Mar. Biotechnol. 5, 116–118, 2003
DOI: 10.1007/s10126-002-0069-y

MARINE BIOTECHNOLOGY

Isolation of an Organic-Solvent-Tolerant Cholesterol-Transforming *Bacillus* species, BC1, from Coastal Sediment

Yogita Sardessai[1,*] and Saroj Bhosle[2]

[1]*Goa College of Pharmacy, Panjim, Goa, India*
[2]*Department of Microbiology, Goa University, Taleigao Plateau, Goa 403206, India*

Abstract: Steroid transformation is of great importance in the pharmaceutical industry. The major limiting factor in this process is the extremely poor solubility of steroids in aqueous media, which lowers their transformation rate and increases costs. This problem can be overcome by using organic-solvent-tolerant bacteria (OSTB), which can carry out the desired bioconversions in an organic-solvent-saturated system. OSTB are a relatively novel group of extremophilic microbes that have developed various adaptations to withstand solvent toxicity. The aim of this study was to isolate marine bacteria producing organic-solvent-stable cholesterol-transforming enzymes. A *Bacillus* species, BC1, isolated from Arabian Sea sediment was found to degrade cholesterol and exhibit excellent solvent tolerance particularly to chloroform. OSTB have tremendous potential in industrial processes involving nonaqueous biocatalysis and transformation in the presence of an organic phase.

Key words: cholesterol, chloroform, organic solvents, tolerance, biotransformation, bacterial.

INTRODUCTION

Organic-solvent-tolerant bacteria (OSTB) are a relatively new group of extremophilic microorganisms, which are capable of thriving in solvent-saturated environments on account of their unique adaptive mechanisms. Most of the reported and well-studied OSTB are gram-negative bacteria, particularly strains of *Pseudomonas* tolerant to toluene. Far less data is available on gram-positive OSTB in comparison (De Bont, 1998). However, most of the gram-positive OSTB reported are of marine origin. In fact, Kato et al. (1996) have stated that OSTB are found in much higher numbers in marine habitats as compared with soil.

Many of these OSTB may prove to be important in industry, particularly in the biotransformation of water-insoluble substrates in biphasic (organic-aqueous) fermentation systems. Steroid biotransformation is a multi-million-dollar industry, and the pharmaceutical uses of steroids are numerous. The major rate-limiting factor in the biotransformation process is the extremely poor dissolution of steroids in water (10^{-2} to 10^{-3} g/ml), which increases costs. However, steroids like cholesterol are completely soluble in some organic solvents such as chloroform and butanol. Hence, a biphasic system wherein the cells are present in the aqueous phase and steroids are dissolved in the organic phase is an ideal setup (Aono et al., 1994; Moriya et al., 1995). The major problem here is the fact that most bacteria and their enzymes are inactivated or destroyed in presence of these toxic organic solvents.

It has been stated that for the adequate production of chemicals by microorganisms in a 2-phase system, it will be necessary to develop microorganisms that contain the relevant enzymes in sufficient amounts and with highly specific activities, even in the presence of the generally destructive apolar phase (Schuller et al., 1984). This can be accomplished by utilizing OSTB having the desired enzymatic activities. The cells and enzymes of solvent-

Received February 14, 2002; accepted September 20, 2002
*Corresponding author: C/o Adv. Nitin Sardessai, Velho Building, 2nd floor, Opp. Muncipal Garden, Panjim-Goa, India; e-mail nnnps@goatelecom.com

tolerant bacteria, particularly extracellular enzymes, can be expected to be active in presence of solvents (Ogino et al., 1994, 1995). The present work was undertaken to isolate cholesterol-transforming OSTB from the marine ecosystem.

Materials and Methods

Several coastal sediment samples from the Arabian Sea were collected and analyzed. One gram of the respective sediment was added to a 50-ml flask containing 8 ml of mineral salts medium. The flask was supplemented with 2 ml of chloroform (20% vol/vol) containing dissolved cholesterol (10 mg). The flasks were incubated at 28°C for 2 days on a rotary shaker, after which 0.1-ml aliquots of the organic layer were plated on mineral salts agar with cholesterol as the sole carbon source. The selected culture was designated as BC1. The culture was identified to the genus level using standard methods (Sneath et al., 1986).

The growth of the culture in mineral medium with 0.5% (wt/vol) cholesterol and in Luria broth was monitored by performing viable counts on Luria agar at periodic time intervals. Organic solvent tolerance was determined by the plate assay (Ogino et al., 1995). Cholesterol transformation was monitored by thin-layer chromatography (TLC) in various solvent systems. The plates were developed by spraying with ferric chloride and in the iodine chamber (Aono et al., 1994).

