determined by measuring the apparent product using a spectrophotometer. Esterase was apparently detected in intracellular cell exposure to ethyl acetate (Table 4.9), while non-exposure cells showed lower specific activities of esterase. Then, esterase was apparent in extra cellular cell both with and with out ethyl acetate but different specific activities. Suggestion, esterase was inducible enzyme.

**Table 4.9** Esterase activity of *D. geothermalis* T27

| Growth conditions | Location of enzyme | Specific activities |
|---|---|---|
| | | Unit/mg protein |
| MSBY | **Extra cellular enzyme** | 0.01 ± 0.001 |
| MSBY+ ethyl acetate | | 0.012 ± 0.002 |
| MSBY | **Intracellular enzyme** | 0.035 ± 0.003 |
| MSBY+ ethyl acetate | | 0.309 ± 0.05 |

### 4.2.2 Factors involving of organic-solvent tolerance

#### 4.2.2.1 Effect of ions on growth and organic–solvent tolerance

The influence of various metal ions on cell growth and organic-solvent tolerance was investigated. Bacterial cells were grown at 45°C for 24 h in HLB medium supplemented with 10 mM divalent ions and monovalent ions ($Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $Ca^{2+}$ and $Na^{+}$). Supplementation of $Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$ and $Ni^{2+}$ inhibited cell growth; therefore, their influences on organic solvent tolerance were not further examined. Divalent ions $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ enhanced cell growth; therefore, the influence of divalent ions on stabilization of organic solvent tolerance was investigated. To investigate the influence of metal ions towards solvent tolerance and cell growth, cells were grown on HLB containing $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ (at 2, 10 and 20 mM) for 6 h then, either toluene (Fig 4.7) and ethyl acetate (Fig 4.8) was added to the final volume of 5% (v/v), 20% (v/v). The result showed that addition of $Ca^{2+}$ at 10 and 20 mM respectively. $Ca^{2+}$ supplementation at all three concentrations tested improved cell tolerance to ethyl acetate. The presence of $Mg^{2+}$ promoted cell growth but did not significantly enhance cell solvent tolerance. The addition of $Na^{+}$ slightly decreased cell yield and did not facilitate the solvent tolerance.

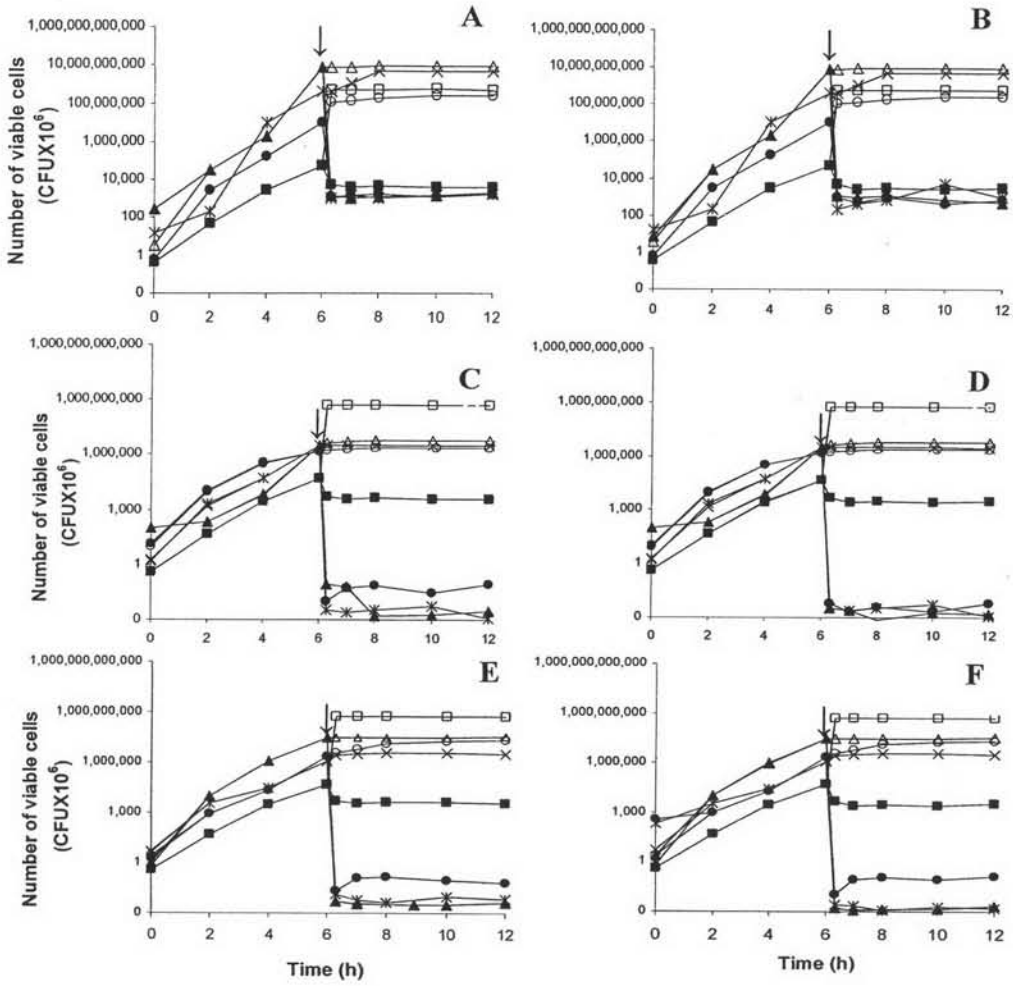


**Fig 4.7** Effect of divalent ion on toluene tolerance in *D. geothermalis* T27. Cells were grown on HLB containing $Ca^{2+}$ (A, B), $Mg^{2+}$ (C, D), $Na^{+}$ (E, F) at 2, 10 and 20 mM for 6 h. Then, solvent was added (↓) to 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F) compared to that in the absence of organic solvent (○, ×, Δ). Symbols of organic solvents are:

**A,C, E**

- ○ 2mM ion, 0% solvent
- ● 2mM ion, 5% solvent
- × 10mM ion, 0% solvent
- * 10mM ion, 5% solvent
- Δ 20mM ion, 0% solvent
- ▲ 20mM ion, 5% solvent
- □ 0mM ion, 0% solvent
- ■ 0mM ion, 5% solvent

**B, D, F**

- ○ 2mM ion, 0% solvent
- ● 2mM ion, 20% solvent
- × 10mM ion, 0% solvent
- * 10mM ion, 20% solvent
- Δ 20mM ion, 0% solvent
- ▲ 20mM ion, 20% solvent
- □ 0mM ion, 0% solvent
- ■ 0mM ion, 20% solvent

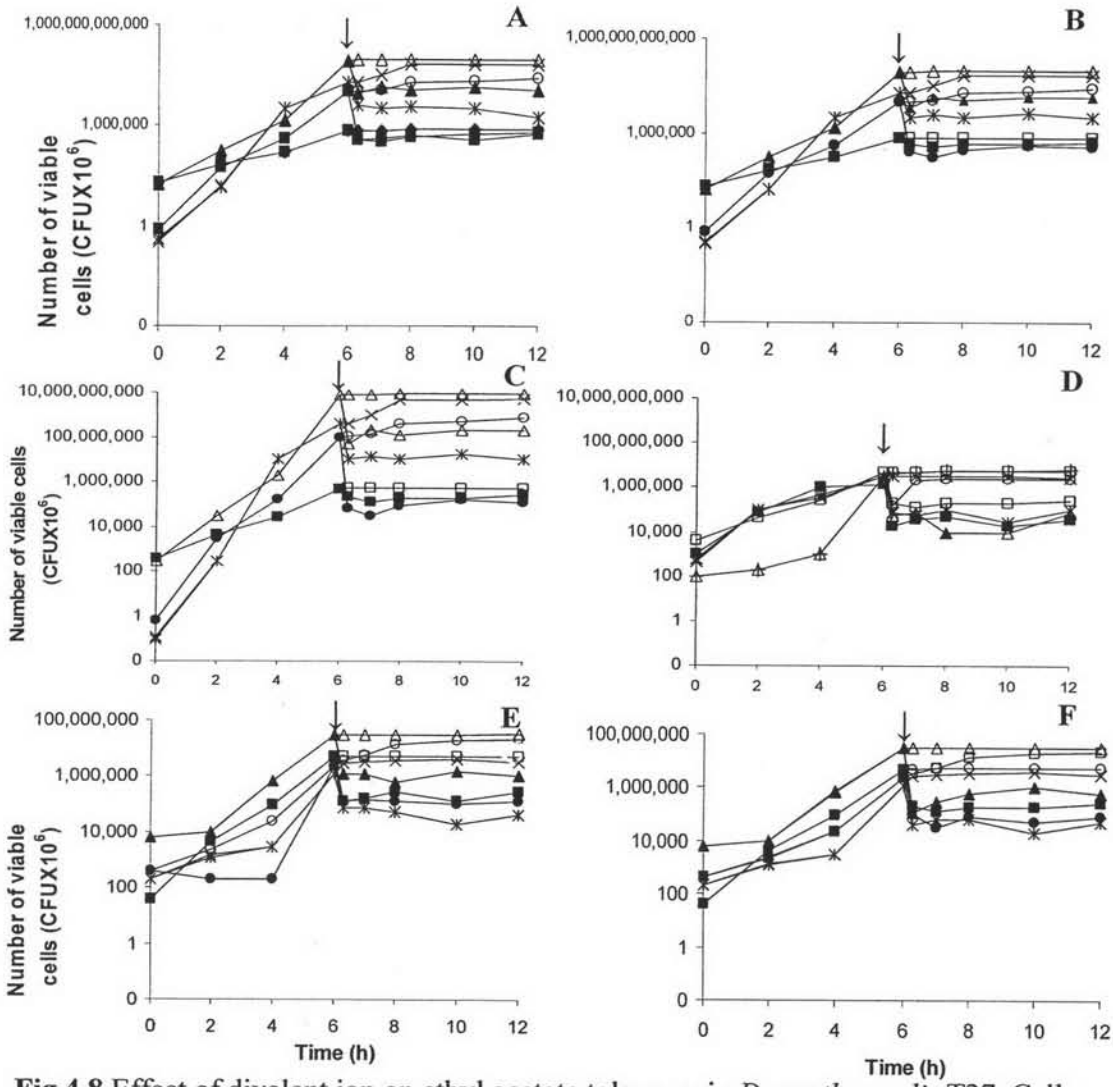


**Fig 4.8** Effect of divalent ion on ethyl acetate tolerance in *D. geothermalis* T27. Cells were grown on HLB containing $Ca^{2+}$ (A, B), $Mg^{2+}$ (C, D), $Na^{+}$ ( E, F) at 2, 10 and 20 mM for 6 h. Then, solvent was added (↓) to 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F) compared to that in the absence of organic solvent (○, ×, Δ). Symbols of organic solvents are:

| | | | | | |
|---|---|---|---|---|---|
| **A,C, E** | ○ | 2mM ion, 0% solvent | **B, D, F** | ○ | 2mM ion, 0% solvent |
| | ● | 2mM ion, 5% solvent | | ● | 2mM ion, 20% solvent |
| | × | 10mM ion, 0% solvent | | × | 10mM ion, 0% solvent |
| | * | 10mM ion, 5% solvent | | * | 10mM ion, 20% solvent |
| | Δ | 20mM ion, 0% solvent | | Δ | 20mM ion, 0% solvent |
| | ▲ | 20mM ion, 5% solvent | | ▲ | 20mM ion, 20% solvent |
| | □ | 0mM ion, 0% solvent | | □ | 0mM ion, 0% solvent |
| | ■ | 0mM ion, 5% solvent | | ■ | 0mM ion, 20% solvent |

#### 4.2.2.2 Effect of nutrient on growth and organic solvent tolerance of *D. geothermalis* T27

The following carbon sources were tested for cell growth and solvent-tolerance enhancement: MSB medium containing 16 mM of glucose (MSBG), fructose (MSBF), galactose (MSBGa), sucrose (MSBS), xylose (MSBX), rhamnose (MSBR), mannitol (MSBM), citrate (MSBC), succinate (MSBSc), 1% of yeast extract (SBY), tryptone (MSBT), LB, HLB. The addition of MSBGa, MSBS, MSBX, MSBR, MSBM, MSBC and MSBSc slightly affected cell growth. *D. geothermalis* T27 showed good growth rate in MSBG, MSBF, HLB, LB, MSBY and MSBT medium (Fig 4.9). To investigate the influence of nutrient towards solvent tolerance, cells were grown on MSBG, MSBY and HLB for 6 h then, either cyclohexane, toluene, benzene and ethyl acetate was added to 5% (v/v), 20% (v/v). Supplementation of glucose apparently improved cell solvent tolerance resulting in the increase of number viable cells, while the improvement of solvent tolerance was not significantly observed in cells grown in the MSBY medium (Fig 4.10).

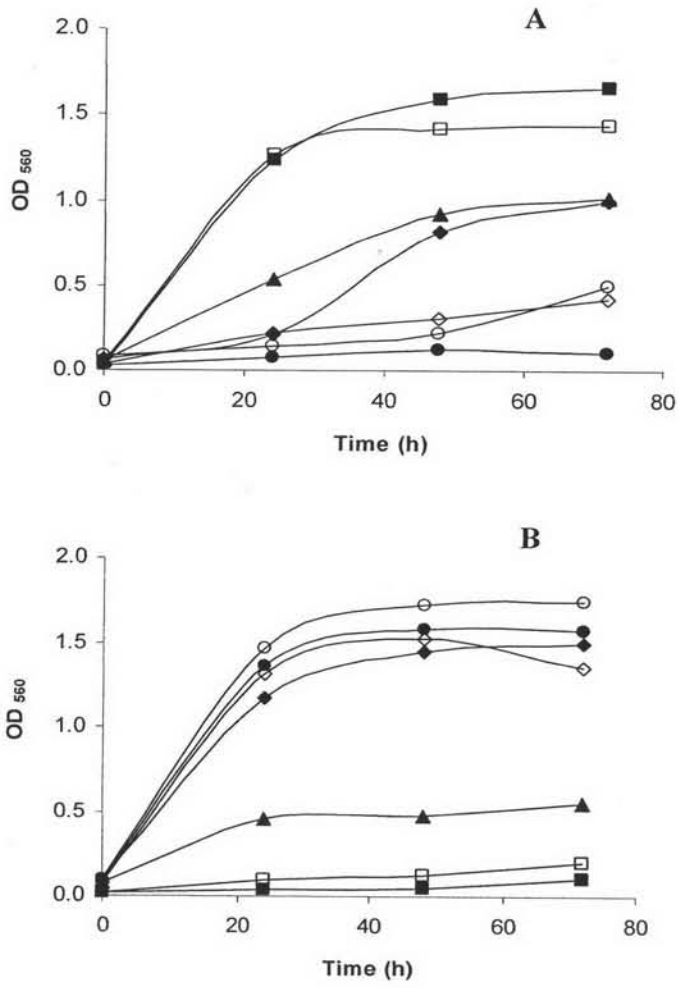


**Fig 4.9** Growth of *D. geothermalis* T27 in various types of nutrients. Symbols of organic solvents are:

| A | | | B | | |
|---|---|---|---|---|---|
| | ■ | Glucose | | ■ | Citrate |
| | □ | Fructose | | □ | Succinate |
| | ● | Rhamnose | | ● | HLB |
| | ○ | Xylose | | ○ | LB |
| | ♦ | Mannitol | | ♦ | MSBY |
| | ◊ | Galactose | | ◊ | MSBT |
| | ▲ | Sucrose | | ▲ | MSBP |

