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ORIGINAL PAPER

Nutrient broth/PEG200/TritonX114/Tween80/Chloroform microemulsion as a reservoir of solubilized sitosterol for biotransformation to androstenedione

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Abstract Microemulsions (ME) can act as a reservoir of solubilized hydrophobic substrates. The biotransformation of hydrophobic sitosterol to androstenedione (AD) with MEs prepared from nutrient broth and PEG 200 (1:1) as aqueous phase, 40 g/l sitosterol dissolved in chloroform as organic phase, Triton X114 and Tween 80 (1:1) as surfactant phase, was investigated. The phase behavior of this system was studied for ten different ratios(w/w), 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10 of the organic phase and surfactant at 30 °C. A pseudoternary phase diagram was constructed to demarcate the region giving stable MEs. The maximum solubility of sitosterol in ME medium was observed to be 8 g/l, which is 3 orders of magnitude higher than the reported sitosterol solubility of 2–4 mg/l in aqueous medium. The ME medium was used for biotransformation studies and a comparative result has been reported. Transmission electron microscopy of cells grown in ME having oil, surfactant and aqueous phase in the ratio of 6:14:80 showed a weakened cell wall structure that permitted production of 465.86 mg/l AD.

Keywords Androstenedione · Biotransformation · Microemulsion · Phase diagram · Sitosterol

Introduction

Androstenedione (AD) is a pharmaceutically important steroid intermediate which is industrially prepared by selective side chain degradation of sterols. Microbial side chain degradation of sterols is a diffusion-limited process due to the poor solubility of substrates and products in the aqueous medium. Solubility of steroids in water ranges from 0.01 to 0.1% [8]. Low solubility of substrates leads to low transport rates to and from the cells, resulting in lower biotransformation yields. Several attempts have been undertaken to increase the soluble substrate concentration some of which includes the use of water miscible organic systems, organic aqueous biphasic systems and cloud point (CP) systems [1, 8, 9]. Among these systems, a substantial increase in interfacial surface area was achieved in cloud point system because the solute becomes extensively dispersed in the coacervate phase. Microemulsion (ME) systems can also increase interfacial surface area by the formation of micelles and may be effectively used in microbial biotransformation.

A ME is a homogenous, transparent and stable dispersion comprising micro-domains of oil or water. These are mainly composed of an aqueous phase, an oil phase and a mixture of surfactant/cosurfactant. These can be oil in water (O/W) or water in oil (W/O) type depending on the nature of surfactant, composition and temperature. Use of such systems as biotransformation medium can overcome the problems of substrate solubility as well as the substrate and product inhibition associated with microbial transformation of steroids. In cloud point systems, the solid substrate is carried in dispersible surfactant vesicles to the cell surface of the microorganism where biotransformation occurs [10]. Whereas, in ME systems the hydrophobic substrate is dissolved in the organic phase and carried as micelles to the microorganism. Since the ME system is a micelle system, a smaller quantity of surfactant is required for dissolving a given amount of substrate compared to the CP system. Both CP and ME systems are currently being

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studied for the development of high conversion biotransformation processes. The temperature and composition dependence of these systems are being examined for the development of rapid and efficient downstream processing methods.

There are very few reports on use of ME as a medium for biotransformation. In 1991, Smolders et al. [7] had reported the use of ME for $\Delta^{1,2}$-dehydrogenation of 16-methyl-Reichstein's compound S-21-acetate (16MRSA) using *Arthrobacter simplex*. More recently, Prichanont et al. [5] reported the studies on solubilization of *Mycobacterium* sp. in ME system and subsequently used this system for chiral epoxide production [6]. In this paper, we report for the first time, a systematic methodology for preparation and application of Nutrient broth/PEG200/TritonX114/Tween 80/Chloroform based ME for biotransformation of *β*-sitosterol to AD.

In the present work, application of ME as a reservoir of solubilized *β*-sitosterol and biotransformation medium has been evaluated. Preparation of ME comprising nutrient broth (NB), polyethylene glycol (PEG) 200, Triton X114, Tween 80 and chloroform as principal components, was carried out. The stability of prepared ME system was examined at different temperatures and different lengths of time. Biotransformation of *β*-sitosterol to AD was used as a model system.

