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Suitable Nonionic Surfactants for the Erythromycin Production by *Saccharopolyspora erythraea*

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Abstract

To find a suitable surfactant for enhancing the solubility of fatty acid, some nonionic surfactants including Tween 20, 40, 60, 80, 85; poly-ethylene glycol 200, 300, 400, 600; di-ethylene glycol di-methyl ether (diglyme), Triton X-100, tetrahydrofuran (THF) and dioxan were added to the fermentation medium (with or without palmitic acid) and their effects were studied on the morphology of *Saccharopolyspora erythraea* and erythromycin production. Tween 20 and Triton X-100 lysed the hyphae, but in the media containing other surfactants, the hyphae remained in a vegetative form. PEG 300, Tween 40 and THF had neither negative nor positive effect on the erythromycin production. But, addition of Triton X-100 and dioxan to the fermentation medium significantly decreased the concentration of erythromycin, 9.9 and 1.3 times less than that of control, respectively. In the media containing PEG 200, PEG 400, PEG 600, Tween 60, Tween 80, Tween 85 and diglyme, erythromycin concentration was 1.3, 1.5, 2.0, 1.4, 1.4, 1.3 and 1.3 times higher than that of control, respectively. Production of erythromycin in the palmitic acid containing medium was 1.62 times lower than that of control. However, the effect of palmitic acid on the erythromycin production was different when surfactants were added to media. Erythromycin production in the media containing palmitic acid plus PEG 400 and palmitic acid plus PEG 600 was more than that of palmitic acid containing medium (without any additional surfactant). However, erythromycin concentration in the media containing Tween 40, 60, 80 and 85, THF and diglyme was less than that of medium containing palmitic acid (without any surfactant).

Keywords: *Erythromycin, Fatty acid, Morphology, Nonionic surfactants, Saccharopolyspora erythraea.*

Introduction

Fatty acids are aliphatic carboxylic acid with varying length hydrocarbon chain. Chain-lengths range is from 2 to 80 carbons but commonly from 12 up to 24. Normal fatty acids exhibit appreciable solubility in water compared to the corresponding hydrocarbons due to the presence of the polar carboxyl group. However, increasing hydro-carbon chain length will lead to decreasing the water solubility of fatty acids.

Fatty acids have various effects on the bacteria. Their inhibitory effects have demonstrated on the bacteria such as, *Helicobacter pylori* (Bergsson *et al.* 2002) meat spoilage organisms, including *Lactobacillus curvatus*, *Lactobacillus sake, Carnobacterium pisciola, Brochothix thermosphacta, Pseudomonas fluorescens* and *Serratia liquifaciens* (Outtara *et al.* 1997), spores of *Clostridium sporogenes*, *C. botulinum* and *Bacillus cereus* (Ababouch *et al.* 1992). On the other hand, palmitic acid was suitable for tylosin production by *Streptomyces frediae* (Lee *et al.* 1997) and actinorhodin production by *Streptomyces lividans* (Peacock *et al.* 2003).

Because of poor distribution of fatty acids in water, it is difficult to compare the effect of various fatty acids on the bacteria. For enhancing the distribution of fatty acids in aqueous media, ethanol (Outtara *et al.* 1997, Bergsson *et al.* 2002) and chloroform (Boyaval *et al.* 1995) have been used. But even at very low concentration, palmitic and stearic acids are not soluble in ethanol. They are soluble in propanol and chloroform but precipitate in the culture medium (Boyaval *et al.* 1995). Also, these solvents are toxic for bacteria.

Surfactants have been used to improve solubility of fatty acids (Peacock *et al.* 2003). Nonionic surfactants are more hydrophobic than

ionic ones, but they are efficient emulsifiers in biological systems (Rege *et al.* 2002) and have less toxicity to biological membranes (Davis *et al.* 1970), even Tween 80 increased the rumen microbial culture (Goto *et al.* 2003). However, to our knowledge, there is no study on the effect of surfactants on the actinomycetes or other bacteria.

The aim of this study is to examine the effect of nonionic surfactants on the growth of *Saccharopolyspora erythraea* and erythromycin production and the solubility of palmitic acid, as a model non-soluble solid fatty acid in water and finding suitable non-toxic surfactants to make similar condition for the effect of fatty acids on the bacteria.

Materials and methods

Surfactants: Tween 20, 40, 60, 80, 85; polyethylene glycol 200, 300, 400, 600; di-ethylene glycol di-methyl ether (diglyme), Triton X-100, tetrahydrofuran (THF) and dioxan were used. Minimum concentrations that caused to homogenous distribution of 7 g/l palmitic acid were used (g/l): Tweens 18, PEGs 105, diglyme 22.8, Triton X-100 6.6, THF 12.7, dioxan 15.38.