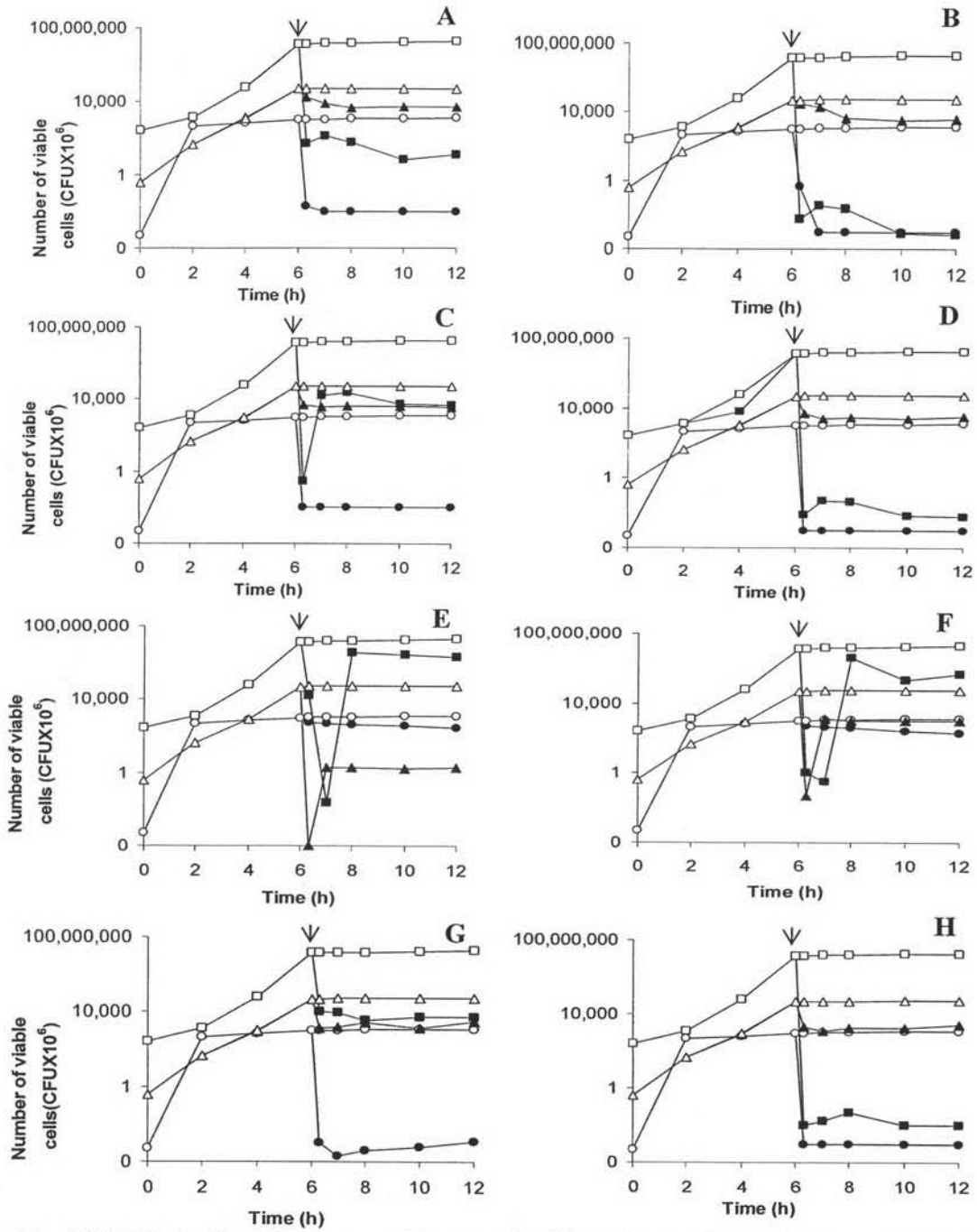


**Fig 4.10** Effect of nutrient on cyclohexane (A, B); toluene (C, D); benzene (E, F); ethyl acetate (G, H) tolerance in *D. geothermalis* T27**.** Cells were grown on MSBG (■), MSBY (●) and HLB (▲) medium at 45°C for 6 h. Then, organic solvent was added (↓) to 5% (v/v) (A, C, E, G, I) and 20% (v/v) (B, D, F, H, J); compared to absent organic solvent □, ○, Δ, respectively.

## 4.3 *Bacillus cereus* strain 4/1

### 4.3.1 Characterization of organic solvent tolerant bacteria

#### 4.3.1.1 Effect of types and concentration of organic solvent on growth and tolerance

Growths of were monitored after organic solvent shock with various types and quantities of organic solvent. Cells were grown to late log phase (about 6 h) before the organic solvent added (Method 3.3.5.1). Cells growth and cell survival was assessed by viable cell number ($CFU.ml^{-1}$). Cell survival after organic solvent shock are displayed in Fig 4.11, showing a relationship between cell number and the amount of solvent added (5% and 20%, v/v) to the media compared to that in the absence of organic solvent. When exposed to toluene, the number of viable cells slightly decreased at both solvent concentrations tested suggesting a relative high tolerance of cells. When exposed to toluene cells have a good growth but styrene and toluene high toxicity to cells. Cells increase when exposure to low concentration of chloroform. Besides exposed to low concentration of 1-heptanol and to both concentration of toluene, the decrease of cell was observed after 30 min. However increase of within 1h of 1-heptanol and toluene addition suggested that cells could adapt to the 1-heptanol and toluene.

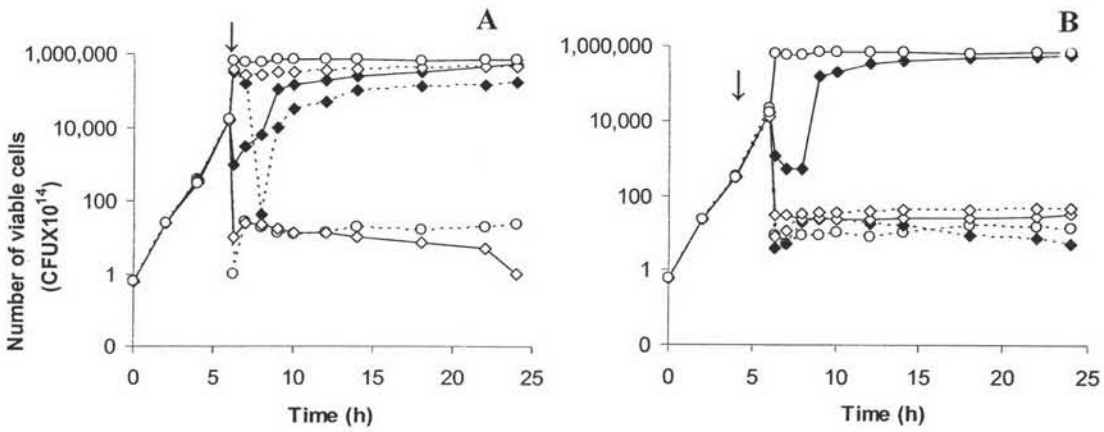


**Fig 4.11** Growth inhibition of *B. cereus* strain 4/1 when exposed to organic solvent. Organic solvent was added (↓) to the final volume 5% (A) and 20% (B). Cell growth in the absence of organic solvent (○) was carried out as a positive control. Symbols of organic solvents are:

| | | | |
|---|---|---|---|
| --○-- | styrene | ◊ | benzene |
| ♦ | toluene | --◊-- | chloroform |
| --♦-- | 1-heptanol | | |

#### 4.3.1.2 Effect of organic solvent on cell morphology of *B. cereus* strain 4/1

*B. cereus* strain 4/1 demonstrated unique ability to tolerate chloroform. Effect of organic solvent stress on morphology of *B. cereus* strain 4/1 was determined. Cells were grown in HLB medium at 45°C for 6 h, then chloroform was added to medium (20%, v/v) and further incubated for 6 h. The control was cells grown in the absence of organic solvent. The morphology of the bacterium exposed to organic solvent was analysis by gram strain and TEM (Fig 4.12). As compared to the morphology of cells grown with and without organic solvent expose, *B. cereus* strain 4/1 cells in a direct contract to chloroform appears to not different significance of statistics at confidence 95% with compared control (Table 4.10). *B. cereus* strain 4/1 exposed to chloroform did not exhibit distorted cell cells structure. The outer and cytoplasmic membrances were

apparently well defined even after the solvent shock. Cells dimensions of cylindrical bodies were directly measured from the TEM photographs to calculate cell volume and cell surface area (APPENDIX C).

**Table 4.10** Cell size of *B. cereus* strain 4/1 exposed and not exposed to chloroform

| Condition | Surface area ($\mu m^2$) | Volume ($\mu m^3$) |
|---|---|---|
| Non expose | 0.18 ± 0.126 | 2.01 ± 0.059 |
| Chlorofrom expose | 0.20 ± 0.170 | 2.21 ± 0.056 |

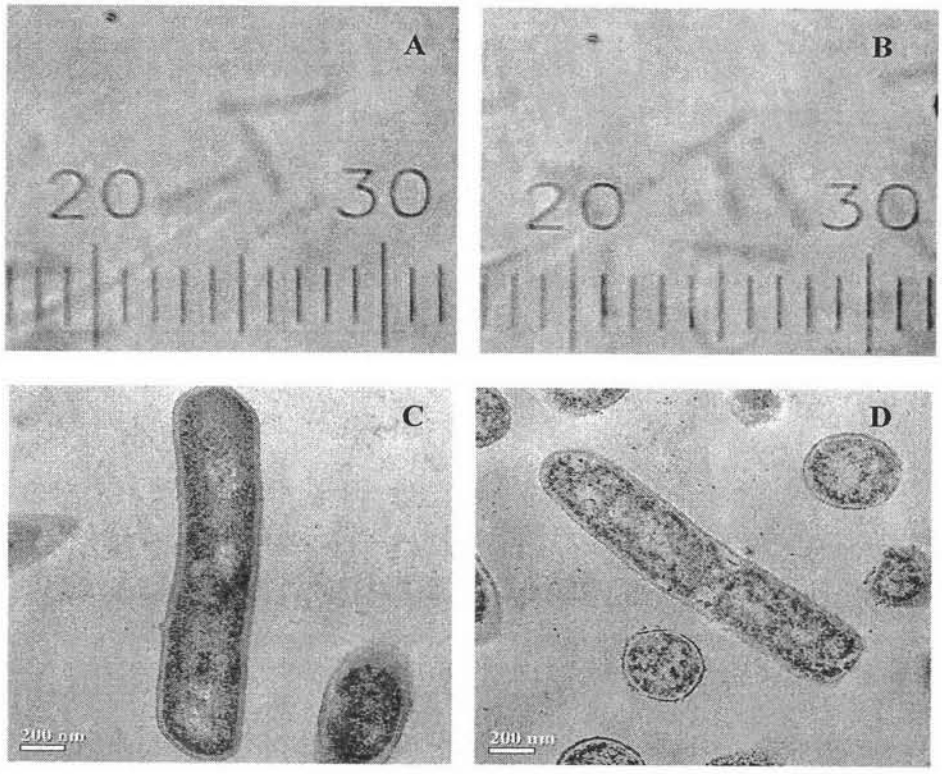


**Fig 4.12** Cell morphology of *B. cereus* strain 4/1. Gram, transmission electron micrographs of cell were grown in HLB medium for 12 h at 45°C (A, C). Gram, transmission electron micrographs of cell were grown in HLB medium for 6 h followed by 6 h chloroform (B, D).

#### 4.3.1.3 Effect of organic solvent on fatty acid composition

The effect of chloroform on the fatty acid composition of cells exposed to chloroform for 6 h was determined (Table 4.11). When, cells exposure with chloroform the fatty acid composition (C16:1) 1.67 time increase.

**Table 4.11** Fatty acid composition of *B. cereus* strain 4/1 non-exposed and exposed to chloroform

| Fatty acid | Bacterial cell fatty acid (% wt) | |
|---|---|---|
| | Non-exposure | Chloroform-exposure |
| C14:0 | 0.515 | - |
| C15:0 | 32.962 | 29.255 |
| C15:1 | 15.056 | 15.138 |
| C16:0 | 5.555 | 5.613 |
| **C16:1** | **11.839** | **19.719** |
| C17:0 | 22.662 | 19.239 |
| C17:1 | 0.234 | - |

#### 4.3.1.4 Organic solvent utilization

The ability of *B. cereus* strain 4/1 to utilize of organic solvent as a carbon source was investigated using resting cell technique in minimal medium containing 0.678 mM of toluene. *B. subtilis* strain 4/1 can degrade 46.73% of toluene at 0.678 mM (Fig 4.13) in MSB liquid medium within 6 h.

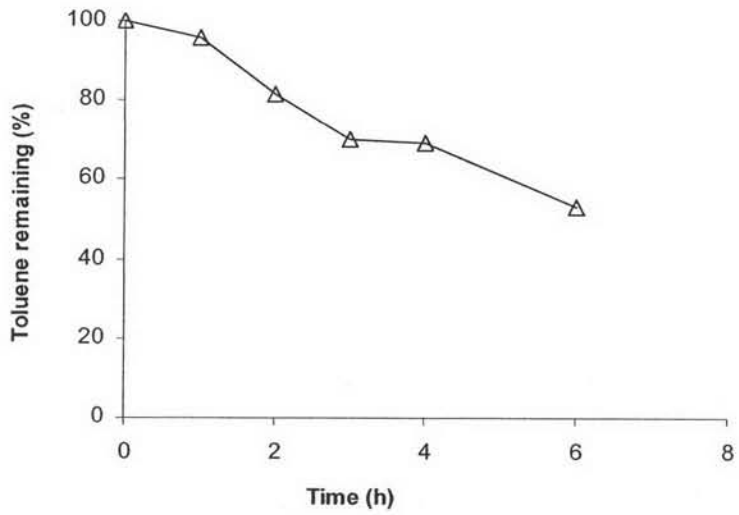


**Fig 4.13** Utilization of toluene by *B. cereus* strain 4/1. Cells were grown in minimal medium containing 0.678 mM (Δ) of toluene.

### 4.3.2 Factors involving of organic-solvent tolerance

#### 4.3.2.1 Effect of ions on growth and organic–solvent tolerance

The influence of various metal ions on cell growth and organic solvent tolerance was investigated. Bacterial cells were grown at 45°C for 24 h in HLB medium supplemented with 10 mM divalent ions and monovalent ions ($Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $Ca^{2+}$ and $Na^{+}$). Supplementation of $Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$ and $Ni^{2+}$ inhibited cell growth; therefore, their influences on organic solvent tolerance were not further examined. Divalent ions $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ enhanced cell growth; therefore, the influence of divalent ions on stabilization of organic solvent tolerance was investigated. To investigate the influence of metal ions towards solvent tolerance and cell growth, cells were grown on HLB containing $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ (at 2, 10 and 20 mM) for 6 h then, either styrene (Fig 4.14) and toluene (Fig 4.15) was added to the final volume of 5% (v/v), 20% (v/v). The result show that addition of $Ca^{2+}$ at all three concentrations tested improved cell toluene tolerance. While, the presence of $Mg^{2+}$ and $Na^{+}$ at all three concentrations were not significantly affected to styrene and toluene tolerance.