Materials and methodology

Microorganisms and chemicals

Mycobacterium sp. DSM 2966 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany and maintained on nutrient agar slants. A mutant strain obtained from *Mycobacterium* sp. DSM 2966 was used in the present study. For development of a mutant strain, the parent strain was initially acclimatized to 20 g/l sitosterol concentration by growing them on increasing concentration (2–20 g/l) of sitosterol in a step wise manner. The strain acclimatized to 20 g/l sitosterol concentration, was subjected to NTG mutagenesis. The strain tolerant to 1 g/l AD concentration and exhibiting highest sitosterol biotransformation ability was used in experiments.

β-sitosterol and Triton X114 was purchased from Acros organics (USA). Reference grade 4-androstene-3,17-dione (AD) was purchased from Sigma. Nutrient broth (NB) was purchased from Himedia (India). HPLC grade Chloroform, Polyethyleneglycol 200 (PEG) and Tween 80 was purchased from Merck (India). Double distilled deionized water was used for the preparation of ME.

Preparation and characterization of microemulsions

For ME preparation, NB and PEG (1:1 w/w) was used as aqueous phase, while chloroform carrying 40 g/l sitosterol was used as oil phase. Triton X114 and Tween 80 (1:1 w/w) was used as surfactant phase. Calculated quantities of different components were measured (w/w) and mixed individually in a beaker with constant stirring on a magnetic stirrer. All compositions of oil: surfactant ratio ranging from 10:0 to 0:10 with corresponding amount of aqueous phase were tried. The optimized stirring time was 45 min at a constant temperature of 30 °C during ME preparation. The different compositions which gave stable ME are presented in Table 1. A pseudoternary phase diagram was constructed and the region giving stable ME system has been shown in Fig. 1.

These ME systems were characterized for sitosterol solubilizing capacity and stability at 4, 25, 30 and 35 °C. Effective diameter of ME particles was determined, using a particle size analyzer (Brookhaven Instruments Corporation, USA). Sitosterol solubility in prepared ME system was analyzed by HPTLC (CAMAG, Switzerland).

Application of microemulsion as biotransformation medium

Biotransformation studies were performed in five different ME preparations (Table 2), along with a control. The mutant *Mycobacterium* sp. DSM 2966 cells required for biotransformation was grown in NB medium containing 0.2% (v/v) Tween 80; the culture was prepared in 50 ml of this medium taken in 250 ml flasks and incubated for 48 h at 30 °C with agitation at 200 rpm. Ten milliliters of this culture was centrifuged at 10,000 rpm for 10 min. Cell precipitate was used for inoculating 20 ml of ME preparation to carry out the biotransformation experiments. Control experiments were carried out in similar experimental conditions with 20 ml NB having 0.2% (v/v) Tween 80 and 2 g/l *β*-sitosterol. Biotransformation experiments were carried out for 48 h at 30 °C with constant shaking at 200 rpm in an orbital shaking incubator (Orbitek, India).

Extraction and analysis

Two milliliter of sample was withdrawn from culture broth and was extracted by 8 ml of methanol for 2 h. The extracts were then filtered through a 0.45 μm nylon syringe filter and transferred to a sampling vial.

The extracts were spotted in 5 μl aliquots along with internal standards, onto Kieselgel 60 F_{254} fluorescent thin layer chromatography (TLC) plates (Merck, Germany) using a Linomat V sample applicator (Camag, Switzerland). Linear ascending development, with 1:1 v/v composition of

