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Cholesterol biotransformation in monophasic systems by solvent tolerant *Bacillus subtilis* AF 333249

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New developments in biotechnology are focused on new products or the introduction of new processes as a substitute to the conventional way, or improvements in performance of existing biotechnological applications. The examples of application of non-conventional media are organic solvents, supercritical solvents or aqueous two phases systems. Steroid biotransformation is a multi million-dollar industry and the pharmaceutical uses of steroids are numerous. Steroids like cholesterol are completely soluble in some organic solvents like benzene, toluene and butanol. Biphasic systems wherein the cells are present in the aqueous phase and steroids dissolved in the organic phase is considered an ideal set-up. The major problem here is the fact that most bacteria and their enzymes are inactivated or destroyed in the presence of these toxic organic solvents, also it is necessary that the microbes contain relevant enzymes in sufficient amounts and in high specific activities, even in the presence of the generally destructive apolar phase. This can be very well accomplished by using organic solvent tolerant bacteria having desired enzyme activities. One such application is being discussed.

Keywords: cholesterol, organic solvents, tolerance, solubility, bio-transformation, monophasic systems
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Introduction

Steroids, a diversified class of oxygenated tetracycline isoprenoid derivatives bearing ring systems, are essential for membrane stability, growth and proliferation in living systems. Precursors of steroids are of great importance. Degradation and utilization of cholesterol, compesterol and β-sitosterol by the species of *Nocardia*, *Pseudomonas*, *Mycobacteria*, *Rhodococcus*, *Arthrobacter* and *Streptomyces* has been studied[1-5].

Steroid transformation is of great importance in the pharmaceutical industry. Cholesterol analogues are therapeutically more active. Cholesterol oxidase, usually present in body fluids, is being commercially produced. Since pharmaceutical uses of steroids are numerous, sterol conversions by microbial systems is more effective in steroid and drug industries. The major limiting factor in this process is the extremely poor solubility of steroids in aqueous media, which lowers the transformation rate and increases costs. Methods of enhancing steroid solubility in bioconversion media include substrate derivatization or micronization, sonication or the use of detergents, water miscible co-solvents, cyclodextrins, polymers and liposomal or aqueous biphasic media[6].

Cholesterol has a maximum solubility in water of 1.8 mg/L (4.7 m*M*) and undergoes a thermodynamically reversible monomer micelle equilibrium with a critical micelle concentration of 25 to 40 m*M* at 25°C. The utilization of water immiscible organic solvents is an efficient way of enhancing substrate solubility. However, organic solvents have not been frequently used in the fermentation industry because of their toxicity for many microorganisms. This problem can be overcome by using organic solvent tolerant bacteria, which can carry out the desired bioconversions in an organic solvent saturated system.

For bioconversion of organic compounds with low solubility in water, large volumes of appropriate medium are required for solubilization of compounds. This consumption of medium or water and the inevitable treatment of the wastewater constitute one of the major cost factors in bioconversion fermentations. If the water insoluble compounds were suspended in a small volume of the medium, however, a long reaction period would be required to complete the conversion. Although such compounds can be dissolved in media by emulsifying with

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surfactants, it is difficult to separate conversion products from the media. These difficulties could be solved by the appropriate use of organic solvent tolerant microbes. Several steroid bioconversions have been carried out in the presence of organic solvents in biphasic systems with such solvent tolerant organisms[1-5,7-10].

Most of the reported and well-studied organic solvent tolerant bacteria are Gram-negative bacteria, particularly the strains of *Pseudomonas.* In comparison, the data available on Gram-positive organic solvent tolerant bacteria is meagre[5]. Recently, an organic solvent tolerant cholesterol transforming *Bacillus* sp. *BC1*, isolated from coastal sediment has been reported[11]. Buckland *et al* demonstrated that cholesterol could be transformed to 4-cholestene-3-one very efficiently by *Nocardia* sp. *NC1B 10554* in the presence of high concentrations of carbon tetra chloride. The enzymes involved-cholesterol oxidase and catalase continued to function under these conditions[3].

In the present paper our findings on bio-transformation of cholesterol in monophasic system, using organic solvent tolerant, *Bacillus subtilis* AF 333249 culture isolated from the soil in industrial area has been reported.

Materials and Methods

Isolation and Growth of Bacteria

For the isolation of bacteria, enrichment technique was used[10]. Liquid medium containing poly peptone (0.5% w/v), yeast extract (0.25% w/v) and glucose (0.1% w/v) was used. The *p*H was adjusted to 7.5 with NaOH. The tubes with the medium were inoculated and overlaid with the solvent-toluene. Screw capped tubes were used to avoid evaporation. The source of inoculum was the soil from industrial area. The tolerance and growth of the organisms was tested to different concentrations of the solvent. The solvent concentration was raised stepwise from 0.1% v/v to 100% v/v. The incubation temperature was 30°C and incubation time 48-60 h. In the 100% solvent concentration tube, there was only solvent in the tube, without any other nutrients. The organisms tolerant to 100% solvent (toluene) concentration were selected[12]. The organism did not show any appreciable growth at 100% concentration, but the cells were found to be viable when inoculated and incubated on normal nutrient agar medium. The viable count almost remained the same even after a month. The growth of the cells in aqueous phase was determined by observing the OD at 600 nm, using a spectrascan (Chemito make). In case where 100% solvent was present, the solvent was allowed to evaporate and the cells were suspended in aqueous medium. All the chemicals used were of Analar grade and the solvents of HPLC grade.