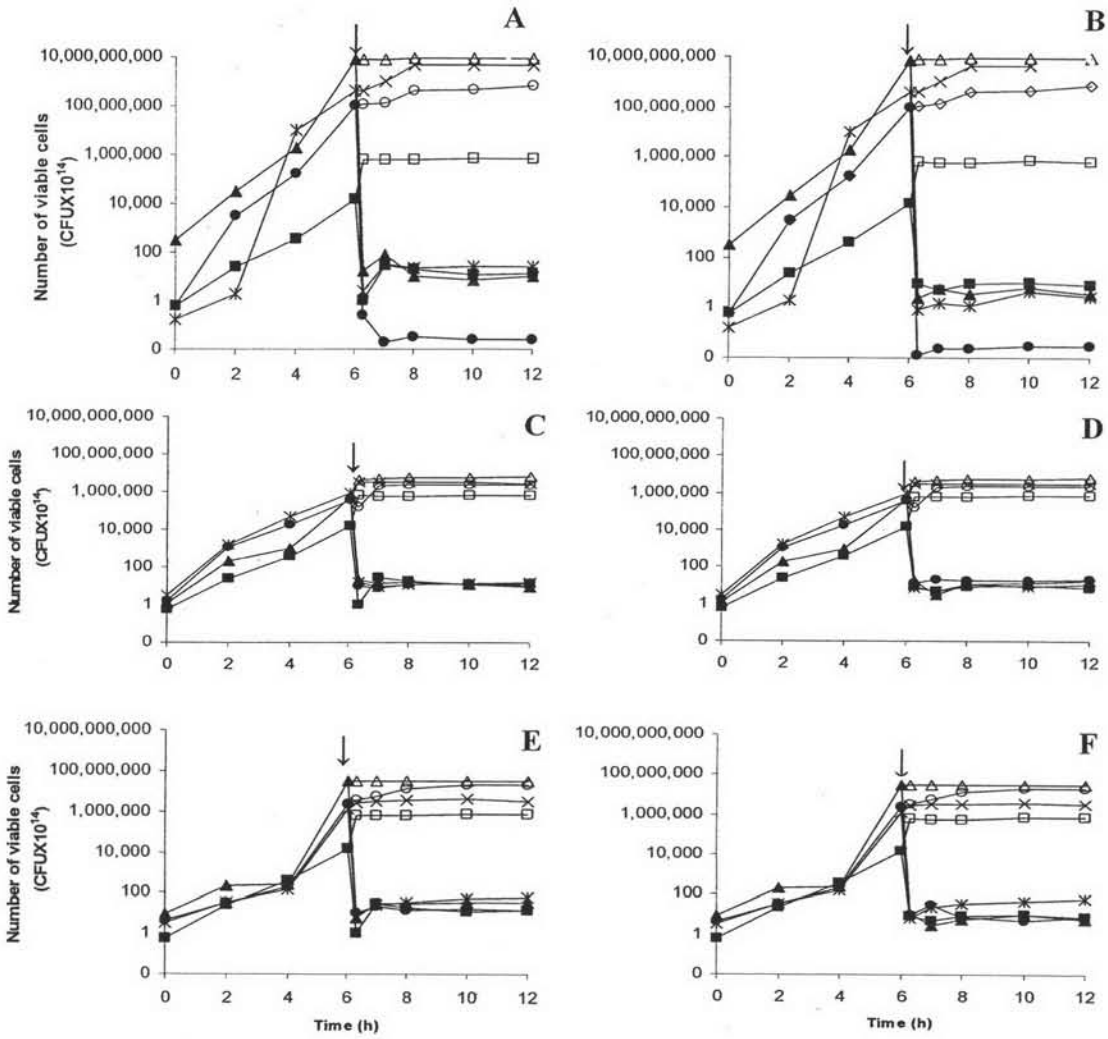


**Fig 4.14** Effect of divalent ion on styrene tolerance in *B. cereus* strain 4/1. Cells were grown on HLB containing $Ca^{2+}$ (A, B), $Mg^{2+}$ (C, D), $Na^{+}$ (E, F) at 2, 10 and 20 mM for 6 h. Then, organic solvent was added (↓) to 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F) compared to that in the absence of organic solvent (○, ×, Δ). Symbols of organic solvents are:

| | | | | | |
|---|---|---|---|---|---|
| **A,C, E** | ○ | 2mM ion, 0% solvent | **B, D, F** | ○ | 2mM ion, 0% solvent |
| | ● | 2mM ion, 5% solvent | | ● | 2mM ion, 20% solvent |
| | × | 10mM ion, 0% solvent | | × | 10mM ion, 0% solvent |
| | * | 10mM ion, 5% solvent | | * | 10mM ion, 20% solvent |
| | Δ | 20mM ion, 0% solvent | | Δ | 20mM ion, 0% solvent |
| | ▲ | 20mM ion, 5% solvent | | ▲ | 20mM ion, 20% solvent |
| | □ | 0mM ion, 0% solvent | | □ | 0mM ion, 0% solvent |
| | ■ | 0mM ion, 5% solvent | | ■ | 0mM ion, 20% solvent |

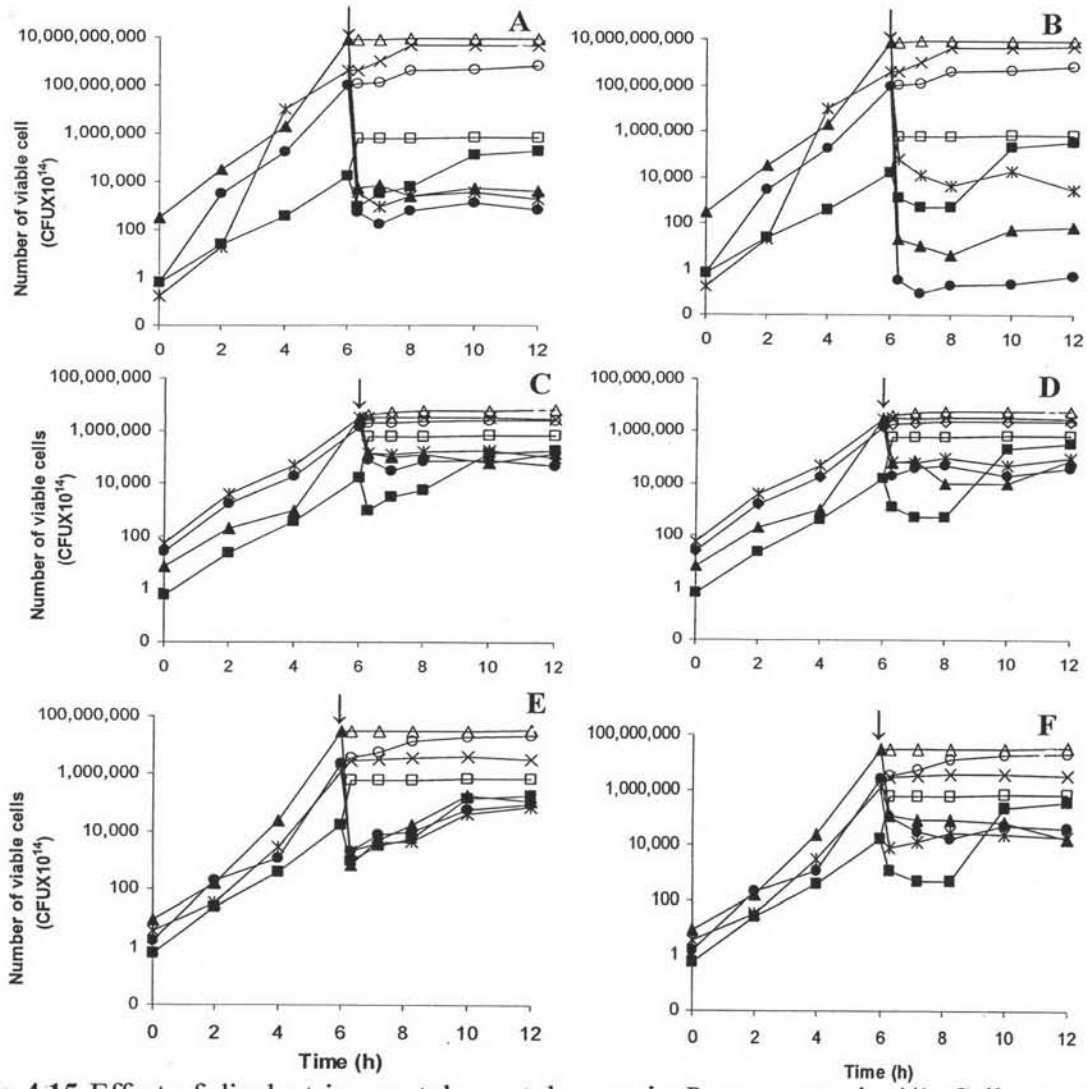


**Fig 4.15** Effect of divalent ion on toluene tolerance in *B. cereus* strain 4/1. Cells were grown on HLB containing $Ca^{2+}$ (A, B), $Mg^{2+}$ (C, D), $Na^{+}$ (E, F) at 2, 10 and 20 mM for 6 h. Then, organic solvent was added (↓) to 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F) compared to that in the absence of organic solvent (○, ×, Δ). Symbols of organic solvents are:

| | | | | | |
|---|---|---|---|---|---|
| **A,C, E** | ○ | 2mM ion, 0% solvent | **B, D, F** | ○ | 2mM ion, 0% solvent |
| | ● | 2mM ion, 5% solvent | | ● | 2mM ion, 20% solvent |
| | × | 10mM ion, 0% solvent | | × | 10mM ion, 0% solvent |
| | * | 10mM ion, 5% solvent | | * | 10mM ion, 20% solvent |
| | Δ | 20mM ion, 0% solvent | | Δ | 20mM ion, 0% solvent |
| | ▲ | 20mM ion, 5% solvent | | ▲ | 20mM ion, 20% solvent |
| | □ | 0mM ion, 0% solvent | | □ | 0mM ion, 0% solvent |
| | ■ | 0mM ion, 5% solvent | | ■ | 0mM ion, 20% solvent |

### 4.3.2.2 Effect of nutrient on growth and organic–solvent tolerance

The following carbon sources were tested for cell growth and solvent-tolerance enhancement: MSB medium containing 16 mM of glucose (MSBG), fructose (MSBF), galactose (MSBGa), sucrose (MSBS), xylose (MSBX), rhamnose (MSBR), mannitol (MSBM), citrate (MSBC), succinate (MSBSc), 1% of yeast extract, tryptone, LB, HLB. *B. cereus* strain 4/1 chowed good growth rat in HLB, LB, MSBY, MSBT and MSBP medium (Fig 4.16). To investigate the influence of nutrient towards organic solvent tolerance, cells were grown on MSBY, MSBT and HLB for 6 h at 45°C then, either styrene, toluene and chloroform added to 5% (v/v) and 20% (v/v) (Fig 4.17). Supplementation of yeast extract and tryptone was not significantly solvent tolerance.

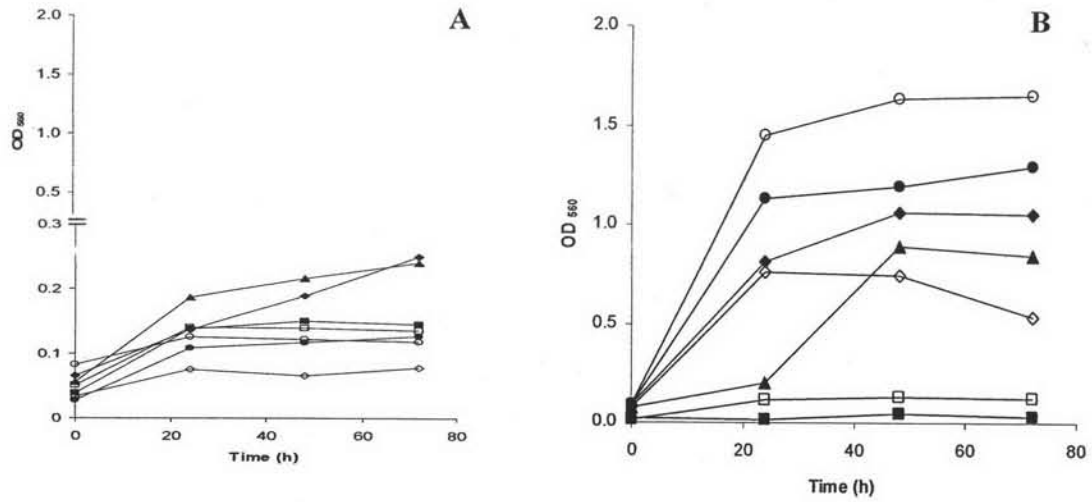


**Fig 4.16** Growth of *B. cereus* strain 4/1 in various types of nutrients. Symbols of organic solvents are:

| A | | | B | | |
|---|---|---|---|---|---|
| | ■ | Glucose | | ■ | Citrate |
| | □ | Fructose | | □ | Succinate |
| | ● | Rhamnose | | ● | HLB |
| | ○ | Xylose | | ○ | LB |
| | ♦ | Mannitol | | ♦ | MSBY |
| | ◊ | Galactose | | ◊ | MSBT |
| | ▲ | Sucrose | | ▲ | MSBP |

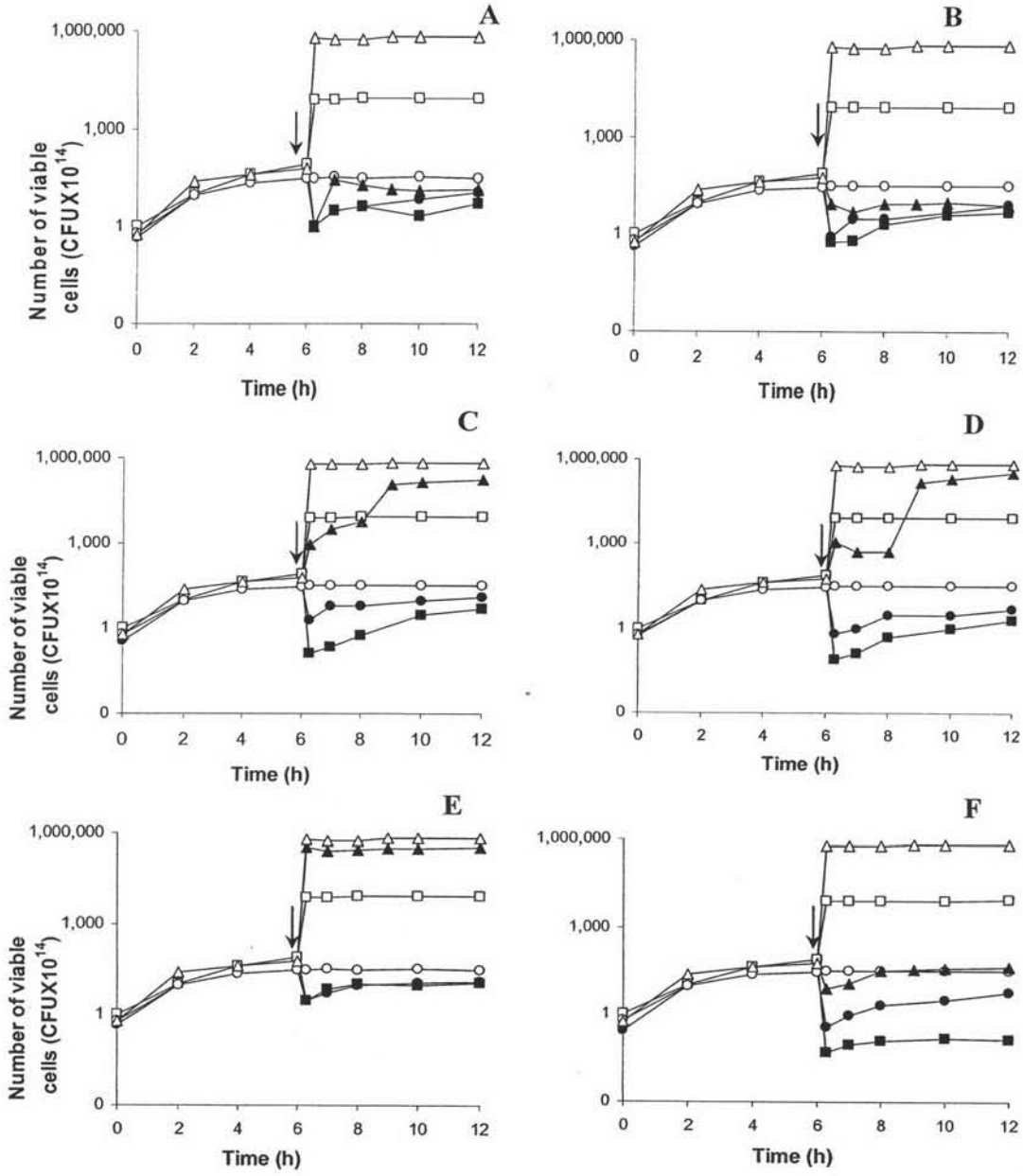


**Fig 4.17** Effect of nutrient on styrene (A, B); toluene (C, D); chloroform (E, F) tolerance in *B. cereus* strain 4/1. Cells were grown on MSBT (■), MSBY (●) and HLB (▲) medium at 45°C for 6 h. Then, organic solvent was added (↓) 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F); compared to absent organic solvent □, ○, Δ, respectively.

