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Enhancing the effect of soybean oil refinery by-products on erythromycin production by *Saccharopolyspora erythraea*

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ABSTRACT

Erythromycin production is clearly enhanced by the addition of vegetable oils. However, whether this positive effect is brought about by the triglyceride or non-triglyceride fraction, is not clear. The non-triglyceride portions of the oils accumulate in the waste during the refining process leaving mainly triglycerides in the refined oil. In this research, sub-fractions of soybean oil, including lecithin, soap stock, free fatty acid, deodorizer distillate and unsaponifiable matter were supplemented by batch and fed-batch to chemically defined and complex fermentation media. The fermentation flasks were incubated at 30°C for 8 days at 220 rpm. It resulted in the production of low concentrations of oil by-products (1-5 g/l) at the beginning of fermentation, increased antibiotic production and had a greater enhancing effect than using crude oil. However, some fractions of oil such as free fatty acids considerably reduced antibiotic production. The results of this study highlight the advantages of substitution of vegetable oil with its refining by-products in the fermentation media.

Keywords: deodorizer distillate, erythromycin, lecithin, oil refining waste, *Saccharopolyspora erythraea*, soybean oil.

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Introduction

Erythromycins are the most important secondary metabolites of *Saccharopolyspora erythraea* produced at an industrial scale for more than five decades. The commercial and clinical importance of erythromycins, have led to continuous efforts to optimize their production. Genetic manipulation (1-4), bioprocess optimization (5-9), whole genome sequence of the wild strain of *S. erythraea* NRRL 23338 (10), and industrial hyper producer strong mutator *S. erythraea* strain D have been identified (11). Oils are the essential components of industrial fermentation media and have been routinely supplemented into media for the production of secondary metabolites, including erythromycin (12, 13). Usually, crude oils are used in fermentation media (14). Crude vegetable oil consists of two main fractions of triglycerides and non-triglycerides (free fatty acids, phosphatides, pigments, sterols, tocopherols, glycerol, hydrocarbons, vitamins, proteins, trace amounts of metals, glycolipids, pesticides, resinous and mucilaginous materials) (15).

Crude vegetable oil is refined to food grade oil which has a purity of over 99% triglyceride content. The refining process removes elements that contribute to undesirable effects and some valuable non-triglyceride components of the oil through degumming, neutralization and deodorization steps. Gum, soap stock and deodorizer distillate are three important by-products of the refining process (15, 16). Lecithins and free fatty acids are the main components of the gum and soap stock, respectively. Sterols, tocopherols and free fatty acids are the major components of deodorizer distillate (15, 16).

The effects of vegetable oil refinery wastes on erythromycin production are unknown. In this study, batch and fed-batch supplementation of oils, refining wastes and their main ingredients in the fermentation medium of *S. erythraea* and their impact on growth and secondary metabolite (erythromycin) production were evaluated. The aim of this experiment was to answer two fundamental questions: 1. which fraction(s) of vegetable oil plays a significant role in secondary metabolite production?, and 2. can oil refinery by-products be substituted in fermentation media in place of crude vegetable oil to diminish the cost of production?

Materials and Methods

Crude soybean oil and processing fractions

Crude, neutralized, bleached and refined soybean oil, lecithin, soap stock and soybean oil deodorizer distillate were obtained from Savola Behshahr Co. (Tehran, Iran). Free fatty acids were removed from the soap stock by acidification with sulfuric acid. The organic phase was then washed with deionized water, oven dried at 103°C, and maintained at 4°C until use (17). The unsaponifiable matter was obtained from deodorizer distillate, by saponification with KOH. After the addition of deionized water and diethyl ether, the organic phase was separated, and the solvent was removed under reduced pressure using a rotatory film evaporator (AOCS Ce 8-89) (18). The organic phase was stored at 4°C for up to 2 days and was used as unsaponifiable matter of deodorizer distillate.

Bacterial strain and media

Saccharopolyspora erythraea UTMC00026 (from the University of Tehran Microorganisms Collection) was used throughout this study. The composition of sporulation and seeding medium used was the same as previously described (13).