Identification of Strain

The culture so isolated was Gram positive, aerobic and endospore forming. The culture was identified up to the genus level by Classical method[13] and was identified by the 16S RNA phylogenesis using NCBI blasts, at the National Centre for Cell Sciences, Pune, India. The isolated culture was identified as the one showing nearest homology to *B. subtilis AE333249* [10].

Study of Cholesterol Biotransformation

In 25 mL flasks, 1 mg cholesterol was dissolved in 10 mL solvent–toluene. It was inoculated with the organism and incubated at 30°C for 5 d on a rotary shaker at 100 rpm. Then the incubated flask contents were centrifuged to remove the cells at 10,000 rpm for 5 min. The viability and the Gram nature of the organism were confirmed. The supernatant was analysed to see if the initial cholesterol concentration has decreased, using HPLC (Merck Hitachi DADL 7455). The Gradient HPLC was carried out using a photodiode array detector. The composition of the mobile phase was *n*-hexane and isopropanol in the ratio of 1:0.02; the wavelength and scanning range being 215 and 200 to 250 nm, respectively. Flow rate of mobile phase was 0.8mL/min and the temperature 30°C. Silica-based column (make–SGE, Australia) with a length of 25 cm, and with stationary phase-C18 was used.

Results and Discussion

The bacteria in the solvent-toluene with dissolved cholesterol, were incubated for 5 d. These samples were centrifuged and the supernatant was subjected to HPLC. The chromatograms with standard (cholesterol+solvent; Fig. 1a) and the test (cholesterol + solvent +bacterium, after incubation; Fig.1b) were compared. A decreased area in the cholesterol peak in the test sample indicated cholesterol transformation and its loss in the test sample chromatogram indicated the disappearance. The results show the efficiency of the isolated strains in bringing about cholesterol transformation.

The results indicate that *Bacillus subtilis* AF333249 degrades cholesterol very efficiently when