A biphasic system for cholesterol degradation was prepared as follows. Cells of BC1 grown in Luria broth overnight were harvested by centrifugation, washed, and resuspended in phosphate buffer (pH 7) to give an absorbance of 1.5 at 600 nm. The cells were supplemented with mineral salts medium devoid of nitrogen source 2% (vol/vol). This aqueous layer (25 ml) was overlaid with 25 ml (50% vol/vol) of chloroform containing 0.5 mg/ml cholesterol. The flask was incubated on a rotary shaker for 24 hours, and the chloroform layer was analyzed periodically by chromatography. The intermediates obtained were purified by preparative TLC. The proton nuclear magnetic resonance (NMR) and U/V spectra of the partially purified intermediate was determined. To determine the viability of cells in the presence of chloroform, viable cell counts were made on Luria agar at periodic time intervals.

Results and Discussion

Ten bacterial isolates capable of withstanding chloroform and producing colonies on cholesterol agar were obtained, most of which were gram-positive bacteria. One of the sediment samples was found to yield a culture producing predominantly white colonies on cholesterol agar. This culture, designated as BC1, was chosen for further studies as it grew faster (2 to 3 days) and produced larger colonies in comparison with other cultures, which took 5 to 7 days to show growth. BC1 was found to be an aerobic gram-positive, endospore-forming rod and was identified as a strain of *Bacillus.*

BC1 had a doubling time of 20 and 162 minutes in Luria broth and mineral medium with 0.5% (wt/vol) cholesterol, respectively. The culture exhibited excellent tolerance to a wide range of organic solvents besides chloroform, such as hexane, benzene, toluene, and xylene, in the plate assay. Solvent toxicity is graded based on their log *P* values, wherein *P* is the partition coefficient of the given solvent in an equimolar mixture of octanol and water. Solvents with log *P* values below 4 are considered extremely toxic because their degree of partitioning into the aqueous phase and from there into the bacterial membranes is very high, causing cell death in most of the ordinary bacteria (De Bont, 1998).

Every organism has a limiting log *P* value below which it cannot grow, called the index value. (*)In almost all of the reported *Pseudomonas* strains, this value is 2.5 (toluene), whereas in the few gram-positive solvent-tolerant bacteria such as *Bacillus* and *Arthrobacter* sp., it is 2 (benzene) (Kato et al., 1996). Chloroform and benzene have similar log *P* values, but the toxicity of chloroform is much higher due to the presence of the chlorine atom. Although *Pseudomonas* sp. strain ST-200 has been found to transform cholesterol in the presence of solvents, its enzymes are inactivated in the presence of chloroform (Doukyo and Aono, 1998). To our knowledge, this is the first report of a culture tolerant to high concentrations of chloroform. This study showed that free resting cells of BC1 were capable of transforming cholesterol in the presence of 50% (vol/vol) chloroform, indicating the organic solvent stability of the enzyme system involved.

The transformation yielded 2 detectable intermediates designated as p1 and p2. Intermediate p1 appeared to be more polar than cholesterol as it was seen below cholesterol in a chloroform-acetone (9:1) system. Generally, more polar compounds are known to bind tightly to silica gel and thus have a slower rate of migration. Also, p1 seemed to be a transient intermediate as it could be detected within 30 minutes to 4 hours, but not after prolonged incubation. If the flasks were incubated for 4 to 24 hours, the second intermediate p2 appeared, which was better resolved in

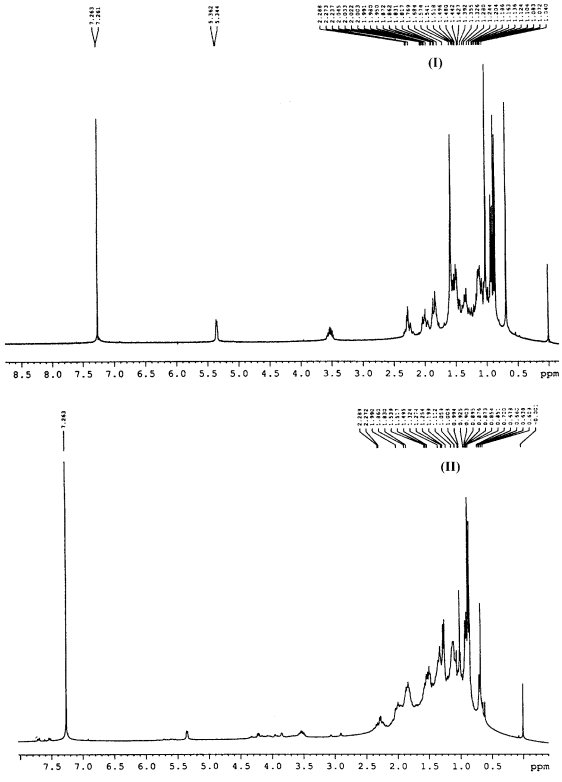

Figure 1. Proton-NMR spectra of cholesterol (I) and the partially purified product p2 (II).

