Isolation and characterization of a solvent tolerant alkaliphilic marine bacteria

Roselin Alex*, and Sarita G. Bhat**

Abstract. Organic solvents are extremely toxic to microbial cells even at very low concentration of 0.1% (v/v). They disrupt the bacterial cell membrane, affecting the functional and structural integrity of the cells. However some microorganisms are known to tolerate highly toxic chemicals and hold potential for application in industrial production of toxic chemicals. Majority of the reported solvent tolerant bacteria are Gram negative. Here we report the isolation of a Gram positive bacterium capable of tolerating various organic solvents. The bacteria was identified as *Lysinibacillus fusiformis* and it showed tolerance to both aromatic and aliphatic solvents with maximum tolerance for acetone, benzene and octane and minimum tolerance towards dichloromethane. Bacteria showed maximum growth at 45 ^{0} C, pH 10.0 and tolerated up to 800mM of NaCl.

Index Terms— Alkaliphilic, Bacteria, Characterization, Enzyme, Isolation, solvent – tolerance**.**

1 INTRODUCTION[1]

Contamination of the environment with toxic chemicals causes great damage to all living things. Ability of microorganisms to transform and degrade many types of pollutants has been widely recognized [1], [2]. Organic solvent tolerant bacteria are a group of organisms which have specific mechanisms to thrive in toxic solvent saturated environments [3]. Inoue and Horikoshi 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. When the polarity of the solvent is higher the log P values decreases and the value of solvent toxicity increases. The solvents with log P values below 4 are considered to be extremely toxic. Every microorganism has limiting log P values below which it is unable to grow. This property is determined by genetic and environmental factors. The most toxic solvent to which a given microorganism is tolerant is called the index solvent and the log value of the index solvent is the index value of that particular solvent [4]. There are different mechanisms which enable solvent tolerant bacteria to grow in the presence of hydrocarbons. They involve either modification of the membrane and cell surface properties, changes in the overall energy status or activation of active transport system for extracting hydrocarbons from the membranes in to the environment [5]. It has been proposed that the mechanisms of solvent tolerance of the benzene tolerant *Bacillus DS*-1906 and the toluene tolerant *P.putidaIH-2000* are different, due to differences in cell surface components. Many of these benzene tolerant bacteria also have the potential to degrade this solvent. It is believed that organic solvent emulsifying; deactivating, solubilising enzymes/substances could play a very important role in diminishing solvent toxicity in Gram-positive bacteria [1]. There are three reported approaches to assess the solvent tolerance of bacteria. The first one involves overlaying agar medium which was previously inoculated with the test organism with the solvent .[7]. The second one involves growing the bacteria in presence of a solvent in liquid medium and third approach involves exposing a high density suspension of cells in exponential phase to a solvent and counting the number of colony forming units. The present work was undertaken to identify and characterize an organic solvent tolerant bacterium from the sediments collected from Munakkal beach, in Kerala, India. The bacterium was preserved by lyophhilization.

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2 MATERIALS AND METHODS

2.1 Isolation and screening of solvent tolerant Bacteria.

10 gm of sediments collected from the Munakkal beach in Kerala, India was added to 90 ml of sterilized sea water and mixed thoroughly by keeping it in a shaker at 150 rpm for 1 hr. Then 1 ml of soil solution was inoculated into 100ml Marine- Zobell broth medium in a 250 ml conical flask and incubated at 37^{0}C for 24 hrs in a shaker at 130 rpm. All the bacteria isolated from the medium were used for screening of solvent tolerant bacteria. Bushnell-Haas medium (B.H medium) was used in the enrichment technique (ref). B.H medium was supplemented with 1 % v/v of organic solvents. The enrichment medium contained equivalent mixture of octane, cyclohexane and hexane and an equivalent mixture of benzene, toluene, ethyl benzene and xylene. After one week of incubation on a rotary shaker at 37 ^{0}C, 1 ml of culture from primary enrichment medium was transferred to fresh Bushnell – Hass medium containing the same components and continued to incubate for one more week. After second enrichment, 0.1 ml was plated on a Marine- Zobell agar and incubated at 37^{0}C for 24 hrs. The potential strains capable of utilizing organic solvents as sole source of carbon were isolated and stored at -20^{0}C in 50mM KH_2PO_4 /K_2 HPO_4 buffer at pH 7..2 containing 20 % (v/v) glycerol. Working cultures were maintained by sub culturing in every week on mineral salt agar slants containing crude oil at 1%(v/v) concentration and permanent stocks were maintained by lyophilisation.