Chemically defined medium (CDM) was used for the production of erythromycin with the following composition (per liter of deionized water): starch 30 g, K_2HPO_4 7 g, KH_2PO_4 3 g, $NaNO_3$ 2.38 g, $CaCO_3$ 10 g, $MnSO_4$ 1 g, pH was adjusted at 7.1±0.1 by NaOH (0.5 N) or HCl (0.5 N). It was supplemented with crude soybean oil, degummed oil, neutralized oil, bleached oil, lecithin and mixed fatty acids obtained from soap stock, deodorizer distillate and unsaponifiable matter from deodorizer distillate.

The complex medium (CM) containing CDM plus 25 g/l crude soybean oil was used as fermentation medium to evaluate the effect of testing components on erythromycin production as well.

CDM with zero concentrations of testing materials was used as the control, throughout the experiments.

The ability of *S. erythraea* UTMC00026 to use lecithin, soap stock, free fatty acids, deodorizer distillate and unsaponifiable matter as the sole carbon source at concentration of 1% w/v was studied in the medium with the following ingredients: $(NH_4)_2SO_4$ 2.64 g, KH_2PO_4 2.38 g, K_2HPO_4 4.31 g, $MgSO_4.7H_2O$ 1 g, $CuSO_4.5H_2O$ 0.64 µg, $FeSO_4.7H_2O$ 0.11 µg, $MnCl_2.4H_2O$ 0.79 µg, $ZnSO_4.7H_2O$ 0.15 µg) (19).

Culture method

A volume of 1 ml of a spore suspension (ca.10^7-10^8 spores/ml) of *S. erythraea* UTMC00026 was inoculated in a 500 ml Erlenmeyer flask, containing 100 ml of seed medium. The flasks were incubated at 30°C for 48 h on a rotary shaker at 220 rpm. Then, 5% (v/v) of the seed culture was inoculated into each 250 ml Erlenmeyer flask, containing 30 ml of fermentation medium and was incubated at 30°C for 8 days at 220 rpm.

For batch culture, the test material was added to the medium at the beginning of the fermentation and for the fed-batch culture, optimum concentrations of testing materials were fed to the flasks after 48, 96 and 144 h of start of fermentation.

Assays

Determination of tocopherols, phosphatide, free fatty acids and peroxide content of various fractions of soy bean oil

The analysis of the tocopherol content was done by separation of, unsaponifiable matter of crude, neutralized, bleached, refined soybean oil, soap stock and deodorizer distillate as described in section 2.1. The unsaponifiable matter was diluted in hexane and was subjected to direct high-performance liquid chromatography (20) by an HPLC system (Shimadzu-10, Japan) equipped with a UV detector at 292 nm. Other conditions were: GHRC-SIL column (250×4 mm, Japan), isopropanol/hexane as mobile phase at 1.0 ml/min, column temperature at 40°C (column oven, CGO-10A, Japan), and sample injection volume of 20 µl. Calibration factors were determined for each tocopherol from the chromatography of solutions of standard tocopherols (α, β, γ, δ tocopherol).

The Nephelometric method based on the turbidity of oil-acetone mixtures was used for the determination of phospholipids. The phosphorous level for a given oil type was determined according to the AOCS Ca 19-86 protocol (20).

The amounts of free fatty acid (as oleic) was determined based on AOCS method (21) by titration with standard sodium hydroxide solution.

Peroxide value was analyzed based on oxidation and titration of potassium iodide (22).

Determination of total sterols and tocopherols contents of unsaponifiable matter

Analysis of the sterol contents of unsaponifiable matter was done according to ISO 12228:1999 (E) protocol (23). The deodorizer distillate was saponified with ethanolic potassium hydroxide. The reaction solution was passed from an aluminum oxide glass column. The column was rinsed with ethanol and then with diethyl ether. The elute was collected, and the solvent was evaporated at low pressure. The sterol fraction of the unsaponifiable matter was separated by thin-layer chromatography on silica gel plate. Hexane/diethyl ether (1/1 v/v) and methanol were used as developing solvent and visualizer, respectively. The qualitative and quantitative composition of the sterol fraction were determined by gas chromatography.