petroleum ether–ethyl acetate (8:2) and hexane–diethyl ether (3:10) systems. Further, p2 was stable and persisted in the flasks even after continued incubation. p2 appeared to be a less polar compound as its Resolution factor (*Rf*) value was greater than that of cholesterol. Cholesterol had *Rf* values of 0.80, 0.62, and 0.48, respectively, in the chloroform-acetone, hexane–diethyl ether, and petroleum ether–ethyl acetate systems, whereas *Rf* values of p2 were 0.87, 0.70, and 0.58, respectively. Its UV absorption maxima (255 nm) suggested the presence of conjugated double bonds, which are absent in cholesterol (207 nm). The UV absorption spectra and TLC patterns of p2 were similar to those of cholest-4-ene-3,6-dione, a ketonic derivative of cholesterol (Aono et al., 1994). The proton-NMR spectrum of the partially purified product p2 showed splitting of the peaks in the region from 0.2 to 1.2 ppm. Also, additional signals were seen in the region between 2.5 and 5.5 ppm, besides the signals at 3.5 and 5.5 ppm exhibited by cholesterol, which indicates transformation (Figure 1).

Transformation, however, was not 100%, and residual cholesterol persisted in the flasks after 24 hours of incubation. It is noteworthy that the cell count remained significantly high even after 24 hours of incubation in the presence of 50% (vol/vol) chloroform. From 7×10^6 at the start, the cell count becomes 1×10^5 after 4 hours and 4.8×10^3 after 24 hours.

The same products, p1 and p2, were found in growing cells when inoculated in mineral salts medium with cholesterol in the absence of solvent as were found in resting cells without solvent. However, the time taken was much longer (several days), indicating that the biphiasic system with resting cells was more efficient. Bacteria like BC1 that possess solvent-stable enzymes may prove to be extremely useful in a variety of industrial processes.

References

Aono, A., Aibe, K., Ingue, A., and Horikoshi, K. (1991). Preparation of organic solvent tolerant mutants from *E. coli* K-12. *Agric Biol Chem* 55:1935–1938.

Aono, R., Doukyu, N., Kobayashi, H., Nakajima, H., and Horikoshi, K. (1994). Oxidative bioconversion of cholesterol by *Pseudomonas* sp. strain ST-200 in water-organic solvent two phase system. *Appl Environ Microbiol* 60(3):2598–2523.

De Bont, J. (1998). Solvent tolerant bacteria in biocatalysis. *TIBTECH* 16:493–499.

Doukyu, N., and Aono, R. (1998). Purification of an extracellular cholesterol oxidase with high activity in presence of organic solvents from *Pseudomonas* sp. strain ST-200. *Appl Environ Microbiol* 64(5):1929–1932.

Kato, C., Inoue, A., and Horikoshi, K. (1996). Isolating and characterising deep-sea marine micro-organisms. *TIBTECH* 14:6–12.

Moriya, K., Yanigitani, S., Usami, R., and Horikoshi, K. (1995). Isolation and some properties of an organic solvent tolerant marine bacterium degrading cholesterol. *J Mar Biotechnol* 2:131–133.

Ogino, H., Miyamoto, K., and Ishikawa, H. (1994). Organic solvent tolerant bacterium which secretes organic solvent stable lipolytic enzyme. *Appl Environ Microbiol* 60(10):3884–3886.

Ogino, H., Yasui, K., Shiotani, T., Ishiwara, T., and Ishikawa, H. (1995). Organic solvent tolerant bacteria which secretes an organic solvent stable proteolytic enzyme. *Appl Environ Microbiol* 61(12):4258–4262.

Schuller, C., Van Der Meer, A., Van Lelyveld, V., and Joosten, G. (1984). Long term stability of immobilised *Pseudomonas oleovorans* cells in the production of fine chemicals. In: *Innovations in Biotechnology, Progress in Industrial Microbiology*, Hounich, E., and Van Der Meer, R., eds. New York, N.Y.: Elsevier Science Publishers, 20:85–92.

Sneath, P., Mair, N., Sharp, M., and Holt, I. eds. (1986). *Bergey's Manual of Systematic Bacteriology, Vol. 2.* Baltimore, Md.: Williams and Wilkins.