Organic solvent-tolerant bacteria in mangrove ecosystem

Organic solvents are known to be extremely toxic to cells. They dissolve and accumulate in the bacterial cell membrane, resulting in changes in structural and functional integrity and cause cell lysis[1,2]. Organic solvent-tolerant bacteria are a newly discovered group of micro-organisms with novel tolerance mechanisms, which enable them to thrive in solvent-saturated environments[1–3]. These bacteria are significant due to their immense potential in non-aqueous bio-catalysis[3], industrial processes[4,5] involving biphasic organic-aqueous fermentation systems, effluent treatment and bioremediation in hydrocarbon-saturated environments[6–9].

Inoue and Horikoshi[1] used the parameter log *P* as a measure of solvent toxicity, where *P* is the partition coefficient of the given solvent in an equimolar mixture of octanol and water. The greater the polarity of a solvent, the lower its log *P* value and the greater its toxicity. In general, solvents with log *P* values below 4 are considered extremely toxic. Solvent tolerance is a strain-specific property and every micro-organism has a limiting log *P* value below which it is unable to grow. This intrinsic tolerance level is determined genetically and is also influenced by environmental factors[10]. The most toxic solvent to which a given microbial strain is tolerant is called the index solvent and the log *P* value of the index solvent is called the index value of that particular organism[11].

Many micro-organisms are known to degrade organic solvents, but their tolerance to these solvents is less than 0.3% (v/v)[1]. This has been a major limiting factor in biodegradation in natural habitats[6]. Organic solvent-tolerant micro-organisms with the required enzymes could help in degrading pollutants like benzene and toluene which are carcinogenic in ppm amounts[6–8]. Hydrocarbon-oxidizing bacteria and fungi play an important role in bioremediation of oil in the sea and represent one of the primary mechanisms by which petroleum and other hydrocarbon pollutants are eliminated from the environment[12]. It has been demonstrated that the level of organic solvent tolerance of a given strain in soil is related to its level of tolerance to the solvent in liquid medium. The higher the tolerance in liquid media, the faster the recovery of the strain in soil after solvent shock. Therefore, in sites heavily contaminated with aromatic hydrocarbons, solvent-tolerant strains would be expected to establish first, colonize the site and become predominant in the removal of these compounds[7].

The present study was undertaken primarily to determine the existence of bacteria which possess the traits of organic solvent tolerance and hydrocarbon degradation in natural ecosystems. To achieve this goal, mangrove sediment and water samples from various sites of the Mandovi estuary in Goa were collected and analysed. Our studies led to the isolation of a unique strain of *Bacillus* from mangrove sediment which tolerates the organic solvent *n*-butanol having a log *P* value of 0.8, conferring on it the lowest ever reported index value for any organic solvent tolerant bacterium. In addition to this, the culture also has the potential to degrade aliphatic and aromatic hydrocarbons and hence could play a significant role in waste-water treatment and bioremediation.

This culture was enriched from a mangrove sediment sample by a stepwise process. Initially, the sediment was kept soaked in *n*-butanol for a month. One gram of this sample was transferred to a flask containing artificial sea water supplemented with 20% (v/v) butanol. The flasks were incubated at 30°C on a rotary shaker for one week after which the organic layers from these flasks were transferred to a nutrient-rich medium (LBMG, i.e. modified Luria broth containing 1% tryptone, 0.5% yeast extract, 1% NaCl, 10 mM $MgSO_4$ and 0.1% glucose)[11] and overlaid with 50% (v/v) of the organic solvent. After 2 days of incubation on a shaker, 0.1 ml of the organic layer was plated on LBMG agar and the plates were incubated. A single bacterial culture producing white wrinkled colonies was obtained. This culture, designated as SB-1, was found to be an aerobic endospore forming Gram-positive rod and was identified as a strain of *Bacillus* species[13].

Young culture was inoculated in flasks in a medium containing mineral salts in which the organic solvents (1% v/v) such as *n*-butanol, benzene and toluene served as the only carbon source. SB-1, being a hydrocarbon degrader, was capable of growth under these conditions. It also formed colonies on mineral medium plates overlaid with solvents serving as the sole carbon source.