Tocopherol content of unsaponifiable matter was determined as described previously (18).

Erythromycin

The concentration of total erythromycin produced was measured by the modified colorimetric method after removing the biomass and insoluble ingredients. The fermentation broth was diluted with 0.2 M carbonate/bicarbonate buffer, pH 9.6 and extracted with chloroform. Extracted erythromycin was mixed with the bromophenol blue reagent (0.008% bromophenol blue in 0.2 M citrate-phosphate buffer, pH 4.2). The organic fraction was separated with great care. The absorbance of this organic fraction was measured at 415 nm with a spectrophotometer. The concentration of erythromycin A was determined by HPLC. The system (Adept 4900, Cecil, UK) was equipped with a UV detector (CE4200, Cecil, UK) at 205 nm. Other conditions were: C18 column (250×4.6 mm, Hichrom, UK), acetonitrile: methanol: 0.2 M ammonium acetate: water (45:10:10:35) mobile phase at 1.0 ml/min, column temperature at 40°C (column oven, CE4601, Cecil, UK), and sample injection volume of 50 µl.

Biomass

The ratio of the packed cell weight to the wet weight of the culture medium was measured after centrifuging the fermentation broth samples at 4,000 rpm for 20 min.

Reproducibility of experiments and statistical analysis

Each experiment was done in triplicates in three independent batches (nine replications). The significance of the results was analyzed by SPSS ver. 16 software (IBM Co).

Results and Discussion

Oils have been used in erythromycin production for decades, there are, however, no reports on the details of the process and impact of oil ingredients on antibiotic production. This study evaluated the effect of the non-triglyceride components of soybean oil on erythromycin production.

Selected chemical ingredients of soybean oil fractions

The chemical ingredients of the soybean oil used throughout the study are shown in Table 1 and Figure 1. The free fatty acids of oil accumulate in soap stock and deodorizer distillate. Also, the tocopherol content of the deodorizer distillate is high.

Table 1. Free fatty acids, peroxide, phosphatide, and tocopherols levels in presence of various additives.

Type	Free fatty acid (%)	Peroxide value (Meq/Kg *)	Phosphatide (%)	Tocopherol (%)
Crude oil	1.12	3.26	0.27	1.51
Neutralized oil	0.07	4.22	0.01	NA**
Bleached oil	0.10	1.19	ND***	1.33
Refined oil	0.02	0.53	ND	0.80
Soap stock	NA	0.93	1.99	NA
Deodorizer distillate	36.3	6.00	ND	7.10