## 4.4 *Bacillus subtilis* strain 45

### 4.4.1 Characterization of organic-solvent tolerant bacteria

#### 4.4.1.1 Effect of types and concentrations of organic solvent on growth and tolerance

Growths of *B. subtilis* strain 45 were monitored after solvent shock. Cells were grown to late log phase (about 6 h) before the solvent was added (Method 3.3.5.1). Cell growth and cell survival was assessed by viable cell number (CFU-$ml^{-1}$). Cells survival after solvent shock are displayed in Fig 4.18, showing a relationship between cell number and the amount of solvent added (5% and 20% v/v) to the media compared to that in the absence of organic solvent. Organic solvents were re-classified into three groups of log $P_{ow}$ value,

1) log $P_{ow}$ >3.5 (*n*-decane (5.6), *n*-heptane (4.7), *n*-octane (4.5));
2) log $P_{ow}$ >3.2-3.5 (*n*-hexane (3.5), diethylphthalate (3.3), cyclohexane (3.2);
3) log $P_{ow}$ 3.1 (ethylbenzene (3.1), *o*-xylene (3.1), *m*-xylene (3.1), *p*-xylene (3.1)).

The results demonstrated that *B. subtilis* strain 45 exhibited tolerance to log $P_{ow}$ value > 3 of organic solvent. When exposed to solvent with log $P_{ow}$ greater than 3.5 (Fig 4.18 A, B) and log $P_{ow}$ >3.2-3.5 (Fig 4.18 C, D) the number of viable cells only slightly decreased at both solvent concentrations tested suggesting a relative high tolerance of cells. The exposure of log $P_{ow}$ 3.1 (Fig 4.18 E, F), although the log $P_{ow}$ value of ethylbenzene is equal to isomers of xylene, cells were adversely affected. The exposure of isomers of xylene showed difference results in that they exhibited toxicity to cells.

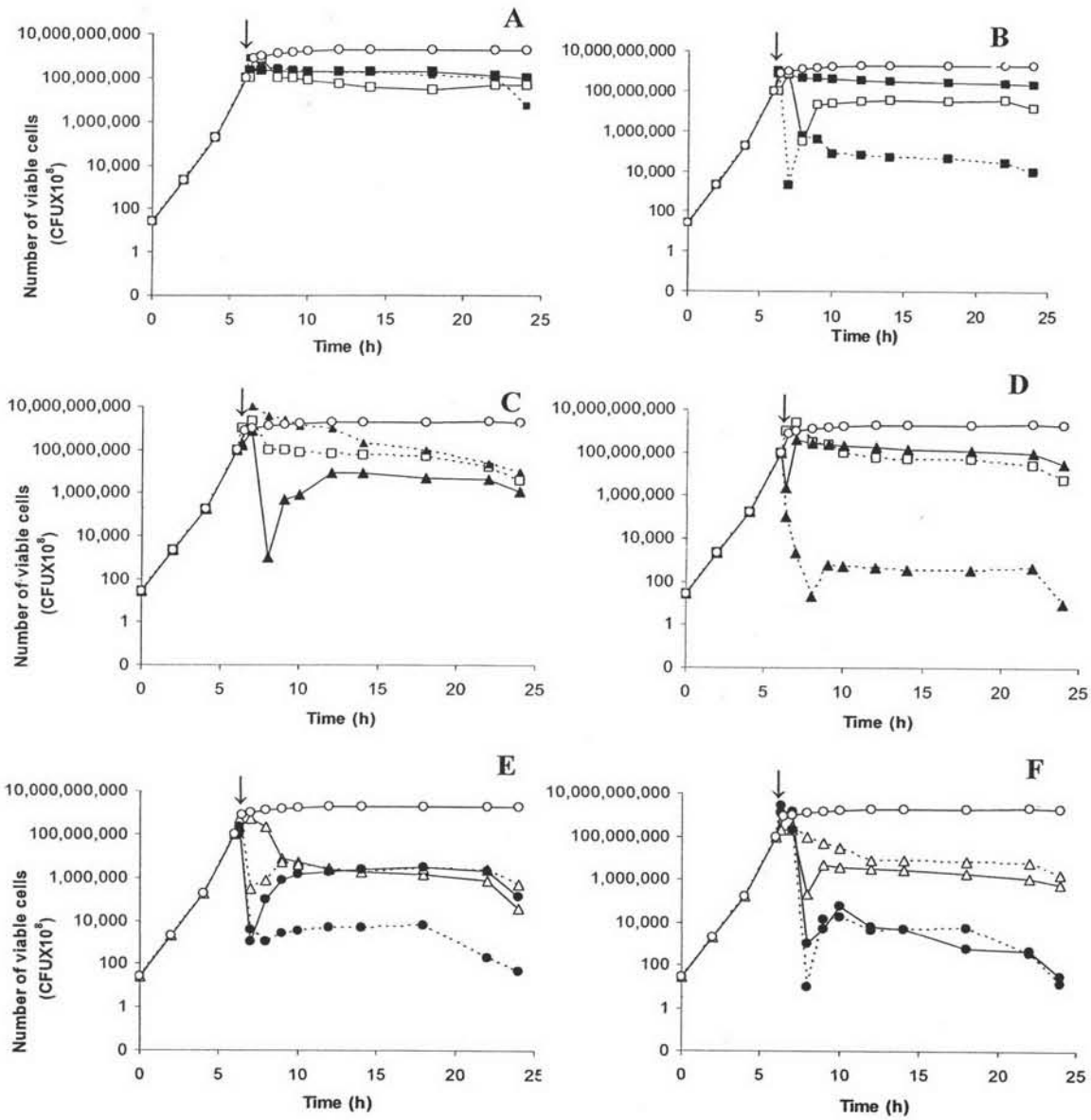


**Fig 4.18** Growth inhibition of *B. subtilis* strain 45 when exposed to organic solvent with log $P_{ow}$ > 3.5, log $P_{ow}$ 3.2-3.5 and log $P_{ow}$ 3.1. Organic solvent was added (↓) to the final volume of 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F). Cell growth in the absent of organic solvent (○) was carried out as a positive control. Symbols of organic solvents are:

| **A, B**: log $P_{ow}$ > 3.5 | | **C, D**: log $P_{ow}$ > 3.2-3.5 | | **E, F**: log $P_{ow}$ 3.1 | |
|---|---|---|---|---|---|
| ■ | *n*-decane | ---□--- | *n*-hexane | Δ | ethylbenzene |
| ---■--- | *n*-heptane | ▲ | diethylphthalate | ---Δ--- | *o*-xylene |
| □ | *n*-octane | ---▲--- | cyclohexane | ● | *m*-xylene |
| | | | | ---●--- | *p*-xylene |

#### 4.4.1.2 Effect of organic solvent on cell morphology of *B. subtilis* strain 45

*B. subtilis* strain 45 demonstrated unique ability to tolerate *n*-decane. Effect of organic solvent stress on morphology of *B. subtilis* strain 45 was determined. Cells were grown in HLB medium at 45°C for 6 h, then *n*-decane was added to medium (20%, v/v) and further incubated for 6 h. The control was cells grown in the absence of organic solvent. The morphology of the bacterium exposed to organic solvent was analyzed by gram stain and TEM (Fig 4.19). As compared to the morphology of cells grown with and without solvent exposure, *B. subtilis* strain 45 cells in a direct contact to *n*-decane not different significance of statistics at confidence 95% with compared control (Table 4.12). *B. subtilis* strain 45 exposed to chloroform did not exhibit distorted cell cells structure. The outer and cytoplasmic membrances were apparently well defined even after the solvent shock (Fig 4.19 D) Cells dimensions of cylindrical bodies were directly measured from the TEM photographs to calculate cell volume and cell surface area (APPENDIX C).

**Table 4.12** Cell size of *B. subtilis* strain 45 grown with and without *n*-decane

| Condition | Surface area ($\mu m^2$) | Volume ($\mu m^3$) |
|---|---|---|
| non exposed | 0.315 ±0.047 | 2.850 ± 0.261 |
| exposed | 0.354 ± 0.711 | 3.124 ± 0.320 |

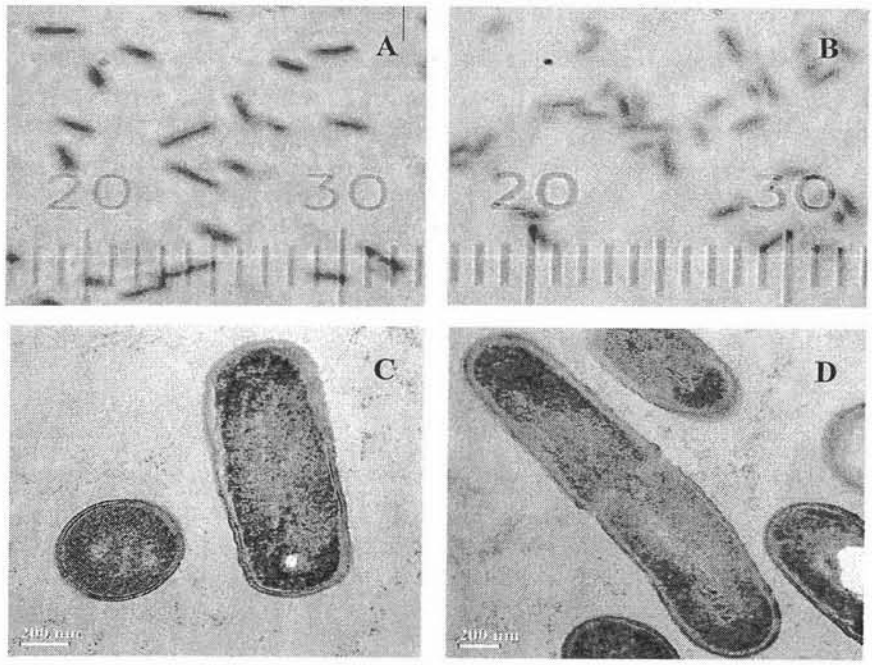


**Fig 4.19 Cell morphology of *B. subtilis* strain 45.** Gram staining and transmission electron micrographs of cell were grown in HLB medium for 12 h at 45°C (A, C). Gram strain and transmission ram strain micrographs of cell were grown in HLB medium for 6 h followed by 6 h *n*-decane (B, D).

#### 4.4.1.3 Effect of organic solvent on fatty acid composition

The effect of *n*-decane on the fatty acid composition of cells exposed to *n*-decane for 6 h was determined (Table 4.13). Fatty acid composition of cells exposed and non-exposed to *n*-decane was determined based on a method previously described (Unagul *et al.*,2007). When *B. subtilis* strain 45 was grown in HLB medium, it contains approximately 22%, 21% and 31% of C15:0, C15:1 and C16:0, respectively, in relative to all compositions. Fatty acid composition analysis of cells exposed to *n*-decane revealed that there was no significant change in the level of fatty acid composition or proportion.

**Table 4.13** Fatty acid composition of *B. subtilis* strain 45 non-exposed and exposed to *n*-decane

| Fatty acid | Bacterial cell fatty acid (% wt) | |
|---|---|---|
| | Non-exposure | *n*-decane-exposure |
| C8:0 | 1.538 | - |
| C14:0 | 1.385 | - |
| C15:0 | 21.769 | 27.314 |
| C15:1 | 20.815 | 24.135 |
| C16:0 | 3.446 | 3.717 |
| C16:1 | 30.603 | 24.027 |
| C17:0 | 14.144 | 17.219. |
| C22:2 | 4.99 | 2.153 |

#### 4.4.1.4 Organic solvent utilization

The ability of *B. subtilis* strain 45 to utilize of organic solvent as a carbon source was investigated using resting cell technique in minimal medium containing 0.878 mM and 1.485 mM of *n*-decane and cyclohexane respectively. *B. subtilis* strain 45 can degrade 54.37% and 32.65% of *n*-decane (Fig 4.20) and cyclohexane respectively (Fig 4.21) in MSB liquid medium within 6 h.

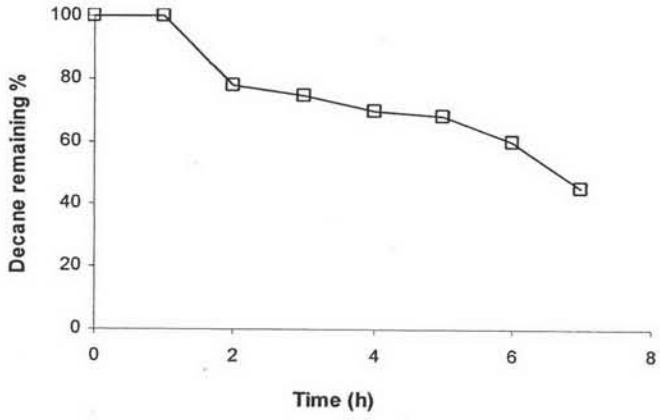


**Fig 4.20** Utilization of *n*-decane by *B. subtilis* strain 45. Cells were grown in minimal medium containing 0.878 mM (□) of *n*-decane.

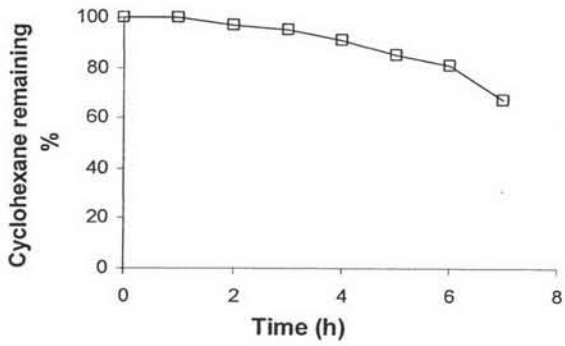


**Fig 4.21** Utilization of cyclohexane by *B. subtilis* strain 45. Cells were grown in minimal medium containing 1.485 mM (□) of cyclohexane.