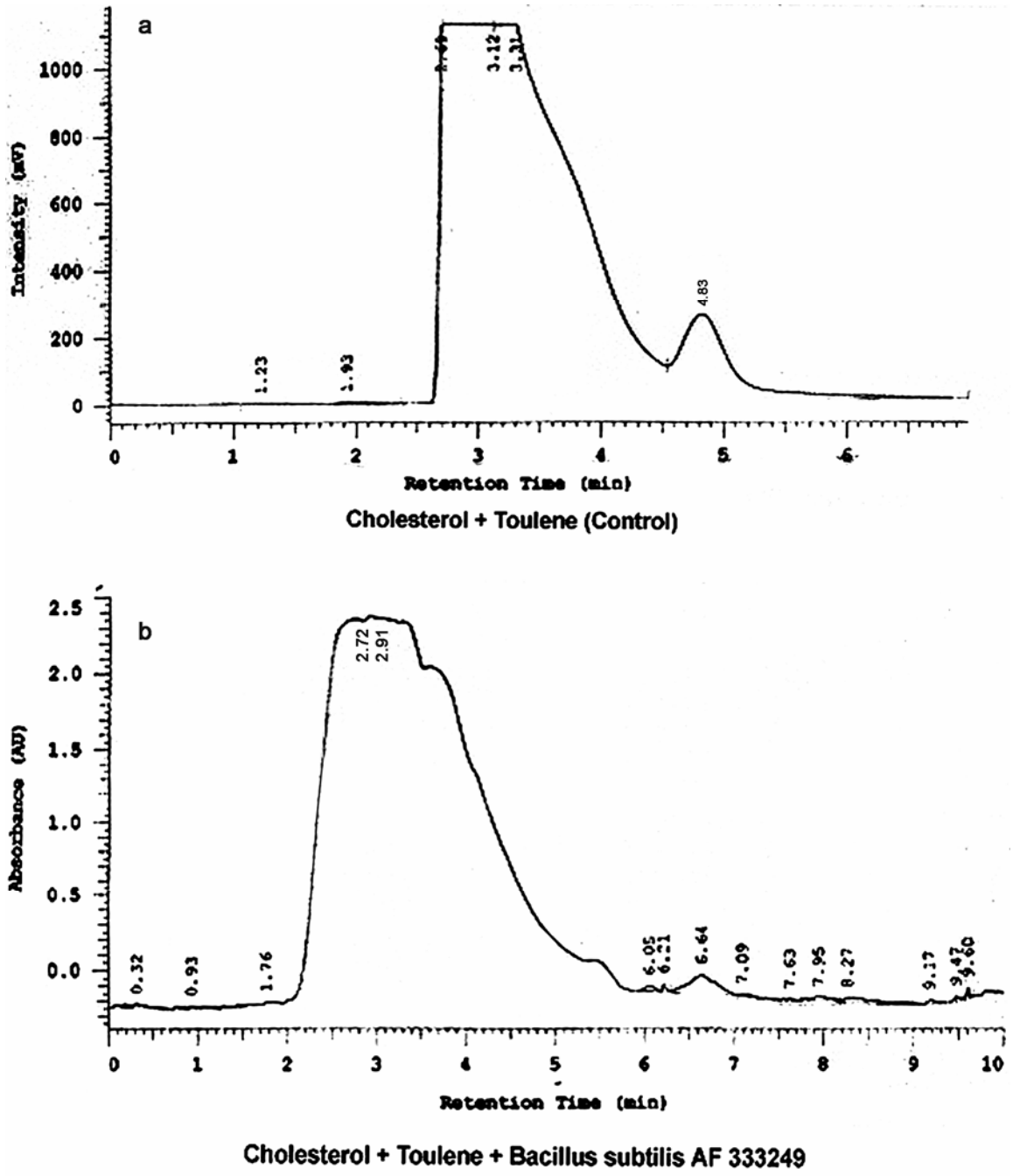

Fig.1— HPLC chromatograms for cholesterol dissolved in the organic solvent toluene without and with the bacterial inoculation a: Bioconversion of cholesterol dissolved in toluene by the organic solvent tolerant *Bacillus subtilis* AF 333249.The organism was grown in the monophasic system for 5 d and the supernatant was analysed by HPLC. Presence of cholesterol (RT-4.83) b: does not show a peak at the same RT but some new peaks indicating biotransformation.

dissolved in toluene. The cholesterol peak (Retention time-4.83) in control i,e; the chromatogram of cholesterol dissolved in toluene was totally lost in the chromatgram with cholesterol dissolved in toluene which was inoculated with the test culture, indicating total cholesterol transformation. The signals seen from retention times from RT 6.05 to 6.64 indicate the transformation. The signal at RT 6.64 appeared to be persistent for a long time indicating its accumulation. The UV absorption spectra and TLC pattern were found to be similar to that of Cholest-4-ene-3,6-dione, a ketonic derivatve of cholesterol[14].

It is well documented that *B. subtilis* exclusively produces extra cellular enzymes. This particular property of the organism was exploited in the present study. The isolated *B. subtilis* AF 333249 was grown in the presence of organic solvent, toluene, must have the solvent tolerant enzyme. The cells and particularly the extra cellular enzymes of solvent tolerant bacteria can be expected to be active in the presence of solvents[15]. *B. subtilis* AF 333249 which possesses solvent stable enzyme*s* can prove to be extremely useful in steroid transformations and various other industrial processes. Though the transformation may not be 100% in some cases, the cells tolerate absolute solvent concentrations and are still efficient in bringing about the biotransformation of cholesterol in the monophasic systems[10]. Most of the reports on cholesterol biotransformations are in biphasic systems and with Gram-negative organisms and Gram-positive actinomycetes. Here we report the cholesterol biotransformation in monophasic system and the bacteria used are Gram-positive organic solvent tolerant bacilli, which to the best of our knowledge has not been reported earlier.

Replacing all of the bulk water (which accounts for 98%) by a water immiscible organic solvent leads to a suspension of the solid enzyme or the cells in a monophasic organic solution. Although the biocatalyst seems to be dry at macroscopic level, it has the necessary residual bound water to remain catalytically active. The reported bacterium also tolerates absolute concentrations of other solvents like benzene.

The growing interest in this biotechnological area has sprung up over the past few years and resulted in various approaches to enzyme stabilization against organic solvents[16]. The use of enzymes in organic solvents significantly extends conventional aqueous based catalysis. Given the membranous parts of a cell are highly non-aqueous in character, the study of enzymes in poorly hydrated media may provide clues as to the function of membrane bound enzymes and their responses[2]. In order to fully exploit the biotechnological opportunities afforded by non-aqueous enzymology, the issue of often drastically diminished enzymatic activity in organic solvents compared with that in water must be addressed and resolved. Recent studies have made great strides towards elucidating causes of this phenomenon of activity loss[15]. None of these causes is unsurmountable.

Practically, many organic molecules of interest for transformation have limited solubility in aqueous media, or in the highly lipophilic solvents most often described in the literature for use with biocatalysts. Moreover, thermodynamic control of normally hydrolysis-favouring equilibria, ease of product recovery and minimization of certain side reactions, decreased chances of contamination, are also important motivations for conducting biocatalylic reactions in organic media[16]. The use of organic media in biocatalysis stems from the fact that in many cases biocatalytic processes can hardly be conducted (if at all) in aqueous solutions because of extremely low solubilities of substrates and/or unfavourable shift of the reaction equilibrium in water. The solvent tolerant microbes, whether used as whole cells or as their enzymes, make an environmentally friendly alternative to conventional chemical catalysts.

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