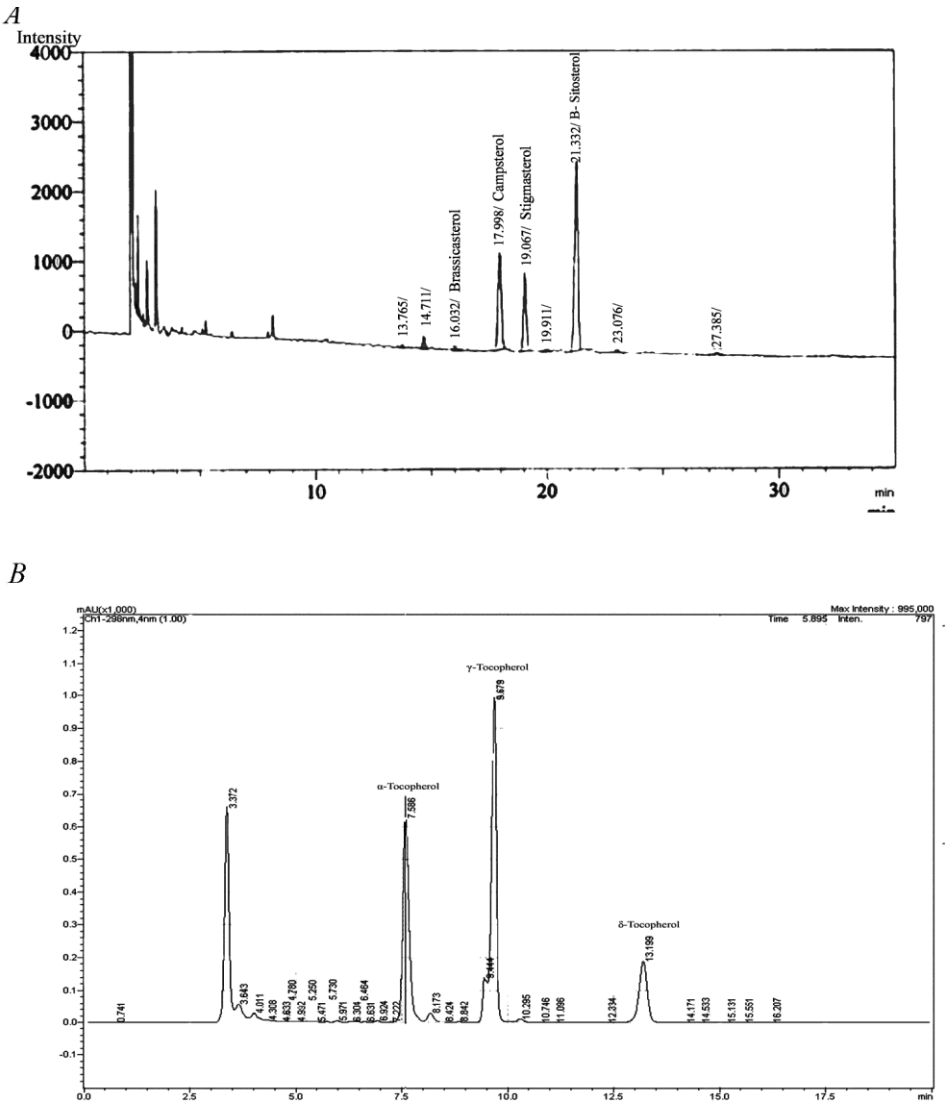

Figure 1. A) GC chromatogram of sterols, total sterols 62.9 mg/l (Brassicasterol, 0.7 mg/l. Campsterol, 15.8 mg/l. Stigmasterol, 10.9 mg/l and β-Sitosterol, 32.3 mg/l). B) HPLC chromatogram of tocopherols of unsaponifiable matter. Total tocopherols 156.2 mg/l (α-Tocopherol, 70.3 mg/l. γ-Tocopherol, 62.4 mg/l. δ-Tocopherol, 23.5 mg/l).

Comparison of erythromycin production in the soybean oil-containing media

The effect of various grades of soybean oil, including crude, neutralized, bleached and refined grades obtained during refining processes on the production of erythromycin is presented in Figure 2a. Addition of 25 g/l crude oil to the CDM increased erythromycin production ($P<0.05$) by 1.5 times. Variation of erythromycin production between the media containing 25 g/l neutralized, bleached and refined oils and control was not significant ($P>0.8$). Advantage of using crude oil may be due to the presence of emulsifier/surfactant (phospholipids), some nutrients (iron, phosphorous, calcium) and antioxidant (tocopherols).