### 4.4.2 Factors involving of organic-solvent tolerance of *B. subtilis* strain 45

#### 4.4.2.1 Effect of ions on growth and organic-solvent tolerance

The influence of various metal ions on cell growth and organic solvent tolerance was investigated. Bacterial cells were grown at 45°C for 24 h in HLB medium supplemented with 10 mM divalent ions and monovalent ions ($Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $Ca^{2+}$ and $Na^{+}$). Supplementation of $Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$ and $Ni^{2+}$ inhibited cell growth; therefore, their influences on organic solvent tolerance were not further examined. Divalent ions $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ enhanced cell growth; therefore, the influence of divalent ions on stabilization of organic solvent tolerance was investigated. To investigate the influence of metal ions towards solvent tolerance and cell growth, cells were grown on HLB containing $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ (at 2, 10 and 20 mM) for 6 h then, either 1-heptanol, cyclohexane and ethylbenzene was added 5% (v/v), 20% (v/v). The result show that addition of $Ca^{2+}$ at 2 mM concentrations tested improved cell *n*-heptane tolerance. The presence of $Mg^{2+}$ and $Na^{+}$ at all three concentrations tested improved cell *n*-heptane tolerance (Fig 4.22-4.24).

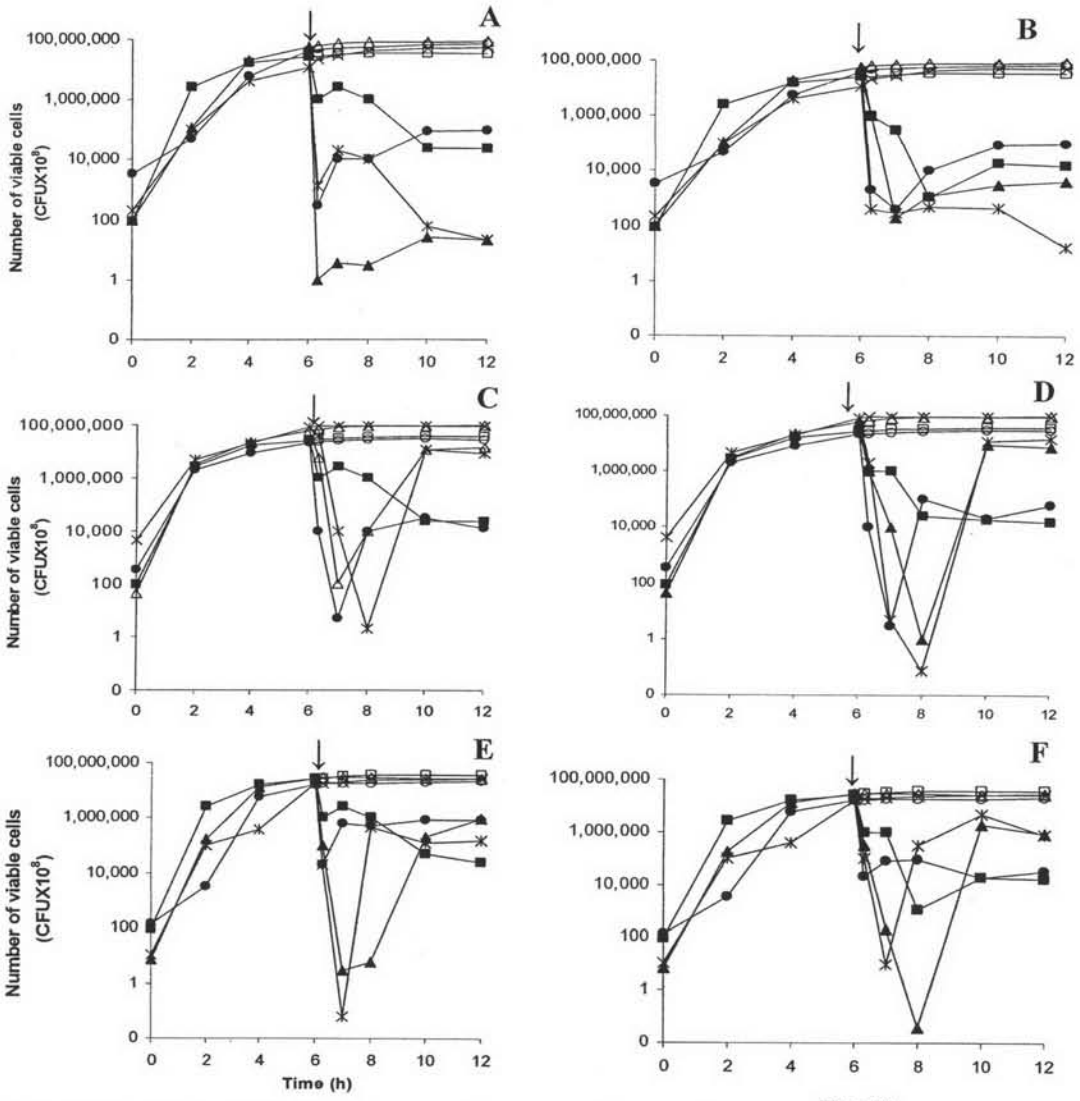


**Fig 4.22** Effect of divalent ion on *n*-heptane tolerance in *B. subtilis* strain 45. Cells were grown on HLB containing $Ca^{2+}$ (A, B), $Mg^{2+}$ (C, D), $Na^{+}$ (E, F) at 2, 10 and 20 mM for 6 h. Then organic solvent was added (↓) to 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F) compared to that in the absence of organic solvent (○, ×, Δ). Symbols of organic solvents are:

| | | | | | |
|---|---|---|---|---|---|
| **A,C, E** | ○ | 2mM ion, 0% solvent | **B, D, F** | ○ | 2mM ion, 0% solvent |
| | ■ | 2mM ion, 5% solvent | | ■ | 2mM ion, 20% solvent |
| | × | 10mM ion, 0% solvent | | × | 10mM ion, 0% solvent |
| | * | 10mM ion, 5% solvent | | * | 10mM ion, 20% solvent |
| | Δ | 20mM ion, 0% solvent | | Δ | 20mM ion, 0% solvent |
| | ▲ | 20mM ion, 5% solvent | | ▲ | 20mM ion, 20% solvent |
| | □ | 0mM ion, 0% solvent | | □ | 0mM ion, 0% solvent |
| | ■ | 0mM ion, 5% solvent | | ■ | 0mM ion, 20% solvent |

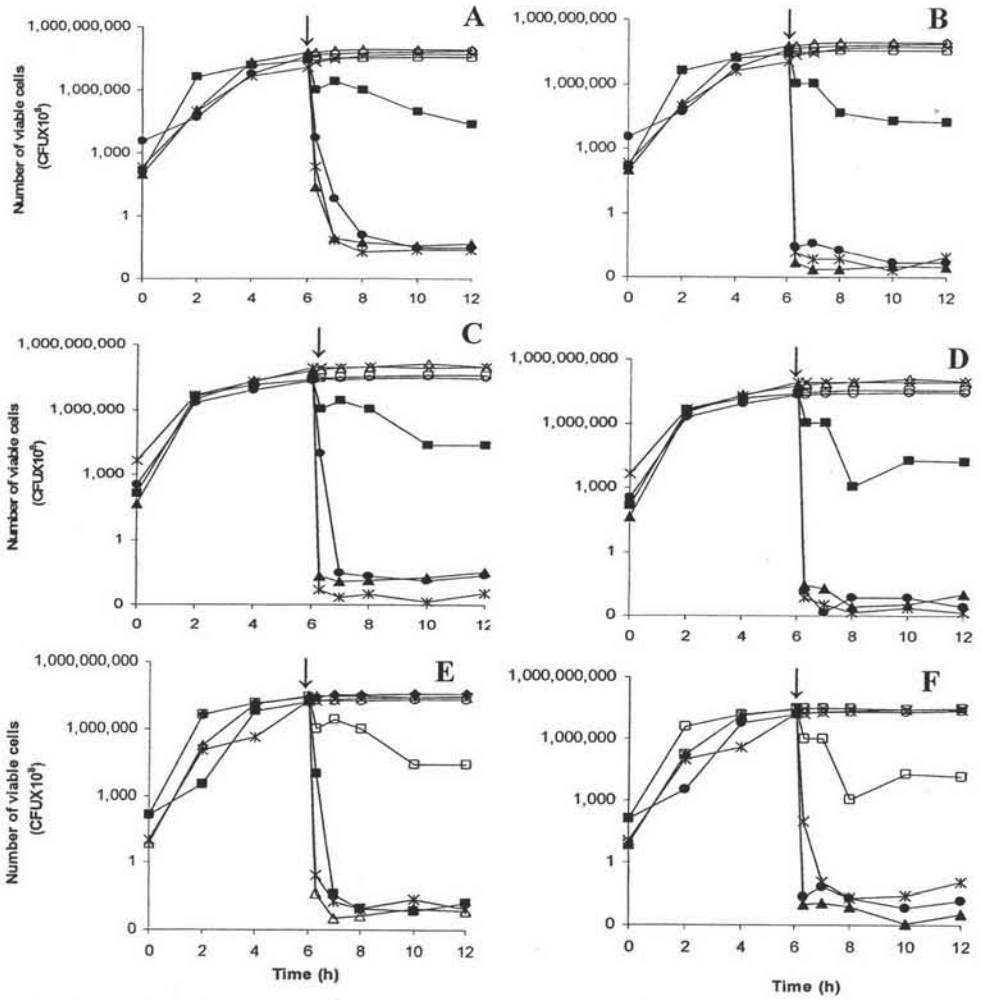


**Fig 4.23** Effect of divalent ion on cyclohexane tolerance in *B. subtilis* strain 45. Cells were grown on HLB containing $Ca^{2+}$ (A, B), $Mg^{2+}$ (C, D), $Na^{+}$ (E, F) at 2, 10 and 20 mM for 6 h. Then, organic solvent was added (↓) to 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F) compared to that in the absence of organic solvent (○, ×, Δ). Symbols of organic solvents are:

| | | | | | |
|---|---|---|---|---|---|
| **A,C, E** | ○ | 2mM ion, 0% solvent | **B, D, F** | ○ | 2mM ion, 0% solvent |
| | ● | 2mM ion, 5% solvent | | ● | 2mM ion, 20% solvent |
| | × | 10mM ion, 0% solvent | | × | 10mM ion, 0% solvent |
| | * | 10mM ion, 5% solvent | | * | 10mM ion, 20% solvent |
| | Δ | 20mM ion, 0% solvent | | Δ | 20mM ion, 0% solvent |
| | ▲ | 20mM ion, 5% solvent | | ▲ | 20mM ion, 20% solvent |
| | □ | 0mM ion, 0% solvent | | □ | 0mM ion, 0% solvent |
| | ■ | 0mM ion, 5% solvent | | ■ | 0mM ion, 20% solvent |

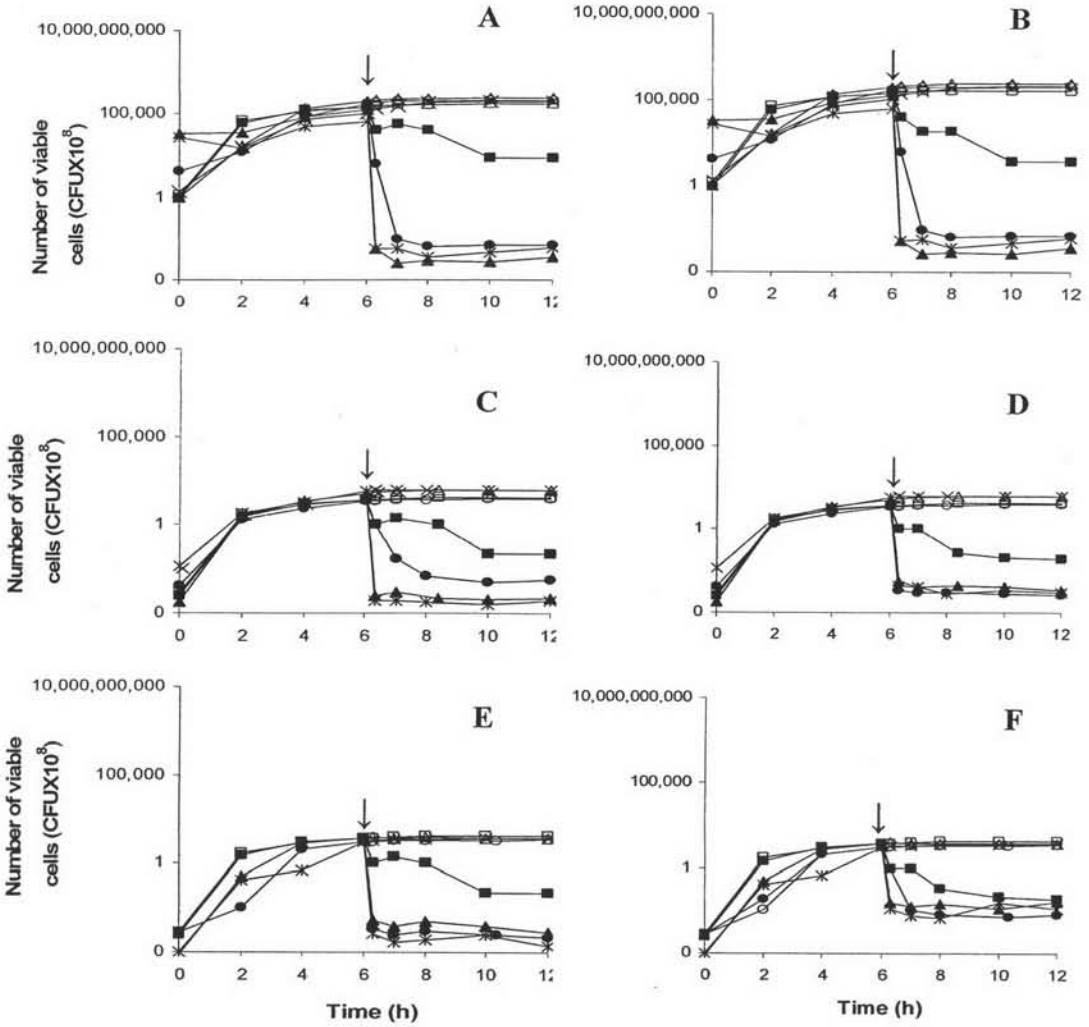


**Fig 4.24** Effect of divalent ion on ethylbenzene tolerance in *B. subtilis* strain 45. Cells were grown on HLB containing $Ca^{2+}$ (A, B), $Mg^{2+}$ (C, D), $Na^{+}$ (E, F) at 2, 10 and 20 mM for 6 h. Then, organic solvent was added (↓) to 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F) compared to that in the absence of organic solvent (○, ×, Δ). Symbols of organic solvents are:

| **A,C, E** | | **B, D, F** | |
|---|---|---|---|
| ○ | 2mM ion, 0% solvent | ○ | 2mM ion, 0% solvent |
| ● | 2mM ion, 5% solvent | ● | 2mM ion, 20% solvent |
| × | 10mM ion, 0% solvent | × | 10mM ion, 0% solvent |
| * | 10mM ion, 5% solvent | * | 10mM ion, 20% solvent |
| Δ | 20mM ion, 0% solvent | Δ | 20mM ion, 0% solvent |
| ▲ | 20mM ion, 5% solvent | ▲ | 20mM ion, 20% solvent |
| □ | 0mM ion, 0% solvent | □ | 0mM ion, 0% solvent |
| ■ | 0mM ion, 5% solvent | ■ | 0mM ion, 20% solvent |

### 4.3.2.2 Effect of nutrient on growth and organic solvent tolerance of *B. subtilis* strain 45

The following carbon sources were tested for cell growth and solvent-tolerance enhancement: MSB medium containing 16 mM of glucose (MSBG), fructose (MSBF), galactose (MSBGa), sucrose (MSBS), xylose (MSBX), rhamnose (MSBR), mannitol (MSBM), citrate (MSBC), succinate (MSBSc), 1% of yeast extract, tryptone, LB, HLB. The *B. subtilis* strain 45 showed good growth rates in HLB, LB, MSBY, MSBT and MSBP medium (Fig 4.25). To investigate the influence of nutrient towards organic solvent tolerance, cells were grown on MSBY, MSBT and HLB for 6 h at 45°C then, either *n*-decane, *n*-heptane, *n*-hexane, cyclohexane and ethylbenzene was added to 5% (v/v) and 20% (v/v) (Fig 4.26). Supplementation of yeast extract and tryptone were not significantly to organic solvent tolerance.