2.2 Identification of the selected Bacterial Strain.

The selected bacterial strain was identified based on morphological and biochemical characteristics as outlined in Bergey's Manual of Systematic Bacteriology [9]. Epifluorescence microscopy was used to study bacterial morphology and endospore. The organism was identified by using 16S rRNA gene sequence analysis

2.3 Enzyme profile of the culture

Enzyme profile of the selected strain was determined as described below.

2.3.1 Protease

The proteolyitic activity was measured according to Nitkowski et al, (1977)[5]. The proteolytic medium consisted of 0.3 % beef extract, 0.5 % peptone, 3% NaCl and 1.5 % agar in distilled water. Casein was provided by adding 1:1 (v/v) solution of evaporated, skimmed milk diluted aseptically in sterile distilled water, the final concentration of milk was 1.5 %. The mixture was swirled gently and poured into Petri plates, which were subsequently dried at room temperature. A loop full of bacterial culture was spot inoculated on the solidified skimmed milk agar plate. The plate was incubated at room temperature (28 ± 2°C) for 24 hr uninoculated media was kept as control. Formation of a clear halo around the bacterial colony was considered as positive for casein hydrolysis and hence proteolysis.

2.3.2 Lipase

Lipase assay was done according to Kim and Hoppe, (1986). The Zobell Marine agar medium supplemented with 1% Tween 80 (sorbitol monooleate; w/v) was used as lipase substrate, autoclaved and poured into sterile Petri plates. A loop full of bacterial culture was spot inoculated and incubated at room temperature (28 ± 2°C) for 24 h. Uninoculated media was kept as control. Appearance of dense opacity around the colony was considered as positive indicating production of extracellular lipase.

2.3.3 Alpha amylase

. Bacterial culture was spot inoculated on the solidified agar plates and incubated at room temperature (28± 2°C) for 24 h. After incubation the plate was flooded with Iodine reagent. Iodine reagent reacts with starch and forms a blue coloured complex. A clearing zone around the bacterial colony is an indication of extracellular alpha amylase production. An uninoculated media was kept as control.

2.4 Effect of temperature on the growth of Bacteria

Optimum temperature for maximum growth was evaluated by incubating the inoculated media at various temperatures viz 25^{0}C, 30^{0}C, 37 ^{0}C, 40^{0}C, 50^{0}C and 55^{0}C, for 24 hrs at 130 rpm in an orbital shaker. Growth was studied by measuring the OD at 600nm in UV- visible spectrophotometer.

2.5 Effect of salinity

Effect of salinity on maximum growth was studied by incubating the media with different concentrations of sodium chloride viz 100 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM, 800 mM, 900 mM and 1 M at 130rpm for 12 hrs in an environmental shaker. All the experiments were conducted in triplicate

2.6 Optimum pH

To find the optimum pH for growth, the bacteria were grown in media with different pH ranging from pH 2 to 12. Growth was studied by measuring turbidity at 600nm in UV-visible spectrophotometer after 12 hrs of incubation in an orbital shaker at 130 rpm at 28^{0}C.

2.7 Utilization of various carbohydrates for growth.

The utilization of various carbohydrate sources for growth was studied by growing bacteria in mineral salt medium (pH 9) supplemented with starch, sucrose, cellulose, maltose, lactose dextrose, galactose, sorbitol, manitol and fructose at 100mM concentration. The inoculated medium was incubated at of 28^{0}C in an orbital shaker at 130 rpm for 12 hrs. Growth was studied by measuring turbidity at 600nm in UV-visible spectrophotometer.

2.8 Study of tolerance of bacteria to various organic solvents.

To study the tolerance of bacteria to various organic solvents *viz* acetone, benzene, toluene, ethylbenzene, dichloromethane, octane, xylene, heptane, hexane and cyclohexane were added at concentrations of 10% and 50% v/v to the mineral salt medium in which bacterial cells were added at a concentration of 1%(v/v). The growth was measured by taking OD at 600 nm in a UV-visible spectrophotometer. Mineral salt medium inoculated with heat killed cells of the same concentration of the culture used for inoculation of samples was used as control.