Table 1 Composition of various components giving stable microemulsions

Experiment	Oil (g)	Surfactant (g)	Water (g)	Oil (%)	Surfactant (%)	Water (%)
1.	2.00 *(1.99)*	3.00 *(3.01)*	5.00 *(5.01)*	20	30	50
2.	1.60 *(1.60)*	2.40 *(2.41)*	6.00 *(6.00)*	16	24	60
3.	1.20 *(1.23)*	1.80 *(1.81)*	7.00 *(7.00)*	12	18	70
4.	0.80 *(0.81)*	1.20 *(1.21)*	8.00 *(8.01)*	8	12	80
5.	1.50 *(1.53)*	3.50 *(3.52)*	5.00 *(5.02)*	15	35	50
6.	1.20 *(1.20)*	2.80 *(2.81)*	6.00 *(6.01)*	12	28	60
7.	0.90 *(0.93)*	2.10 *(2.15)*	7.00 *(7.01)*	9	21	70
8.	0.60 *(0.62)*	1.40 *(1.41)*	8.00 *(8.01)*	6	14	80
9.	0.30 *(0.31)*	0.70 *(0.71)*	9.00 *(9.00)*	3	7	90
10.	0.80 *(0.81)*	3.20 *(3.21)*	6.00 *(6.01)*	8	32	60
11.	0.60 *(0.61)*	2.40 *(2.40)*	7.00 *(7.03)*	6	24	70
12.	0.40 *(0.41)*	1.60 *(1.61)*	8.00 *(8.00)*	4	16	80
13.	0.20 *(0.20)*	0.80 *(0.82)*	9.00 *(9.01)*	2	8	90
14.	0.40 *(0.44)*	3.60 *(3.60)*	6.00 *(6.02)*	4	36	60
15.	0.30 *(0.31)*	2.70 *(2.71)*	7.00 *(7.00)*	3	27	70
16.	0.20 *(0.20)*	1.80 *(1,83)*	8.00 *(8.00)*	2	18	80
17.	0.10 *(0.11)*	0.90 *(0.90)*	9.00 *(9.00)*	1	9	90

The values in normal font denote the points of intersection on the pseudo ternary diagram; the values in italics are actual experimental data corresponding to these points

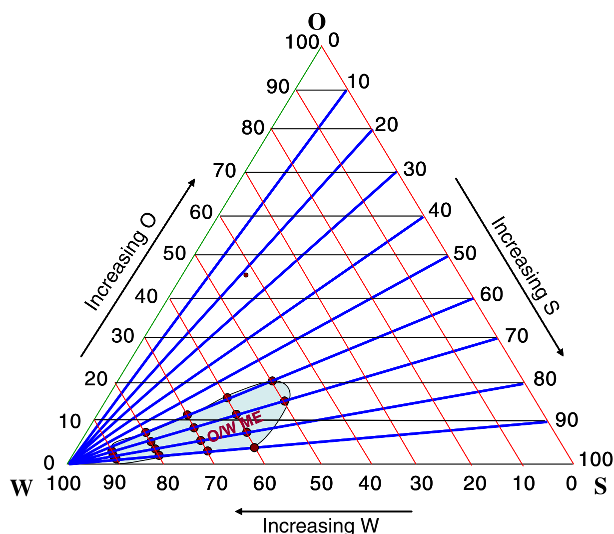

Fig. 1 Pseudoternary Phase Diagram of the system of W (Nutrient broth + PEG)/O (Chloroform)/S (Triton X114 + Tween 80). The *shaded area* indicates the compositions for which stable microemulsions were obtained

Table 2 Particle size and concentration of sitosterol in the ME formulations that were used for biotransformation studies

Experiment	Effective particle diameter (μm)		Sitosterol (mg/l)
	Initial	After 30 days	
5.	25.66	33.95	5,571 ± 272
6.	22.84	33.63	4,614 ± 123
7.	14.25	16.35	3,317 ± 198
8.	10.09	31.48	2,239 ± 176
9.	22.00	33.03	1,058 ± 097

Experiments numbers correspond to those in Table 1

chloroform and diethyl ether as mobile phase, was performed in a 20 cm × 10 cm twin trough glass chamber (Camag, Switzerland). The developing chamber was previously saturated with mobile phase for 30 min at room temperature (25 ± 2 °C) and the plates were developed upto the distance of 80 mm. After development, the TLC plates were dried using a hair dryer. AD was observed as black spots on a yellow green fluorescent background. Densitometric scanning of these TLC plates was performed at 251 nm with a Camag TLC Scanner III in absorbance mode operated by WinCats software (Version 1.2.0). The concentration of AD in the samples was determined from the internal AD standard calibration curve. Sitosterol was also analyzed by HPTLC using Kieselgel 60 F_{254} fluorescent thin layer chromatography (TLC) plates (Merck, Germany). Toluene: Ethyl acetate (8:2) was used as mobile phase. After running the sample loaded plates in mobile phase, these were dipped in anisaldehyde-sulfuric acid reagent (sulfuric acid: methanol: acetic acid:: 1:17:2) for 1 second for derivatization of sitosterol. Plates were then heated at 100 °C for 5 min. Densitometric scanning of these derivatized TLC plates was performed at 366 nm with a Camag TLC Scanner III in absorbance mode operated by WinCats software (Version 1.2.0). The concentration of sitosterol in the samples was determined from the internal sitosterol standard calibration curve.