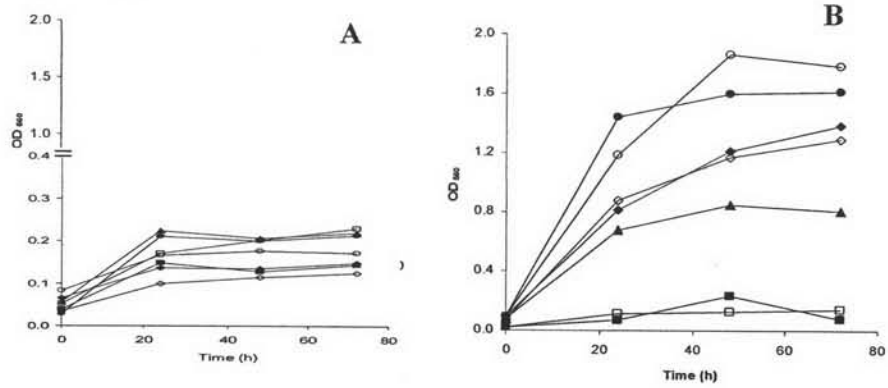


**Fig 4.25** Growth of *B. subtilis* strain 45 in varies type of nutrients. Symbols of organic solvents are:

| A | | | B | | |
|---|---|---|---|---|---|
| | ■ | Glucose | | ■ | Citrate |
| | □ | Fructose | | □ | Succinate |
| | ● | Rhamnose | | ● | HLB |
| | ○ | Xylose | | ○ | LB |
| | ♦ | Mannitol | | ♦ | MSBY |
| | ◊ | Galactose | | ◊ | MSBT |
| | ▲ | Sucrose | | ▲ | MSBP |

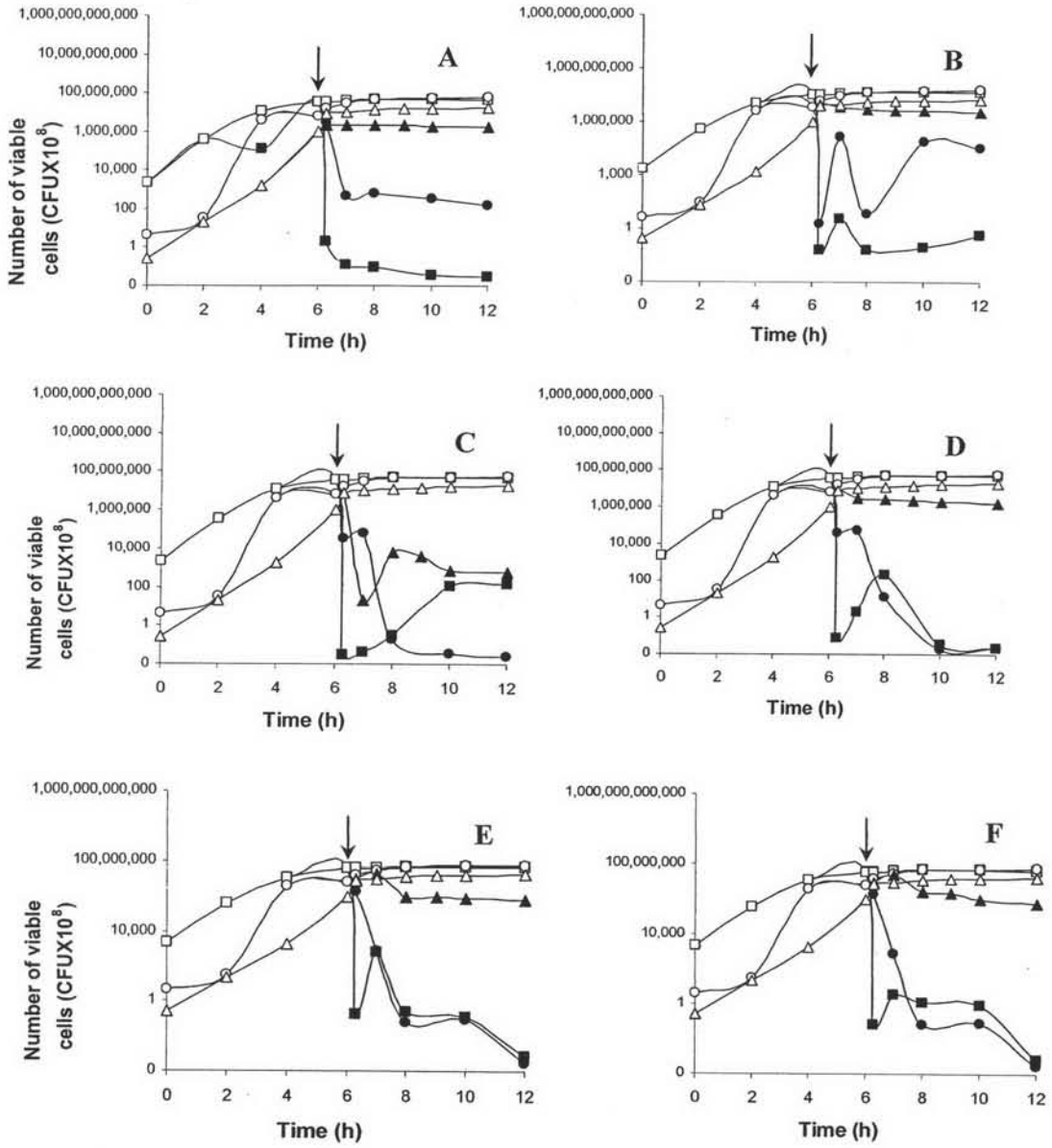


**Fig 4.26** Effect of nutrient on *n*-decane (A, B); *n*-heptane (C, D); *n*-hexane (E, F); cyclohexane (G, H); ethylbenzene (I, J) tolerance in *B. subtilis* strain 45. Cells were grown on MSBT (■), MSBY (●) and HLB (▲) medium at 45°C for 6 h. Then, organic solvent was added (↓) to 5% (v/v) (A, C, E, G, I) and 20% (v/v) (B, D, F, H, J); compared to absent organic solvent □, ○, Δ, respectively.

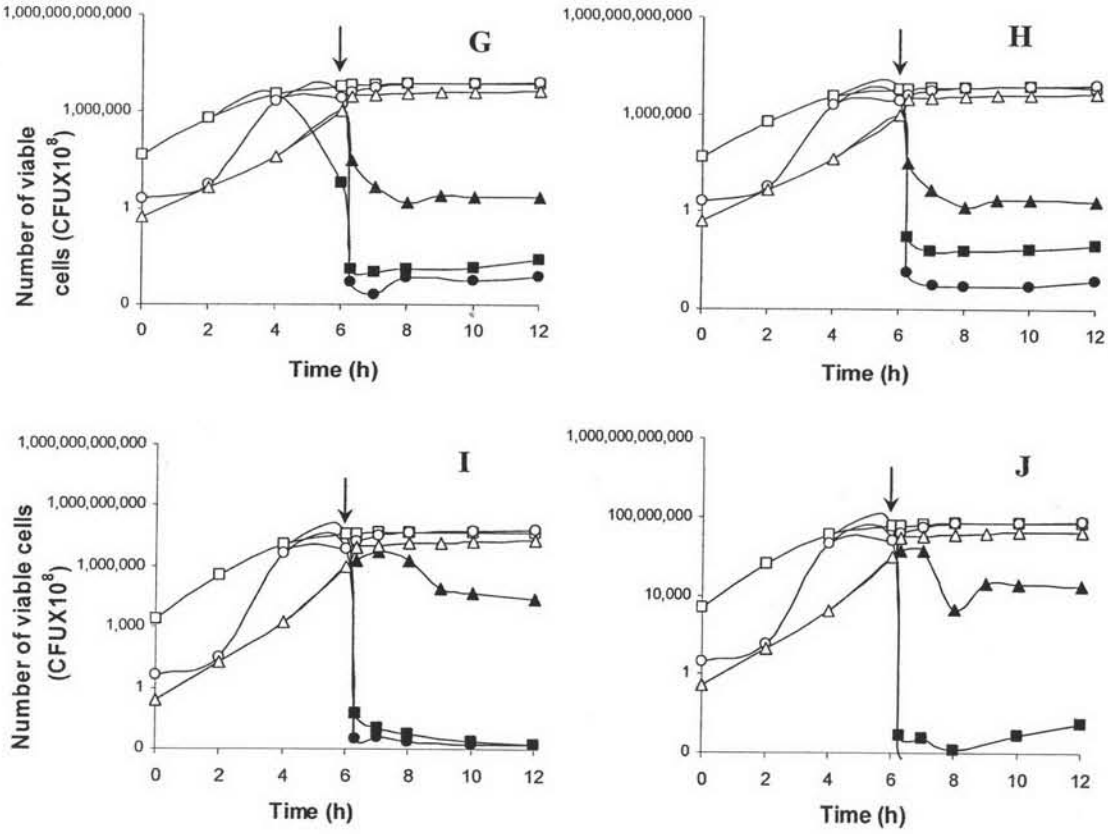


**Fig 4.26** (continue) Effect of nutrient on *n*-decane (A, B); *n*-heptane (C, D); *n*-hexane (E, F); cyclohexane (G, H); ethylbenzene (I, J) tolerance in *B. subtilis* strain 45. Cells were grown on MSBT (■), MSBY (●) and HLB (▲) medium at 45°C for 6 h. Then, organic solvent was added (↓) to 5% (v/v) (A, C, E, G, I) and 20% (v/v) (B, D, F, H, J); compared to absent organic solvent □, ○, Δ, respectively.

## 4.5 *Brevibacillus agri* strain 13

### 4.5.1 Characterization of organic solvent tolerant

#### 4.5.1.1 Effect of types and concentration of organic solvent on growth and tolerance

Growths of *Brevibacillus agri strain 13* were monitored after solvent shock with various types and quantities of organic solvents. Cells were grown to late log phase (about 6 h) before the organic solvent was added (Method 3.3.5.1). Cells growth and cell survival was assessed by viable cell number (CFU.ml$^{-}$). Cell survival after organic solvent shock are displayed in Fig 4.27, showing a relationship between cell number and the amount of solvent added (5% and 20% (v/v)) to the media compared to that in the absence of organic solvent. The ability of *Brevibacillus agri strain 13* to withstand solvent toxicity with log $P_{ow} < 2$ i.e. butyl acetate (1.8), *n*-butanol (0.8) and ethyl acetate (0.7). When exposed to ethyl acetate, the number of viable cells be fast increased at both solvent concentrations tested suggesting a relative high tolerance of cells. Although exposed to *n*-butanol, the number of viable cells slightly increased at 5% (v/v) concentration tested.

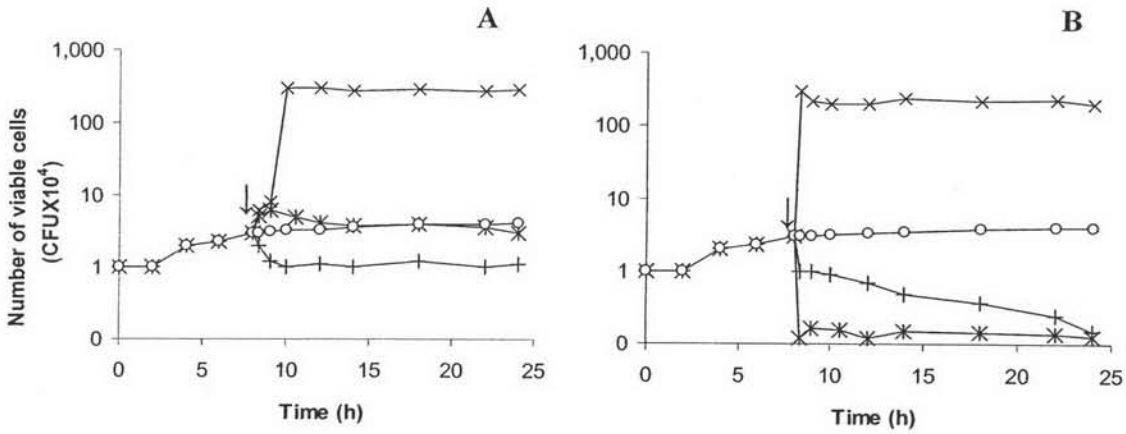


**Fig 4.27** Growth inhibition of *Brevibacillus agri strain 13* when exposed to butyl acetate; +, *n*-butanol; *, ethyl acetate; ×. Organic solvent was added (↓) to the final volume 5% (A) and 20% (B). Cell growth in the absence of organic solvent (○) was carried out as a positive control.

#### 4.5.1.2 Effect of organic solvent on cell morphology

*Brevibacillus agri* strain 13 demonstrated unique ability to tolerant ethyl acetate. Effect of organic solvent stress on morphology of *Brevibacillus agri* strain 13 was determined. Cells were grown in HLB medium at 45°C for 6 h, then ethyl acetate was added to medium (20%, v/v) and further incubated for 6 h. The control was cells grown in the absence of organic solvent. The morphology of the bacterium exposed to organic solvent was analyzed by gram strain and TEM (Fig 4.28). As compared to the morphology of cells grown with and without organic solvent exposure. *Brevibacillus agri* strain 13 cells in a direct contact to ethyl acetate not different significance of statistics at confidence 95% with compared control (Table 4.14).

**Table 4.14** Cell size of *Brevibacillus agri* strain 13 exposed with ethyl acetate

| Condition | Surface area ($\mu m^2$) | Volume ($\mu m^3$) |
|---|---|---|
| non exposed | 0.697 ± 0.073 | 5.167 ± 0.596 |
| exposed | 0.847 ± 0.078 | 5.984 ± 0.443 |

Cell length and radius were estimated by measuring the TEM photographs.