Organic solvent tolerance assays[1,9] with SB-1 were carried out in solid and liquid LBMG media. In the plate assay, the culture was spread over LBMG agar, overlaid with 2 ml each of the organic solvent and incubated at 30°C. The culture showed growth on direct exposure to a wide range of organic solvents (Table 1). In the liquid medium assay, the culture was inoculated in LBMG broth and mineral medium in flasks overlaid with 1, 10, 50 and 90% (v/v) of benzene, toluene and *n*-butanol, and incubated. Active growth and multiplication occurred in the presence of 1% (v/v) of the solvent; however, growth was inhibited at higher concentrations. Viable cells could be isolated by plating on LBMG agar from all the flasks, including the ones with 90% (v/v) of solvent even after 90 days of incubation. Both the assays indicate that *Bacillus* SB-1 exhibits a high level of tolerance to organic solvents.

Since *n*-butanol tolerance is a novel trait, effects of *n*-butanol concentration on growth rate of SB-1 and the minimum inhibitory concentration of *n*-butanol for its growth were determined. The culture was inoculated in different concentrations of *n*-butanol (0.1 to 10% v/v) and growth was monitored by determining the absorbance and by determining the viable counts on LBMG agar at periodic intervals.

Our results show that SB-1 can grow in up to 2% (v/v) of *n*-butanol, whereas growth is severely retarded at 3% (v/v) in LBMG broth. Growth rate increases with increase in butanol concentration (Table 2). Butanol toxicity is due to its amphiphilic nature. Its water solubility being 8%, the degree of partition into the aqueous phase and from there into the cell membrane of bacterial cells is

Table 1. Organic solvent tolerance assay for SB-1 (plate method)

Organic solvent	Log *P* value	Number of colonies of SB-1
n-decane	6	Matt
Iso-octane	4.8	Matt
n-hexane	3.9	154
Cyclohexane	3.4	50
Xylene	3.0	44
Toluene	2.8	52
Benzene	2.1	35
Chloroform	1.9	124
n-butanol	0.8	4

Growth of SB-1 was observed on plates overlaid with all of the above solvents. The control plate (without any organic solvent) showed 312 colonies.

Table 2. Effect of *n*-butanol concentration on growth rate and generation time of SB-1 in LBMG broth

Butanol (% v/v)	Growth rate at 30°C*	Percentage reduction in growth rate at 30°C#	Percentage reduction in growth rate at 48°C
Control flask without butanol	2.5	→	→
1	0.710	71.6	100
2	0.20	92	100
3	0.07	97.2	100
4	No growth	→	→

*There is no variation in growth rate of SB1 when grown at pH 7 and pH 9.
#There is no variation in percentage reduction in growth rate of SB-1 when grown at 30 and 37°C.

quite high. It is known to induce leakage of potassium ions and protons, which in turn affect the proton gradient and interdependent activities like respiration and pH homeostasis. The increased membrane leakage eventually results in cell death[2,14]. The only bacterium reported to exhibit some tolerance to this alcohol is *Clostridium acetobutylicum*, which is the producer organism in acetone–butanol fermentation[15]. However, even this culture shows 99% reduction in growth yield at 1.23% (v/v) of butanol[16].

Extensive work has been done on the organic solvent tolerance mechanisms of Gram-negative bacteria[2,3], but very little is known about the solvent tolerance of Gram-positive bacteria. We tried to determine the effect of factors like composition of the growth medium, its pH and temperature of incubation on the growth of SB-1 in presence of *n*-butanol. It was found that although the culture grows rapidly at 48°C in the absence of *n*-butanol, its growth in the presence of 1% (v/v) *n*-butanol is severely retarded at this temperature. There is no difference in growth rate in the presence of butanol (1% v/v) at pH 7 and 9 (Table 2). In LBMG broth, there is growth at up to 3% (v/v) of *n*-butanol, whereas in mineral medium with *n*-butanol as the carbon source, growth at concentrations above 1.5% (v/v) is severely affected. The presence of Mg^{+} ions is also found to have a beneficial effect on growth in the presence of *n*-butanol. Butanol tolerance is a stable phenotypic property of this culture which is not lost even after repeated sub-cultures in the absence of the organic solvent. To our knowledge, this is the first report of a bacterium tolerant to such high concentrations of *n*-butanol.

It appears that bacteria tolerant to a wide range of solvents with log *P* values as low as 0.8 exist in nature. Solvent tolerance, though a strain-specific property, is influenced by environmental factors and availability of nutrients. *Bacillus* sp. SB1 could serve as a unique model in understanding the solvent tolerance phenomenon in Gram-positive bacteria and play a significant role in bioremediation and biocatalysis.

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Received 23 August 2001; revised accepted 11 January 2002

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