3 RESULTS AND DISCUSSION.

After the preliminary and secondary screening for solvent tolerant bacteria, one isolate was obtained with the ability from the Bussnell Haas medium.The isolate was Gram-positive, motile, facultatively anaerobic, catalase, oxidase, nitrate reductase, indole, gelatin hydroxylase, urease, indole and amylase positive, protease and lipase negative, terminal spore forming bacteria with cream coloured uniform colonies. The organism was identified by using 16SrRNA gene sequencing as *Lysinibacillus fusiformis* .

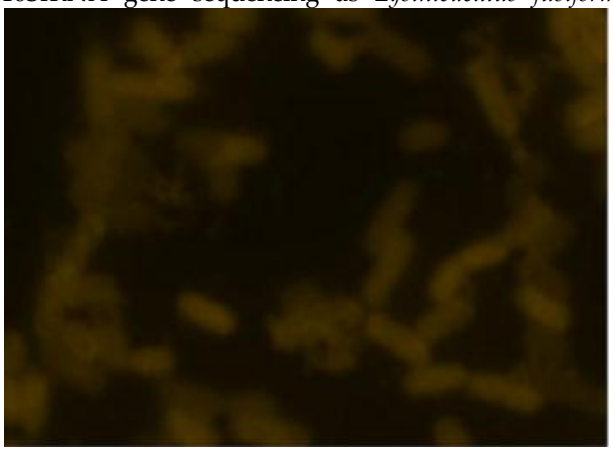

Fig. 1 Epifluorescence microscopic picture of *Lysinibacillus fusiformis*

3.1**. Effect of temperature on the growth of bacteria**

Effect of temperature on the growth of bacteria was studied by incubating the test organism at various temperatures and it was found that the maximum growth was at 45 ^{0}C (Fig.2.).

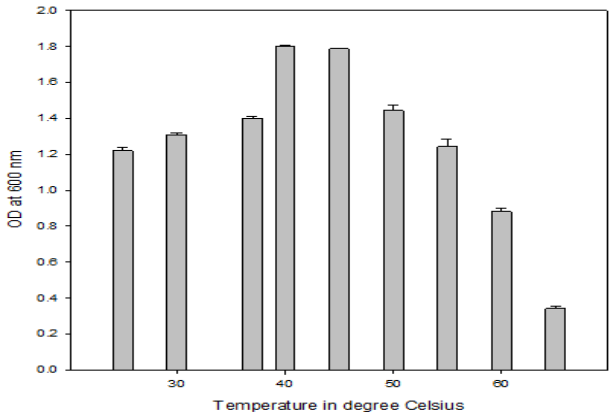

Fig.2.Effect of temperature on the growth of *Lysinibacillus fusiformis*

3.2 Optimum pH for growth

The data showed that *Lysinibacillus fusiformis* is an alkaliphilic organism with optimum pH at for growth pH

10 and the bacteria was also able to tolerate pH of 13(Fig.3). They were unable to grow below pH 6.

3.3 Effect of salinity on growth

Studies on the effect of salinity on the growth of bacteria indicated that the optimum salinity for the growth of bacteria was 200 mM NaCl (Fig.4). Further it was found that the bacteria could tolerate up to 800 mM of NaCl.

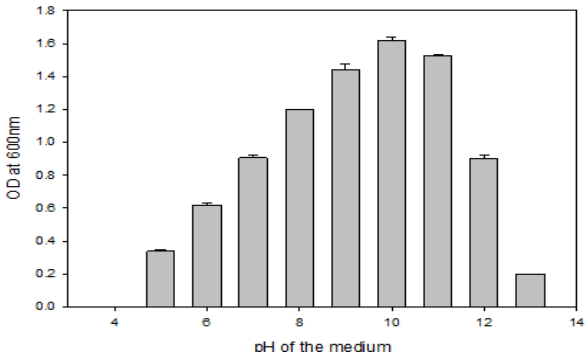

Fig.3 Effect of pH on the growth of the *Lysinibacillus fusiformisBTTS*

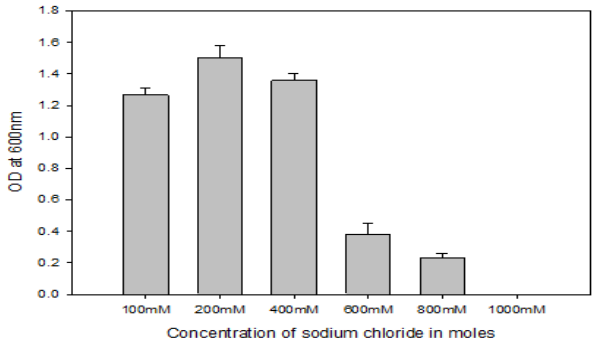

Fig.4.Effect of sodium chloride concentration on the growth of *Lysinibacillus fusiformis*