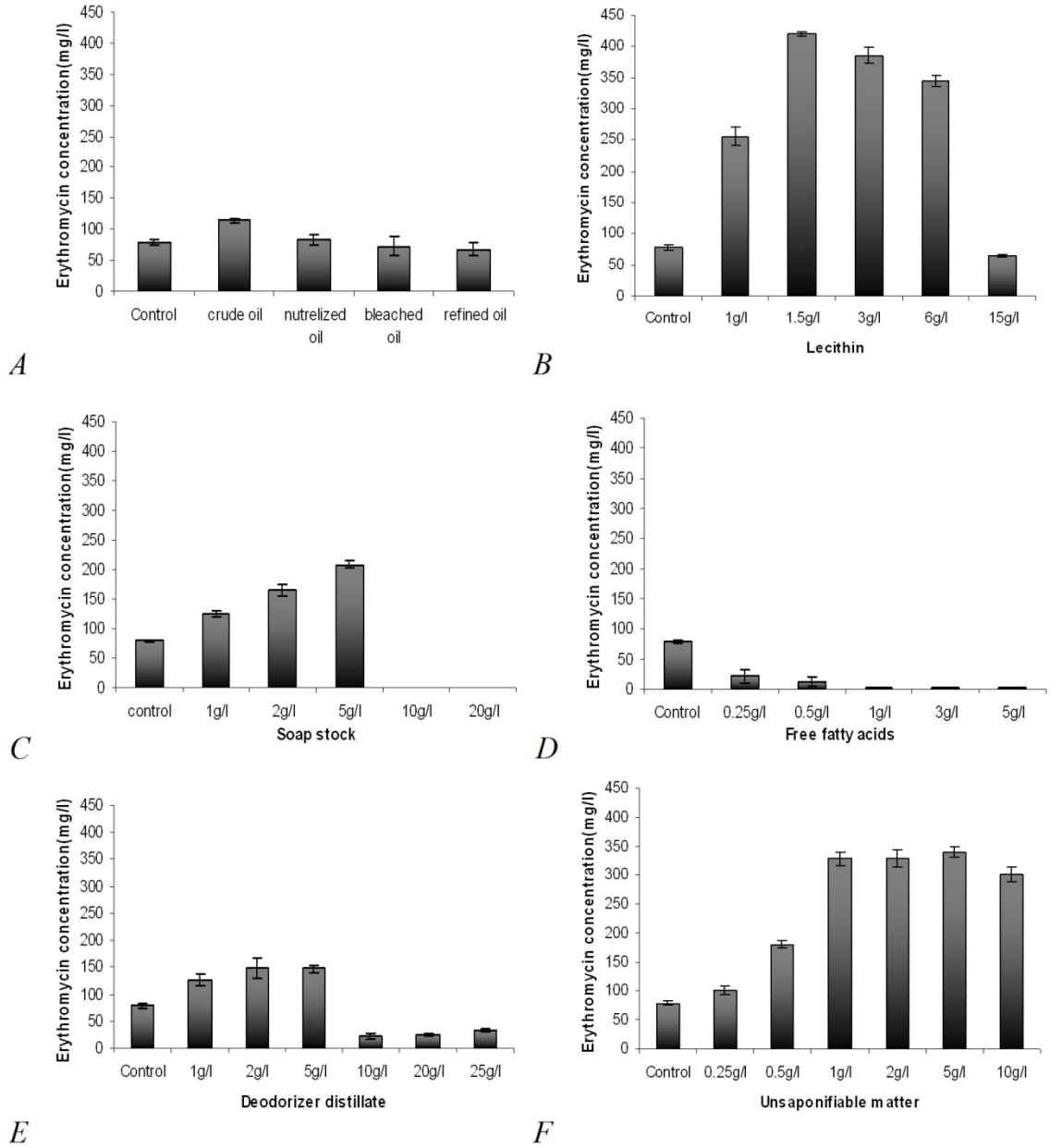

Figure 2. Erythromycin production in presence of various additives: A) soybean oil refining fractions including crude, neutralized, bleached and refined oil. Each fraction is added at 25 g/l, B) lecithin, C) soap stock, D) free fatty acids obtained from soap stock, E) deodorizer distillate and F) unsaponifiable matter in the chemically defined fermentation medium.

Effect of lecithin of soybean oil on erythromycin production

The effect of various concentrations (0–15 g/l) of lecithin on erythromycin production is shown in Figure 2b. Addition of lecithin up to 6 g/l increased erythromycin, significantly ($P<0.05$). However, the addition of higher concentrations of lecithin (>6 g/l) reduced erythromycin production. The maximum concentration of erythromycin (420 mg/l) was observed in the media containing 1.5 g/l lecithin. It was 5.3 times more than that of the control.

Phospholipids are the main components of lecithin. They removed the degumming stage of crude oil refinement. Lecithin is a well-known industrially used component for its emulsifying properties. *S. erythraea* can make use of it as a carbon source. Lecithin is an excellent emulsifier (24) and can increase the dispersion of the fermentation medium and decrease the difficulties raised due to oxygen limitation (12, 25).