Bacterial strain and media: Saccharopolyspora erythraea DSMZ40517 was used throughout the study. The sporulation medium used was oat-meal agar (Shirling & Gottelieb 1966). The composition of the seeding medium used was (g/l): soybean meal 30, glucose 10, glycerol 10, $(NH_4)_2HPO_4$ 1, $(NH_4)_2SO_4$ 3.5, $CaCO_3$ 5 (Hamedi *et al.* 2004). The composition of the basal defined fermentation medium used was (g/l): dextrin 35, $NaNO_3$ 2.38, KH_2PO_4 3, K_2HPO_4 7, $MnSO_4$ 1. The pH was adjusted to 7±0.1 by using 5 M KOH.

Cultural method: One ml aliquots of a spore suspension (ca. 10^7-10^8 spores/ml) of *S. erythraea* DSMZ40517 was inoculated in 1000 ml Erlenmeyer flask containing 100 ml seed medium and incubated at 30°C for 48 h at 220 rpm. Then, 5% (v/v) of the seed culture was inoculated into each 100 ml Erlenmeyer flask containing 20 ml fermentation medium and incubated at 30°C for 5 days at 220 rpm. The fermentations were done are as follows: I) the basal fermentation medium plus appropriate concentration of surfactants, II) the basal fermentation medium plus surfactant and 7g/l palmitic acid, III) the basal fermentation medium plus 7 g/l palmitic acid, and IV) the basal fermentation medium.

Erythromycin assay: Total erythromycin was extracted from fermentation broth with chloro-form and was mixed with bromophenol blue. The absorbance of organic phase was measured at 415 nm. Concentration of erythromycin A was determined by HPLC at 205 nm; column, C18; mobile phase, acetonitrile-methanol-0.2 M am-monium acetate-water (45:10:10:35, by volume) at 1 ml/min; column temperature, 40°C, sample injection volume, 50 µl. In order to confirm the production of biologically active erythromycin, fermentation broth was bioassayed against *Micrococcus luteus* ATCC9341 (Hamedi *et al.* 2004).

Reproducibility of results and data analysis: Each type of fermentation was carried out in three independent batches in triplicate (nine replications). Standard error of the mean was calculated and was showed as error bars on the Fig.s. One way analysis of variance and Tukey HSD test were done with SPSS ver. 10 software (Microsoft, USA).

Results

The effect of surfactants on the growth and morphology of *Saccharopolyspora erythraea*: The morphology of *S. erythraea* DSMZ40517 was affected by the surfactants used. As shown in Fig. 1, in the media containing Tween 20 (Fig. 1a) and Triton X-100 (Fig. 1m), the hyphae were lysed, but in the media containing other surfactants, the hyphae were longer and re-mained in a vegetative form. The alteration in the morphology of the strain in various surfac-tants containing media was not significant.

The effect of surfactants on the erythromycin production: Erythromycin production by *S. erythraea* DSMZ40517 in the media containing various surfactants was shown in Fig. 2.

PEG 300, Tween 40 and THF had not any negative or positive effects on the erythromycin production and concentration of erythromycin in the media containing these surfactants had not significant difference with control ($p<0.05$).