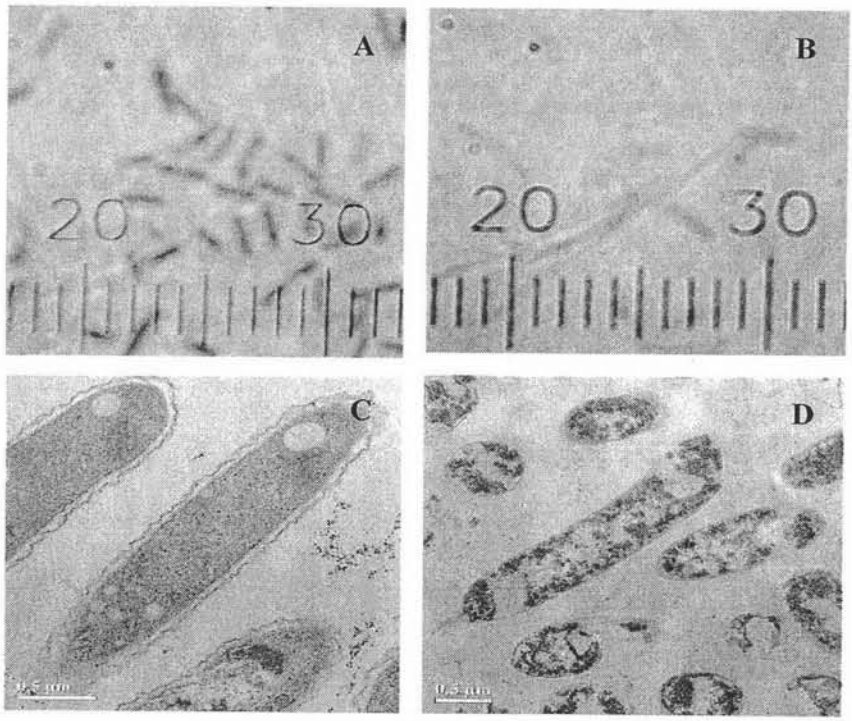


**Fig 4.28** Cell morphologies of *Brevibacillus agri* strain 13. Gram strain and transmission electron micrographs of cell were grown in HLB medium for 12 h at 45C (A, C). Gram strain and transmission electron micrographs of cell were grown in HLB medium for 6 h followed by 6 h ethyl acetate (B, D).

#### 4.5.1.3 Effect of organic solvent on fatty acid composition

The effect of ethyl acetate on the fatty acid composition of cells exposed to ethyl acetate for 6 h was determined (Table 4.15). When *Brevibacillus agri* strain 13 was grown in HLB medium non-expose ethyl acetate, it contains approximately 31% of C15:0 and C15:1; 15 % of C16:1, in relative to all compositions. As shown in Table 4.15, increase in the concentration of unsaturated fatty acids was observed in ethyl acetate-adapted cells. Furthermore, under no growth condition could trans-unsaturated fatty acids be detected.

**Table 4.15** Fatty acid composition of *Brevibacillus agri* strain 13 non-exposed and exposed to ethyl acetate

| Fatty acid | Bacterial cell fatty acid (% wt) | |
|---|---|---|
| | Non-expose | Ethyl acetate-exposure |
| C15:0 | 31.1 | 19.2 |
| C15:1 | 31.1 | 22.2 |
| C16:0 | 2.8 | 2.2 |
| **C16:1** | **14.9** | **39.5** |
| C17:0 | 9.2 | 7.7 |
| C17:1 | 10.8 | 9.3 |

#### 4.5.1.4 Organic solvent utilization

The ability of *Brevibacillus agri* strain 13 to utilize of organic solvent as a carbon source was investigated using resting cell technique in minimal medium containing 1.686 mM and 1.419 mM of *n*-butanol and ethyl acetate respectively. *Brevibacillus agri* strain 13 can degrade 85.89% and 8.53% of (1.686 mM) *n*-butnaol and (Fig 4.29) (1.419 mM) ethyl acetate (Fig 4.30) respectively in MSB liquid medium within 3 h.

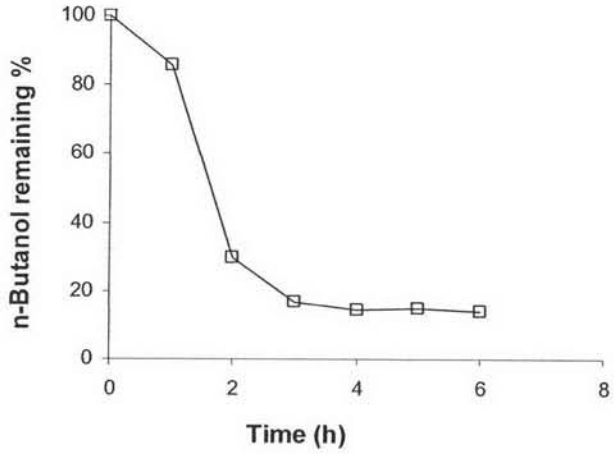


**Fig 4.29** Utilization of *n*-butanol by *Brevibacillus agri* strain 13. Cells were grown in minimal medium containing 1.686 mM (□) of *n*-butanol.

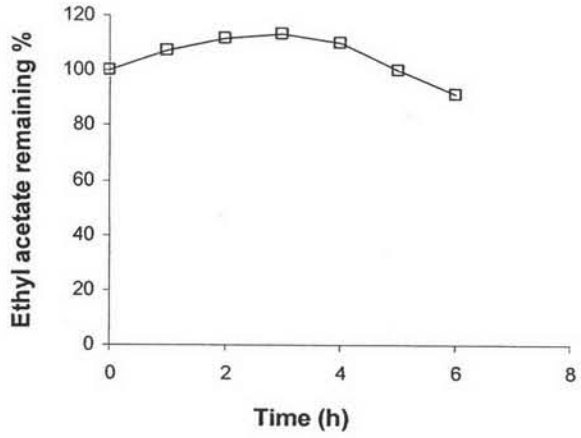


**Fig 4.30** Utilization of ethyl acetate by *Brevibacillus agri* strain 13. Cells were grown in minimal medium containing 1.419 mM (□) of ethyl acetate.

#### 4.5.1.5 Enzymatic activity involving organic solvent utilization

*Brevibacillus agri* strain 13 grown in 2 conditions of liquid medium 1) mineral medium containing 1% yeast extract, 2) mineral medium containing 1% yeast extract and 5% (v/v) of *n*-butanol or ethyl acetate. Cells were grown at 45 ºC for 24 h. The specific activities of these enzymes were determined by measuring the apparent product using a spectrophotometer (Vangnai *et al.*,2004). The results shown in Table 4.16 showed that esterase was apparently detected in both extracellular and intracellular cell at similar level whether or not cells were exposed to ethyl acetate.

Similar experiment was carried out using *n*-butanol as a substrate. Although butanol dehydrogenase activity assay did not yield significant activity level when assayed spectrophotometically, the activity of enzyme towards *n*-butanol was positively detected using native-polyacrylamilnd gel electrophoresis with activity staining. This positive result required lenger incubation period (5 h) in order to detect a band of butanol dehydrogenase. (Fig 4.31)

**Table 4.16** Esterase activity of *Brevibacillus agri* strain 13

| **Growth conditions** | **Location of enzyme** | **Specific activities** |
|---|---|---|
| | | **Unit/mg protein** |
| MSBY | **Extra cellular enzyme** | 0.012 ± 0.0005 |
| MSBY+ ethyl acetate | | 0.018 ± 0.0010 |
| MSBY | **Intracellular enzyme** | 0.034 ± 0.0025 |
| MSBY+ ethyl acetate | | 0.031 ± 0.0008 |

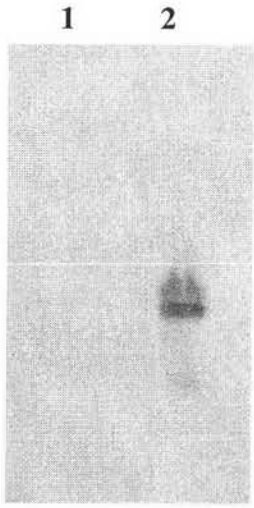


**Fig 4.31** Native- polyacrylamind gel electrophoresis with activity staining of butanol dehydrogenase by *Brevibacillus agri* strain 13 grown in *n*-butanol. The cell-free extracts (100 μg protein) from the cells grown on HLB (lane 1) and MSB with *n*-butanol (5% v/v) (lane 2) loaded on a native PAGE and activity stained with the alcohols.

### 4.5.2 Factors involving of organic-solvent tolerance

#### 4.5.2.1 Effect of ions on growth and organic solvent tolerance

The influence of various metal ions on cell growth and organic solvent tolerance was investigated. Bacterial cells were grown at 45°C for 24 h in HLB medium supplemented with 10 mM divalent ions and monovalent ions ($Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $Ca^{2+}$ and $Na^{+}$). Supplementation of $Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$ and $Ni^{2+}$ inhibited cell growth; therefore, their influences on organic solvent tolerance were not further examined. Divalent ions $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ enhanced cell growth; therefore, the influence of divalent ions on stabilization of organic solvent tolerance was investigated. To investigate the influence of metal ions towards solvent tolerance and cell growth, cells were grown on HLB containing $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ (at 2, 10 and 20 mM) for 6 h then, either butyl acetate (Fig 4.32), *n*-butanol (Fig 4.33) and ethyl acetate (Fig 4.34) was added to the final volume 5% (v/v), 20% (v/v). The result show that addition of $Ca^{2+}$, $Mg^{2}$ and $Na^{+}$ at all three concentrations tested improved cell growth of *Brevibacillus agri* strain 13. While, the presence of $Ca^{2+}$, $Mg^{2+}$ and $Na^{+}$ at all three concentrations were not significantly to butyl acetate, *n*-butanol and ethyl acetate tolerant.

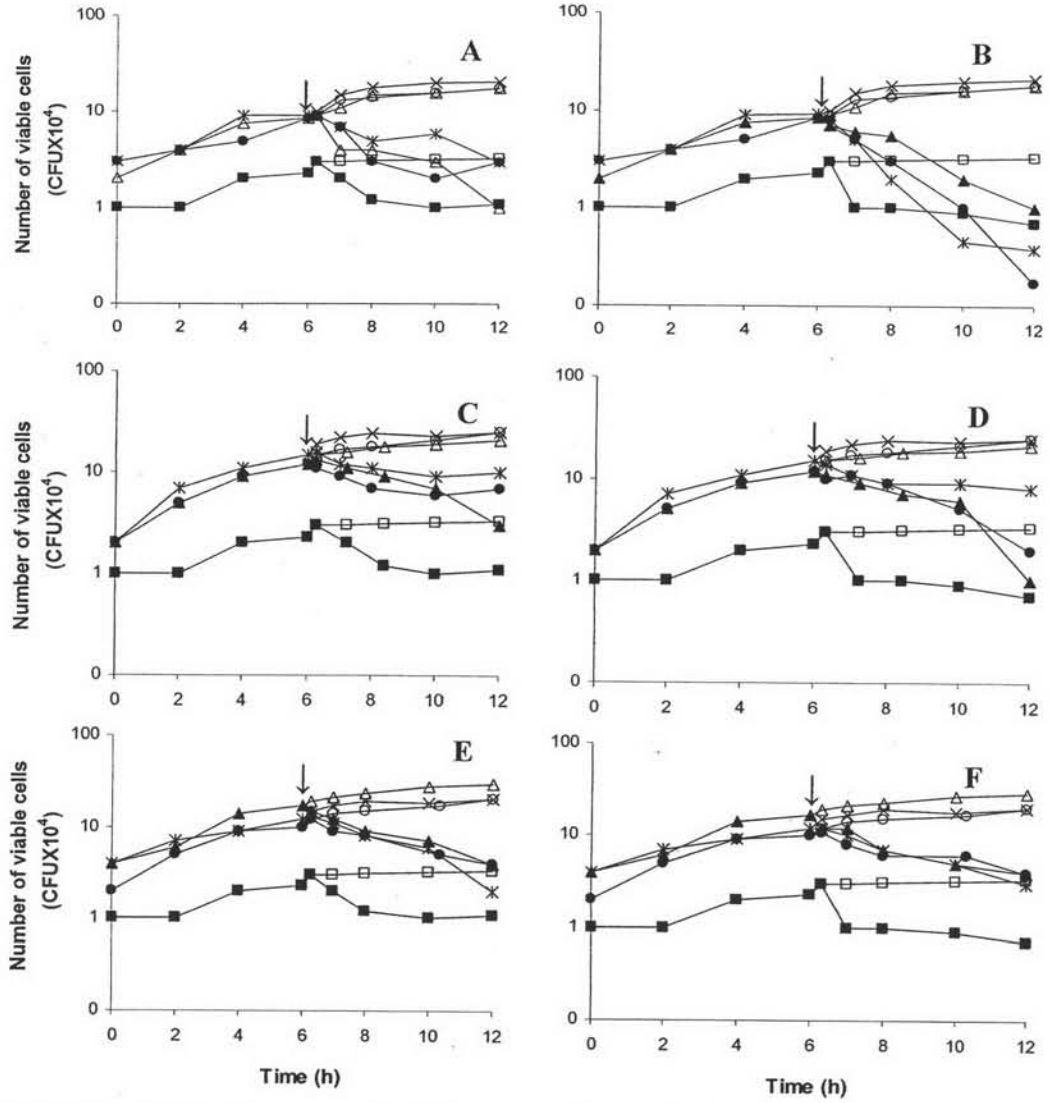


**Fig 4.32** Effect of divalent ion on butyl acetate tolerance in *Brevibacillus agri* strain 13. Cells were grown on HLB containing $Ca^{2+}$ (A, B), $Mg^{2+}$ (C, D), $Na^{+}$ (E, F) at 2, 10 and 20 mM for 6 h. Then, solvent was added (↓) to 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F) compared to that in the absence of organic solvent (○, ×, Δ). Symbols of organic solvent are:

| | | | | | |
|---|---|---|---|---|---|
| **A,C, E** | ○ | 2mM ion, 0% solvent | **B, D, F** | ○ | 2mM ion, 0% solvent |
| | ● | 2mM ion, 5% solvent | | ● | 2mM ion, 20% solvent |
| | × | 10mM ion, 0% solvent | | × | 10mM ion, 0% solvent |
| | * | 10mM ion, 5% solvent | | * | 10mM ion, 20% solvent |
| | Δ | 20mM ion, 0% solvent | | Δ | 20mM ion, 0% solvent |
| | ▲ | 20mM ion, 5% solvent | | ▲ | 20mM ion, 20% solvent |
| | □ | 0mM ion, 0% solvent | | □ | 0mM ion, 0% solvent |
| | ■ | 0mM ion, 5% solvent | | ■ | 0mM ion, 20% solvent |