Effects of soap stock on erythromycin production

The effect of various concentrations of soap stock (0-20 g/l) is presented in Figure 2c. Soap stock up to 5 g/l increased erythromycin, significantly ($P<0.05$), while, higher concentrations of soap stock (>5 g/l) greatly decreased erythromycin production. The maximum concentration of erythromycin (209 mg/l) was observed in the media containing 5 g/l soap stock. A value that is 2.6 times greater than the control.

Soap stock is the main by-product of the vegetable oil refining industry. It is a complex heterogeneous material that typically contains sodium fatty acid soap, glycerides, phosphoglycerides, sterols, organic phosphates, poly-alcohols, carbohydrates and proteinaceous material. There are no reports of the effect of soap stock on antibiotic production in the literature. However, positive effects of soybean soap stock are reported on glycolipids and rhamnolipids synthesis by *Candida* sp. (26) and *Pseudomonas aeruginosa* (27), respectively. Negative effect of soap stock on erythromycin production at high concentrations may be due to the toxic effect of free fatty acids.

Effects of mixed free fatty acids of soap stock on erythromycin production

The effect of 0-5 g/l free fatty acid (mixed soybean oil's fatty acids) on erythromycin production is shown in Figure 2d. Addition of all concentrations of free fatty acid studied reduced antibiotic production in comparison to the control, by a value ranging from 3.8-15.8 times.

The antimicrobial effect of fatty acids is not solely due to the creation of a high extracellular proton concentration, but is also directly related to the concentration of undissociated acid. Undissociated lipophilic acid molecules can pass freely through the membrane. When the pH of the cytoplasm is high, (~7), fatty acid dissociate and the protons produced decrease the pH of the cytoplasm and interrupt the proton motive force (28). Also, the fatty acids that enter the cytoplasm repress *de novo* fatty acid biosynthesis (29, 30). However, the effect of fatty acids on the bacteria is strongly related to the media ingredients. Therefore, it is possible to change the negative effect of fatty acids to positive, by using appropriate chemicals (31).

Effects of deodorizer distillate on erythromycin production

As shown in Figure 2e, the addition of

various concentrations of deodorizer distillate up to 5 g/l increased erythromycin production, significantly ($P<0.05$). However, at a concentration of 10 g/l deodorizer distillate, erythromycin production was reduced 3.4 times less than that of the control. The maximum concentration of erythromycin (148 mg/l) was observed in the medium containing 2 g/l deodorizer distillate (1.9 times more than that of the control). Erythromycin production started in the media containing deodorizer distillate and control on the second and fifth days of fermentation, respectively (data not shown).

The most valuable by-product of edible oil refining is the distillate obtained in the deodorization stage. As shown, the addition of deodorizer distillate has a positive effect on erythromycin production. The increased production may be due to the availability of *S. erythraea* to use soybean oil deodorizer distillate as an auxiliary carbon source and the antioxidative effect of the tocopherol contents of the soybean oil deodorizer [32].

Effects of unsaponifiable matter obtained from deodorizer distillate on erythromycin production

After the extraction of unsaponifiable matter (mainly consists of sterols and tocopherols) from deodorizer distillate, various concentrations (0–10 g/l) of it was supplemented in the fermentation medium. As shown in Figure 2f, the addition of various concentrations of unsaponifiable matter increased erythromycin, significantly ($P<0.05$). The maximum concentration of erythromycin was observed in the medium containing 5 g/l of unsaponifiable matter. It was 4.3 times more than that of the control.

The result also showed that the negative effect of higher concentrations of deodorizer distillate on erythromycin production may be due to the toxicity of high amounts of free fatty acids that are removed while preparing unsaponifiable matter.

Fed-batch effect of lecithin, soap stock, deodorizer distillate and unsaponifiable matter

Considering the enhancing effect of crude soybean oil on erythromycin production, CDM plus 25 g/l crude soybean oil was used as basal complex medium (CM) to investigate the fed-batch effect of soybean oil derivatives. CM hereafter is named as the control (2). The effect of feeding the CDM and CM with the optimal concentrations of soybean oil refinery derivatives over the time (after 48, 96 and 144 h of the start of fermentation) is presented in Figure 3. By doing this, this study could have a better estimation of applicability of these components in pilot-scale productions. The result obtained in CM was in agreement with previous findings in CDM. Lecithin, soap stock, deodorizer distillate and unsaponifiable matter but not free fatty acids increased erythromycin production. However, their positive effect in CM was smaller than those observed in this study (Fig. 3).