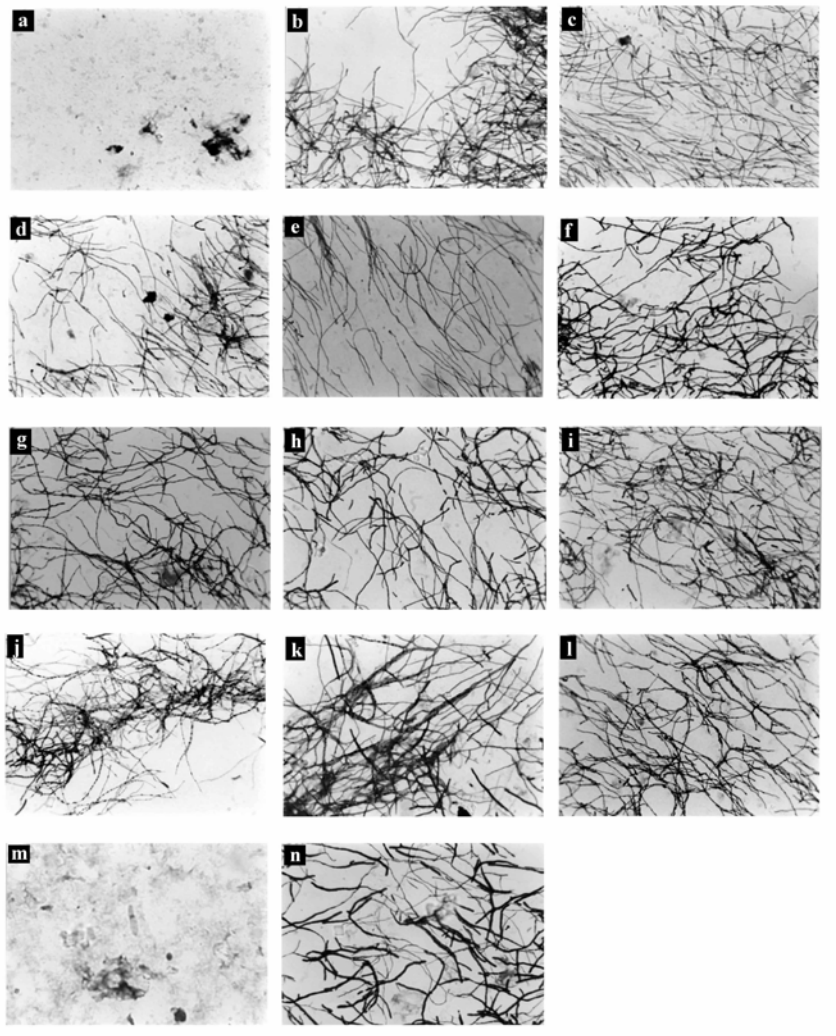

Fig. 1. Effect of surfactants on the morphology of *S. erythraea* DSMZ40517 in the media containing: a) Tween 20, b) Tween 40, c) Tween 60, d) Tween 80, e) Tween 85, f) PEG200, g) PEG300, h) PEG400, i) PEG600, j) di-ethylene glycol di-methyl ether, k) tetrahydrofuran, l) dioxan, m) Triton-X100, n) control (fermentation without any surfactant).

But, addition of Triton X-100 and dioxan to the fermentation medium significantly decreased the concentration of erythromycin, 9.9 and 1.3 times less than that of control, respectively. On the other hand, in the media containing PEG 200, PEG 400, PEG 600, Tween 60, Tween 80, Tween 85 and diglyme, erythromycin concentration was 1.3, 1.5, 2.0, 1.4, 1.4, 1.3 and 1.3 times more than that of control, respectively ($p<0.05$).

The effect of surfactants on the erythromycin production in palmitic acid containing media: Palmitic acid had negative effect on the growth of *S. erythraea* DSMZ40517 (Fig. 2-k) and production of erythromycin (Fig. 3). Production of erythromycin in the fatty acid containing medium was 1.62 times less than that of control. However, the effect of palmitic acid on the erythromycin production was differed when surfactants were added to media. Erythromycin production in the media containing palmitic acid plus PEG 400 and palmitic acid plus PEG 600 was more than palmitic acid containing medium (without any surfactant). However, erythromycin concentration in the media containing Tween 40, 60, 80 and 85, THF and diglyme was less than medium containing palmitic acid (without any surfactant).

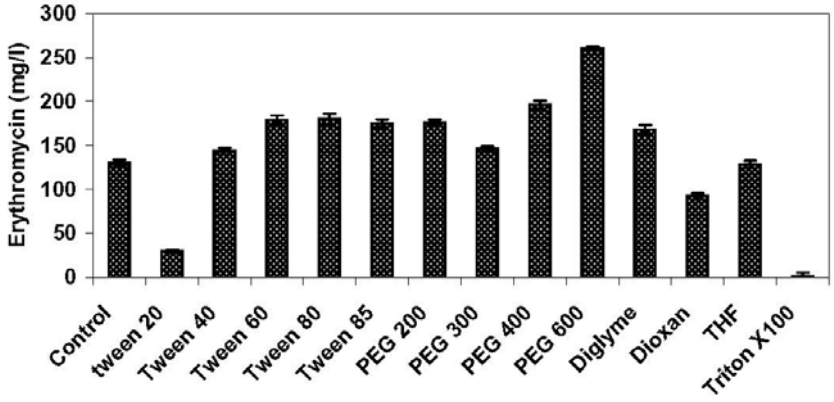

Fig. 2. Effect of surfactants on the erythromycin production by *Saccharopolyspora erythraea* DSMZ40517: a) Tween 40, b) Tween 60, c) Tween 80, d) Tween 85, e) PEG200, f) PEG300, g) PEG400, h) PEG600, i) diethylene glycol dimethyl ether, j) tetrahydrofuran, k) control (fermentation medium without any surfactant).