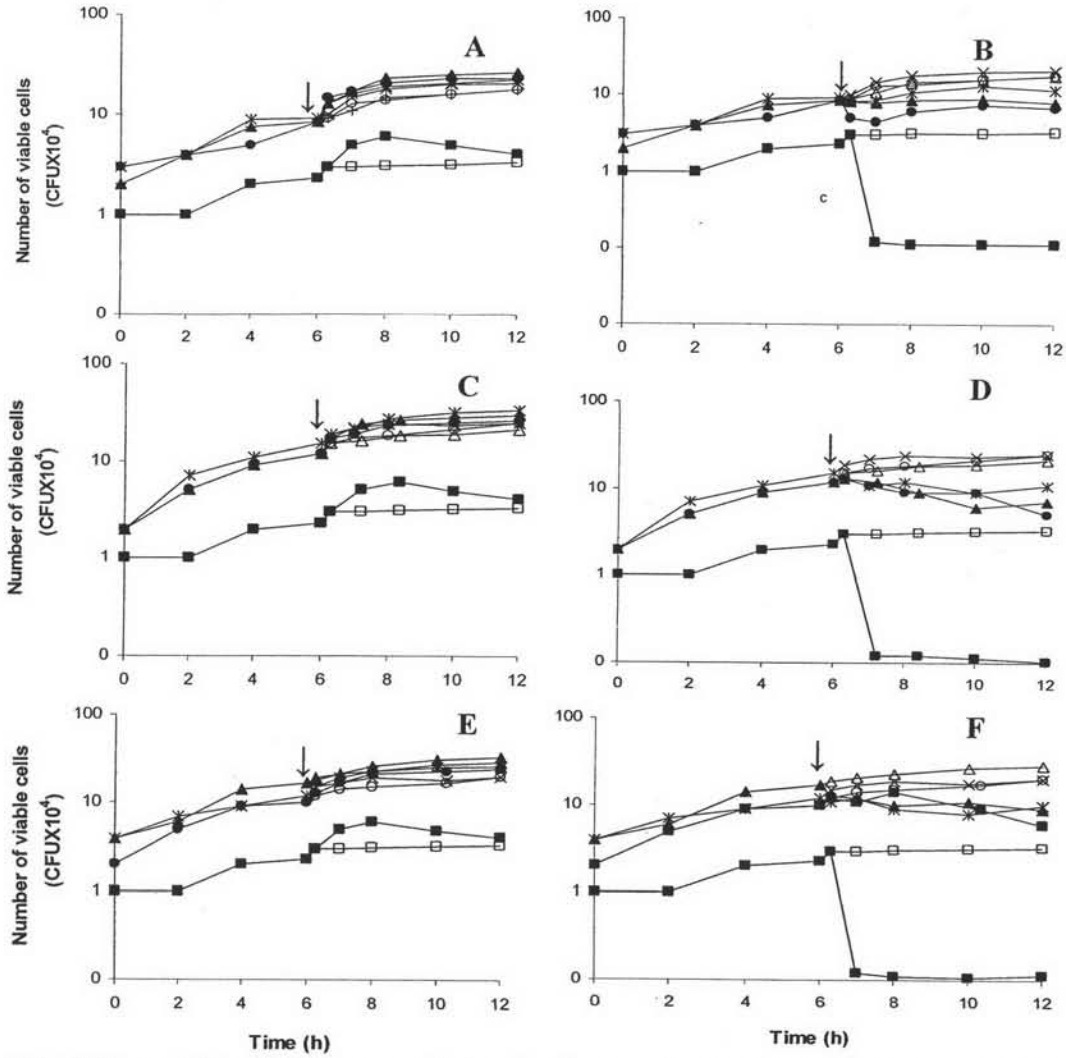


**Fig 4.33** Effect of divalent ion on *n*-butanol tolerance in *Brevibacillus agri* strain 13. Cells were grown on HLB containing $Ca^{2+}$ (A, B), $Mg^{2+}$ (C, D), $Na^{+}$ (E, F) at 2, 10 and 20 mM for 6 h. Then, solvent was added (↓) to 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F) compared to that in the absence of organic solvent (○, ×, Δ). Symbols of organic solvent are:

| **A,C, E** | | **B, D, F** | |
|---|---|---|---|
| ○ | 2mM ion, 0% solvent | ○ | 2mM ion, 0% solvent |
| ● | 2mM ion, 5% solvent | ● | 2mM ion, 20% solvent |
| × | 10mM ion, 0% solvent | × | 10mM ion, 0% solvent |
| * | 10mM ion, 5% solvent | * | 10mM ion, 20% solvent |
| Δ | 20mM ion, 0% solvent | Δ | 20mM ion, 0% solvent |
| ▲ | 20mM ion, 5% solvent | ▲ | 20mM ion, 20% solvent |
| □ | 0mM ion, 0% solvent | □ | 0mM ion, 0% solvent |
| ■ | 0mM ion, 5% solvent | ■ | 0mM ion, 20% solvent |

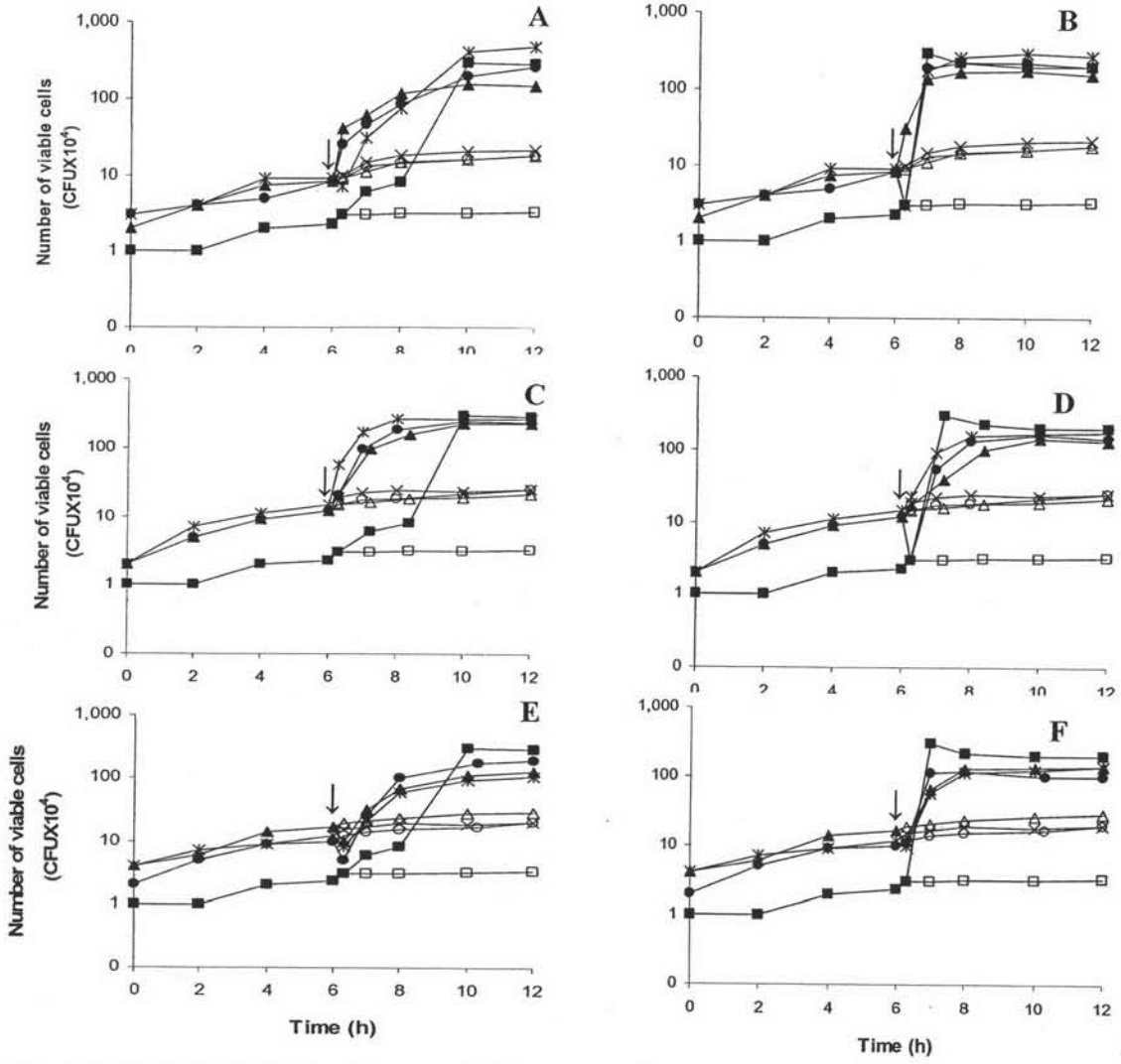


**Fig 4.34** Effect of divalent ion on ethyl acetate tolerance in *Brevibacillus agri* strain 13. Cells were grown on HLB containing $Ca^{2+}$ (A, B), $Mg^{2+}$ (C, D), $Na^{+}$ (E, F) at 2, 10 and 20 mM for 6 h. Then, solvent was added (↓) to 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F) compared to that in the absence of organic solvent (○, ×, Δ). Symbols of organic solvent are:

| | | | | | |
|---|---|---|---|---|---|
| **A,C, E** | ○ | 2mM ion, 0% solvent | **B, D, F** | ○ | 2mM ion, 0% solvent |
| | ● | 2mM ion, 5% solvent | | ● | 2mM ion, 20% solvent |
| | × | 10mM ion, 0% solvent | | × | 10mM ion, 0% solvent |
| | * | 10mM ion, 5% solvent | | * | 10mM ion, 20% solvent |
| | Δ | 20mM ion, 0% solvent | | Δ | 20mM ion, 0% solvent |
| | ▲ | 20mM ion, 5% solvent | | ▲ | 20mM ion, 20% solvent |
| | □ | 0mM ion, 0% solvent | | □ | 0mM ion, 0% solvent |
| | ■ | 0mM ion, 5% solvent | | ■ | 0mM ion, 20% solvent |

### 4.5.2.2 Effect of nutrient on growth and organic–solvent tolerance

The following carbon sources were tested for cell growth and solvent-tolerance enhancement: MSB medium containing 16 mM of glucose (MSBG), fructose (MSBF), galactose (MSBGa), sucrose (MSBS), xylose (MSBX), rhamnose (MSBR), mannitol (MSBM), citrate (MSBC), succinate (MSBSc), 1% of yeast extract, tryptone, LB, HLB. *Brevibacillus agri* strain 13 showed good growths in HLB, LB medium (Fig 4.35). To investigate the influence of nutrient towards organic solvent tolerance, cells were grown on LB and HLB medium at 45°C for 6 h then, either butyl acetate, *n*-butanol and ethyl acetate was added to 5% (v/v), 20% (v/v) (Fig 4.36).The result show that LB medium tested improved cell growth of *B. circulans* strain 13 but not significantly to tolerant.

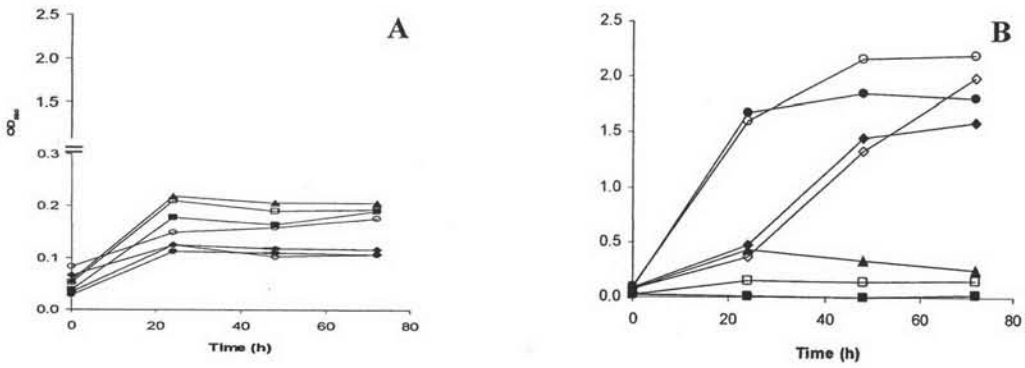


**Fig 4.35** Growth of *Brevibacillus agri* strain 13 in various types of nutrients. Symbols of organic solvents are:

| | | | | | |
|---|---|---|---|---|---|
| **A** | ■ | Glucose | **B** | ■ | Citrate |
| | □ | Fructose | | □ | Succinate |
| | ● | Rhamnose | | ● | HLB |
| | ○ | Xylose | | ○ | LB |
| | ♦ | Mannitol | | ♦ | MSBY |
| | ◊ | Galactose | | ◊ | MSBT |
| | ▲ | Sucrose | | ▲ | MSBP |

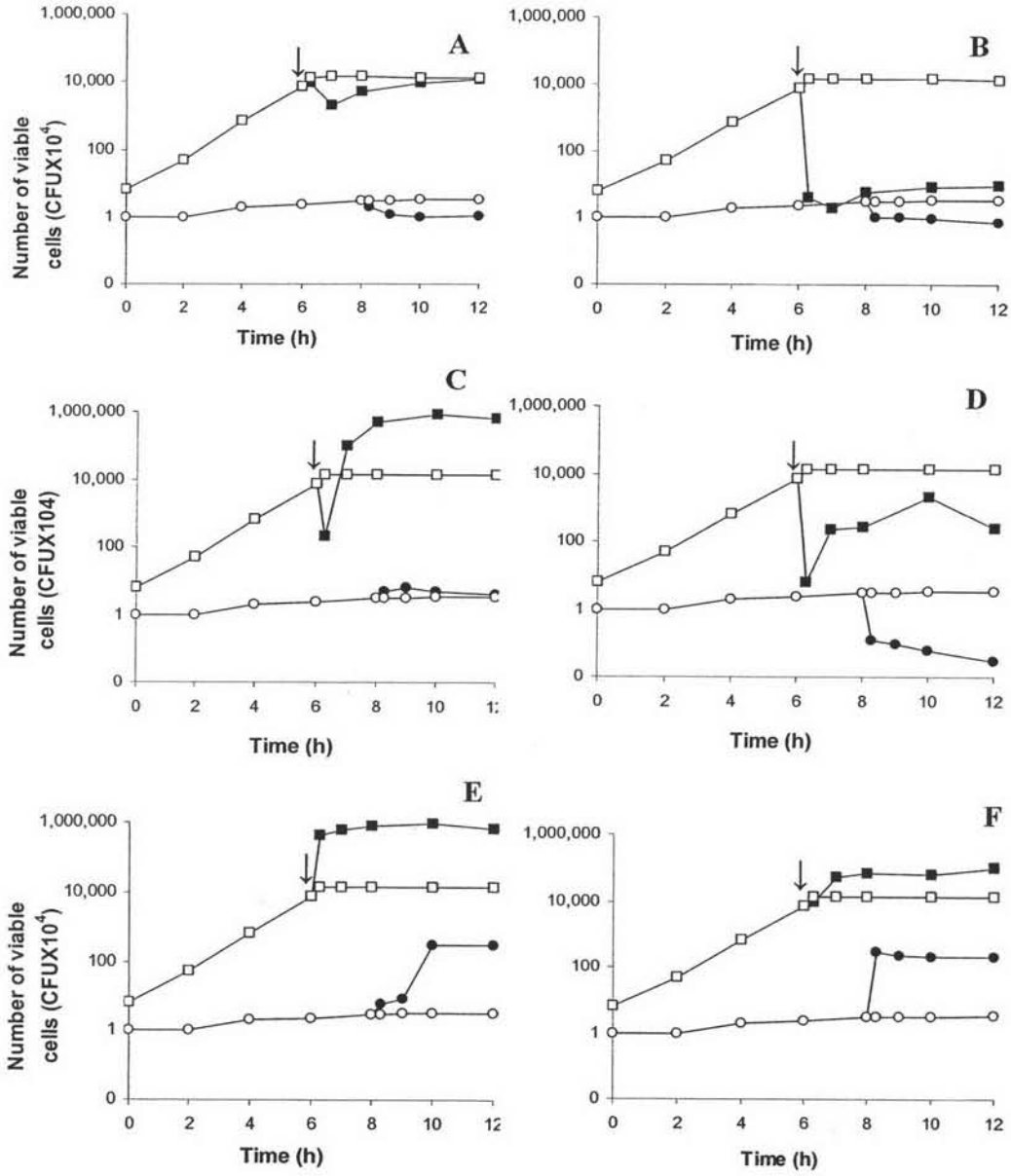


**Fig 4.36** Effect of nutrient on butyl acetate (A, B); *n*-butanol (C, D); ethyl acetate (E, F) tolerance in *Brevibacillus agri* strain 13. Cells were grown on LB (■) and HLB (●) medium at 45°C for 6 h. Then, organic solvent was added (↓) 5% (v/v) (A, C, E) and 20% (v/v) (B, D, F).