Although supplementation of the media with testing material over time showed a positive effect on erythromycin production, maximum quantity of antibiotic production was achieved when the test components were added to the media at the beginning of the fermentation process (0 h). The result obtained in CM is in agreement with those in CDM. Both media showed maximum enhancing effect of the feeds when the testing components were added at the onset of fermentation.

It can be concluded that the addition of oil ingredients to the fermentation medium is better than the addition of crude oil. It did not

only increase the productivity of erythromycin significantly, but also decreased the limiting effects of oil addition to erythromycin including oxygen transfer limitation (33), plugging the membrane filters (34) and interference with organic solvents used for the extraction of erythromycin.

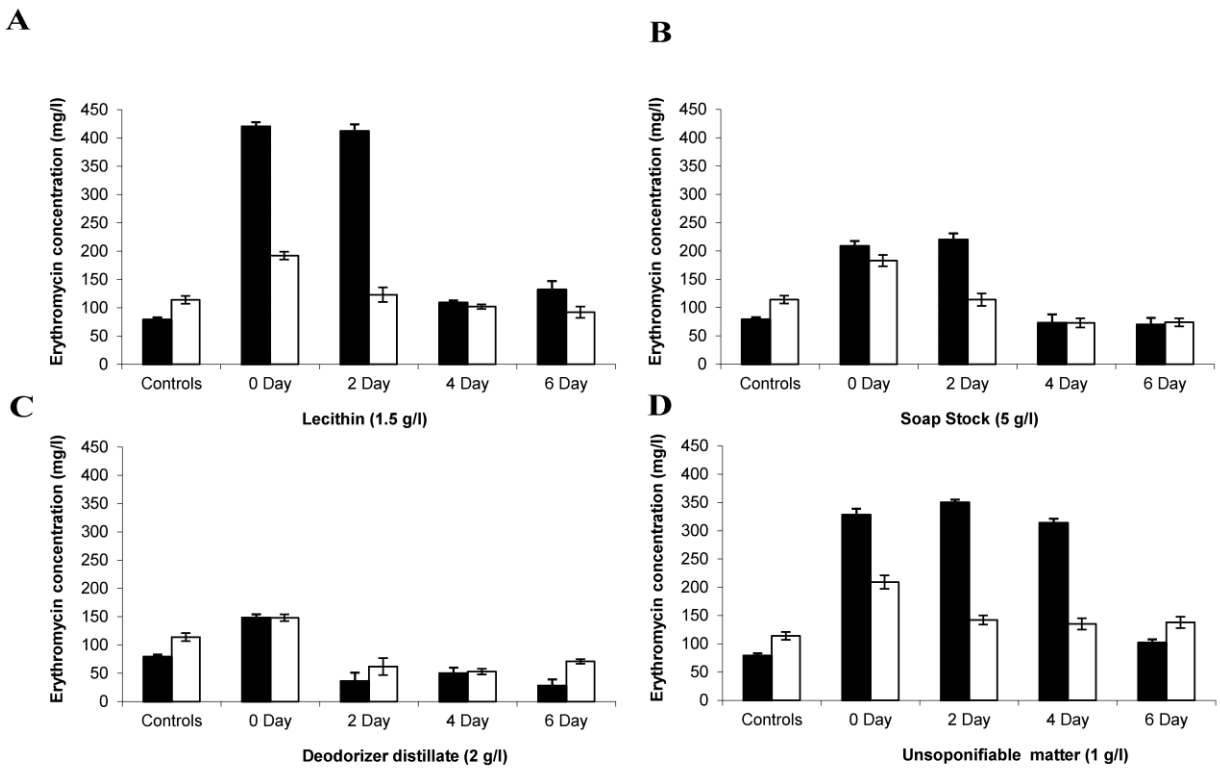

Figure 3. Concentration of erythromycin in fed batch fermentation in presence various feeding:A) lecithin, B) soap stock, C) deodorizer distillate, D) unsaponifiable matter after 0, 2, 4 and 6 days of cultivation in the chemically-defined fermentation medium (CDM) (■) and complex medium (CM) (□). CM is CDM plus 25g/l crude soybean oil. the CDM and CM with zero concentrations of the testing materials was used as controls.