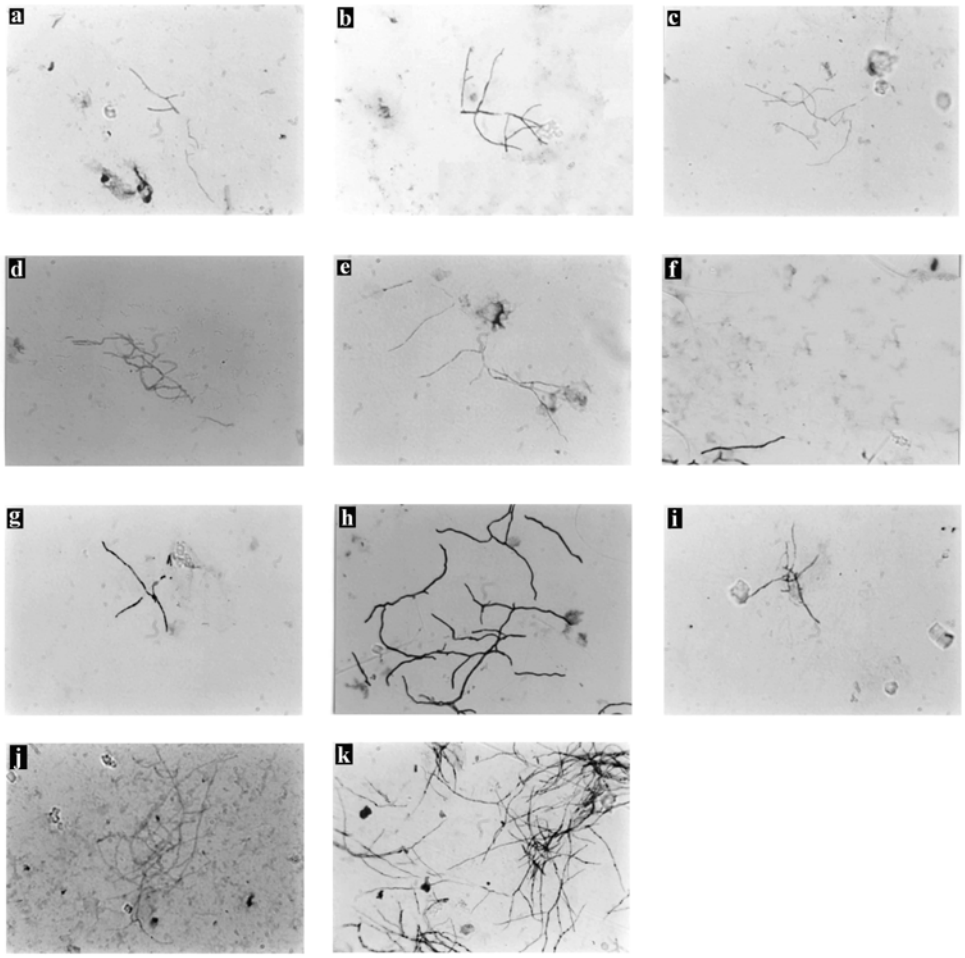

Fig. 3. Effect of surfactants on the erythromycin production by *Saccharopolyspora erythraea* DSMZ40517. A control fermentation conducted in the absence of any surfactant supplement was also included.

Discussion

Antimicrobial effect of fatty acids is not solely due to creation of a high extracellular proton concentration, but it is also directly related to the concentration of undissociated acid. Undissociated lipophilic acid molecules can pass freely through the membrane. At the high pH of the cytoplasm (~7), acid ionizes producing protons which will tend to acidify the cytoplasm and affect of the microbial metabolism by break down the pH component of the proton motive force (Adams & Moss 2000). Also, fatty acids entered to cytoplasm repress *de novo* fatty acid biosynthesis (Partanen *et al.* 2001). On the other hand, fatty acids are used as carbon and energy sources by microorganisms. It seems that difference in solubility of fatty acids in aqueous media relates to their physiological effect on the bacteria. Surfactants adsorb at the fatty acid/water interface, lowering surface free energy, stabilizing the emulsions and thus help us to make similar condition to study the effect of fatty acids on the bacteria.