3.4 Utilization of various Carbohydrate sources

The test organism clearly exhibited the ability to utilise simple sugars effectively when tested for its ability to grow on different carbon sources. Maximum growth was obtained with glucose as C-source (Fig.5).

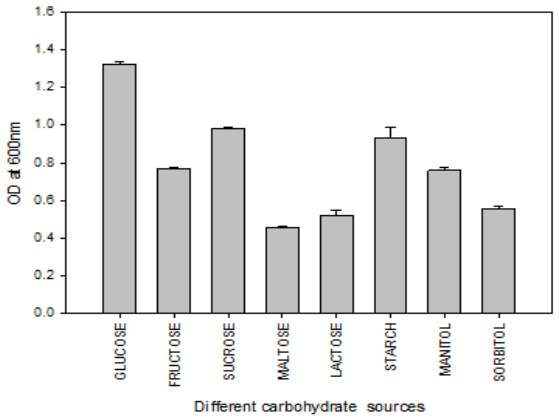

Fig.5. Utilization of different carbohydrates for growth

3.5 Tolerance of bacteria to various organic solvents.

The ability of the bacteria to tolerate and grow in all the various organic solvents tested - acetone, benzene, toluene, ethylbenzene, dichloromethane, xylene, octane, heptane, hexane and cyclohexane, at 10% (v/v) concentration,was observed. (Fig.6) The organism could tolerate both aromatic and aliphatic solvents with maximum tolerance for acetone, benzene and octane. The organism showed minimum tolerance towards dichloromethane. Several Gram-positive bacteria tolerant to organic solvents have been previously identified [10], including Benzene tolerant *Rhodococcus sp*. from a chemical-contaminated site in Australia (6), *Bacillus sp.* strain tolerant to organic solvent butanol from soil (8), and a *Bacillus sp*. from deep sea with a high level of tolerance to benzene [7]. Whereas, bacteria isolated in this report are found to be capable of tolerating both aromatic and aliphatic organic solvents, tolerating ten different solvents

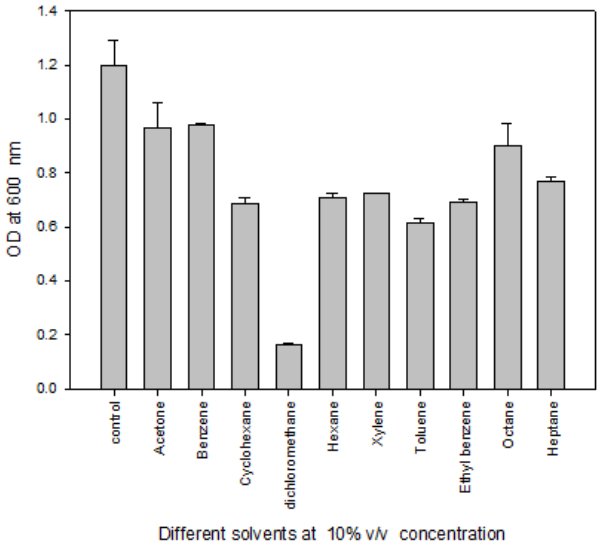

Fig.6. Tolerance of *Lysinibacillus fusiformis* to different solvents

CONCLUSION

Organic solvent tolerant bacteria are an interesting group of extremophiles with specific adaptations for surviving in toxic environment. This property can be exploited in many industrial processes. Studies were conducted mostly on Gram negative bacteria to understand the mechanism of solvent tolerance but there is a great gap in the data available on the mechanism of tolerance in Gram positive bacteria. The bacteria isolated in this report are found to be capable of tolerating both aromatic and aliphatic organic solvents, tolerating ten different solvents. The results of the present study indicated scope for utilizing solvent tolerant bacteria for various industrial applications.

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