Transmission electron microscopy of mycobacterial cells

Effect of ME composition on mycobacterial cells was evaluated by Transmission Electron Microscopy (TEM). Cells

obtained from biotransformation broth of control experiment and experiment exhibiting highest AD concentration (Experiment no. 8) was used for this study. Cells were harvested by centrifuging them at 10,000 rpm for 10 min. These cells were then washed with 0.1 M phosphate buffer (pH 7.4) and fixed in 2.5% v/v glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Cells were further processed as per the procedure of Fraud et al. [2]. Transmission Electron Micrographs were recorded in TEM Philips, CM-10 model.

Results and discussion

Preparation and characterization of microemulsions

During this study of preparation and characterization of ME, different compositions of oil, water and surfactant phase was investigated to locate the stable region of the phase diagram. Stable ME was obtained at 17 different compositions shown in the Table 1. Chloroform concentration in such ME system ranged from 1 to 20%. This meant that the sitosterol concentration in such ME system was in the range of 400 mg/l to 8 g/l. The maximum solubility of sitosterol in ME medium was observed to be 8 g/l (20% chloroform containing 40 g/l sitosterol), which is 3 orders of magnitude higher than the reported sterol solubility (0.01 to 0.1%) in aqueous medium.

Five different ME preparations were evaluated for their stability at different temperatures. Appearance of turbidity or precipitation was treated as an indication of instability. ME compositions found to be stable for more than 30 days at 4, 25, 30 and 35 °C, were used for subsequent biotransformation experiments. The effective diameter of particles in freshly prepared ME system was between 10–25 μm. Over a period of 30 days, particles coalesce and the effective diameter increases but remains below 35 μm (Table 2). These results further confirmed that the ME system used for this study were highly stable.

Sitosterol biotransformation studies in microemulsions

Sitosterol biotransformation results show an enhanced production of AD as compared to the control culture (Fig. 2). While control experiments resulted in an average of 69 mg/l AD production, the AD produced in ME medium was 88.65, 113.06, 126.62, 465.86 and 308.22 mg/l for experiments 5–9, respectively. Higher interfacial area for mass transfer achieved in ME system improves AD production. Interestingly it was observed that although experiment 8 has smallest particle size (10.09 μm) and gives the highest AD concentration, its percentage molar conversion based on the initial moles of solubilized sitosterol is 30.1%. In comparison, experiment 9 has a percentage conversion of 42.2% (particle size 22.0 μm). Similarly, experiment 7 has a percentage conversion of only 5.5% although it has the second smallest particle size (14.25 μm). The oil to surfactant ratios are nearly the same for all the experiments (ca 0.42–0.44) but the surfactant to water ratios are very different (ca 0.07–0.7). Therefore, it appears that, in addition to the particle size, some other factors also influence the sitosterol biotransformation in ME system. Further analysis of biotransformation results revealed a decrease in percent molar conversion with the increase of solvent concentration. Since the particle size of 22 μm obtained for the lowest solvent concentration of 3% is comparable to 25.7 μm obtained for the highest solvent concentration of 15%, the increase in solvent concentration will result in the increase in the number of particles per unit volume of ME. Consequently, the cells will have a higher frequency of interaction with ME particles resulting in a longer duration of contact with the solvent phase. Since the cells of the *Mycobacterium* sp. were adapted to 20 g/l sitosterol concentration and the maximum concentrations of sitosterol used in