Nonionic surfactants with polyoxyethylene chain as the hydrophilic group acquire their water solubility through the formation of hydrogen bonds between oxygen atoms and water molecules. These surfactants thus possess a property termed inverse temperature-dependent water solubility, i.e., they are soluble in water at low temperature but separate from it at a certain higher temperature (the cloud point) on account of the disruption of hydrogen bonds. Polyoxyethylene detergents are available in a variety of structures and under some trade names, such as Triton and Tween. Tweens have three polyethylene-glycol groups and one fatty acid ester group in their molecular structure.

In this category, the chain length of polyethylene glycol groups is same. Among this group, only Tween 20 (polyoxyethylene sorbitanmono-laurate) had negative effect on the growth of *S. erythraea* DSMZ40517 and Tween 40 (polyoxy-ethylene sorbitan-monopalmitate), Tween 60 (polyoxyethylene sorbitanmono-stearate), Tween 80 (polyoxyethylene sorbitanmonooleate) and Tween 85 (poly-oxyethylene sorbitan trioleate) not only had no negative effect on the growth of strain but also increased the erythromycin production.

Toxicity of lauric acid is more than palmitic, stearic and oleic acid to *S. erythraea* (Hamedi 2002). Addition of Tween 80 caused to increase of the growth of *Streptococcus bovis, Slenomonas ruminantium* and some other rumen bacteria (Goto *et al.* 2003).

The basic structure of tritons is similar to polyethylene glycols. The feature of Triton is that the terminal hydroxyl hydrogen atom of its polyoxyethylene ether is replaced by a bulky R group. In general, Tritons are harmful chemicals (Budavari *et al.* 1989), and in our case have negative effects on the growth of *S. erythraea* DSMZ40517. Triton X-100 changed the cytoplasmic membrane and lysed *Yarrowia lipolytica* (Galabova *et al.* 1996).

Polyethylene glycols are members of aliphatic ethers with the terminal hydroxyl groups. The effect of these surfactants on the erythromycin production was similar and all of them increased the erythromycin concentration (Fig. 2). Their effect in the presence of palmitic acid, as shown in Fig. 4, suggests that the increasing of the oxyethylene moiety results in a rapid erythromycin production. Diglyme is half of PEG 200 and has similar behavior. Tetrahydro-furan is a cyclic monoether and had no negative effect on the *S. erythraea* DSMZ40517. Cyclic monoethers such as THF is usually harmless. But dioxan is a harmful cyclic diether (Budavari *et al.* 1989), which also had negative effects on the growth of *S. erythraea* DSMZ40517.

Finally, it can be concluded that the effect of fatty acids on the bacteria is strongly related to the media ingredients, and by using appropriate chemicals, it is possible to change the negative effect of fatty acids to positive. This may also have biotechnological importance, because fatty acids are the precursors in the biosynthesis of antibiotics such as macrolides (Ikeda *et al.* 2002). Also, the data presented herein can provide guidance to suitable nonionic surfactants usage in microbiological applications, such as preparing uniform spore suspension of hydrophobic actinomycetes, such as *Streptomyces clavuligerus* and *Nocardia amarae*.

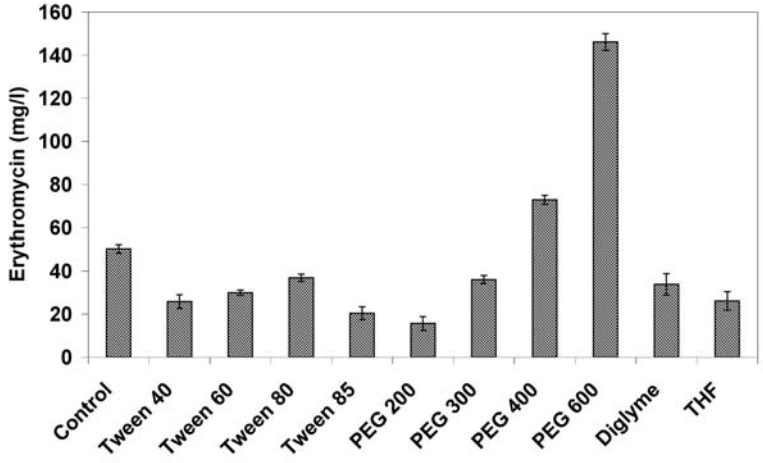

Fig. 4. Effect of surfactants on the erythromycin production by *Saccharopolyspora erythraea* DSMZ-40517 in the media containing palmitic acid (7g/l). A control fermentation conducted in the absence of any surfactant supplement was also included.

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