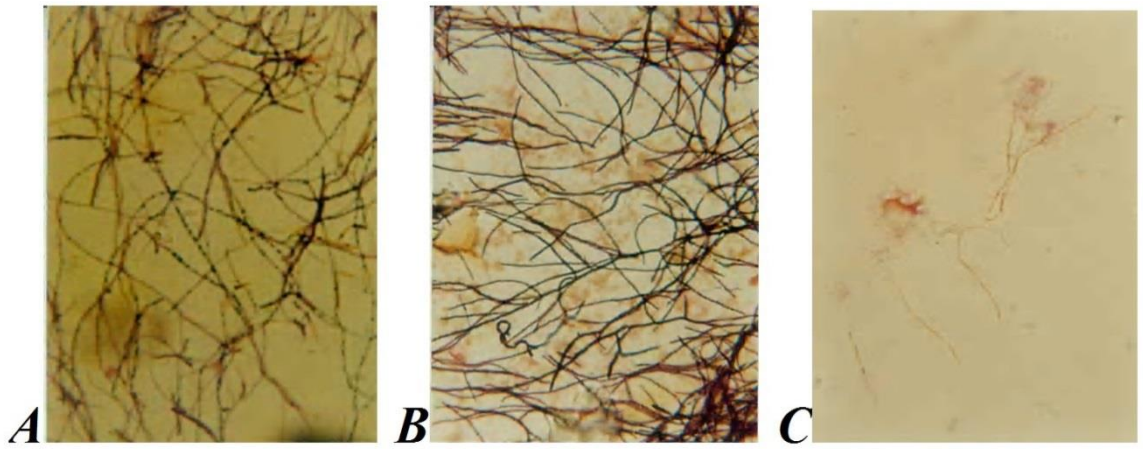

Figure 4. Hyphal morphology of *S. erythraea* UTMC00026 after 8 days. A- Control medium (low concentration of erythromycin produced), B- In the medium containing crude oil, lecithin, soap stock and unsaponifiable matter (higher concentration of erythromycin was produced), C- In the free fatty acids containing media (no erythromycin was produced).

Effect of soybean oil fractions on morphology of *S. erythraea* UTMC00026

The morphology and life-span of *S. erythraea* UTMC00026 were affected by the composition of the medium. As shown in Figure 4b, in the media with greater erythromycin production, hyphae were longer and remained in a vegetative form. However, alteration in the morphology of the strain in the presence of all components that stimulate antibiotic production was not significant. However, in the presence of free fatty acids, and high concentration of soap stock and deodorizer distillate, erythromycin production was reduced, and hyphae were found to be, thin and short (Fig. 4c). Correlation between erythromycin production and morphology of hyphae has also been reported earlier (13, 31). Thus, the morphology of *S. erythraea* hypha can be used as a quick indicator of the fermentation process.

Growth of S. erythraea* UTMC00026 *in the presence of soybean oil fractions as a sole carbon source

S. erythraea UTMC00026 increased in the presence of soybean oil, lecithin and deodorizer distillate as a sole carbon source. However, the soap stock and unsaponifiable matter did not support the growth of the strain.

Conclusions

Based on the findings of the present study, the enhanced production of erythromycin by the addition of oils is in part due to the non-triacylglycerol fractions, including lecithin, soap stock, deodorizer distillate, and unsaponifiable matter. Additionally, the concentration of erythromycin significantly increased after supplementing the fermentation medium with some fractions of soybean oil, including lecithin and deodorizer distillate in comparison to the addition of crude vegetable oil.

Therefore, it seems soybean oil refinery by-products can be used for the production of valuable, useful chemicals. The use of soybean oil refinery by-products rather than crude oil reduces the negative effects of crude oil on upstream and downstream processes of erythromycin, including oxygen limitation, plugging of membrane filters and it interferes with solvent extraction.

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