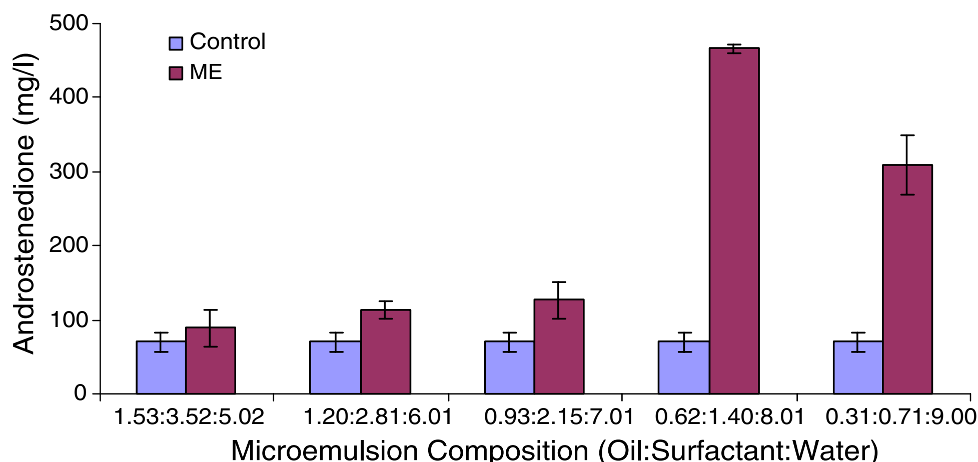

Fig. 2 Results of β-sitosterol to androstenedione biotransformation in different microemulsion compositions. The control experiment was performed in Nutrient broth medium supplemented with 0.2% tween 80 and 2 g/l sitosterol

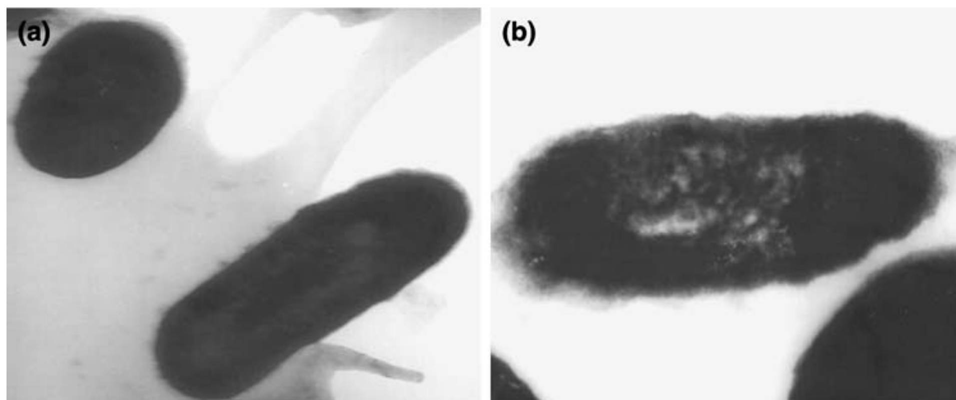

Fig. 3 TEM micrograph of *Mycobacterium* sp. DSMZ 2966. **a** Control cultures grown for 48 h in nutrient broth added with 0.2% tween 80 and 2 g/l sitosterol. **b** *Mycobacterium* sp. grown in ME (6% Chloroform, 14% Surfactant and 80% water) for 48 h

these experiments were far below this value, the toxic effect of substrate may not be the main cause for reduction in biotransformation. Therefore, the effect of the solvent on the mycobacterial cell wall appears to be the primary cause of reduction in biotransformation of sitosterol to AD. Indeed, the values obtained reveal an inverse correlation between chloroform concentration and percentage molar conversion of sitosterol to AD. The log *P* value of chloroform is 2 indicating that its effect on the mycobacterial cells is not predictable [4]. Since the presence of water layer is essential for microbial biocatalysis [3], an increased distortion of this essential water layer surrounding the hydrophobic mycobacterial cell wall as well as partial dissolution of the cell wall, may affect the stability of the cells and hence biotransformation. Therefore, other biocompatible solvents possessing favorable log P and amenable to stable ME production may be investigated to further improve the biotransformation efficiency of such systems.

Transmission electron microscopy of microemulsion grown cells

Effect of components present in ME system on *Mycobacterium* cell wall was evaluated by comparison of the TEM micrograph of cells grown in control culture with those grown in ME giving the highest AD yield. TEM pictures revealed a thin and weakened cell wall of *Mycobacterium* grown in ME, as compared to those of the intact and thick wall of control cells (Fig. 3a, b). This observation indicates that the weakening and partial deformation of mycobacterial cell wall may also contribute to enhanced sitosterol permeability across the cell wall, resulting in a higher yield of AD (Fig. 2).

Conclusion

It was concluded that MEs can be used as a reservoir of solubilized sterol substrates and also as the medium for carrying out sterol biotransformation with higher product yield. The ME medium was stable for more than 30 days at 4–35 °C. The highest percentage molar conversion based on the initial moles solubilized was 42.2% and it was obtained for a 0.03:0.07:0.90 (oil:surfactant:water) ME medium. The large surface area provided by such systems for mass transfer results in higher bioconversion yields. However, the composition of the ME has significant effect on the degree of conversion of sitosterol to AD.

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