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ORIGINAL PAPER

J. Hamedi · F. Malekzadeh · A. E. Saghafi-nia

Enhancing of erythromycin production by *Saccharopolyspora erythraea* with common and uncommon oils

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Abstract The enhancing effect of various concentrations of 18 oils and a silicon antifoam agent on erythromycin production by *Saccharopolyspora erythraea* was evaluated in a complex medium containing soybean flour and dextrin as the main substrates. The oils used consisted of sunflower, pistachio, cottonseed, melon seed, water melon seed, lard, corn, olive, soybean, hazelnut, rapeseed, sesame, shark, safflower, coconut, walnut, black cherry kernel and grape seed oils. The biomass, erythromycin, dextrin and oil concentrations and the pH value were measured. Also, the kinds and frequencies of fatty acids in the oils were determined. The productivity of erythromycin in the oil-containing media was higher than that of the control medium. However, oil was not suitable as a main carbon source for erythromycin production by *S. erythraea*. The highest titer of erythromycin was produced in medium containing 55 g/l black cherry kernel oil (4.5 g/l). The titers of erythromycin in the other media were also recorded, with this result: black cherry kernel > water melon seed > melon seed > walnut > rapeseed > soybean > (corn = sesame) > (olive = pistachio = lard = sunflower) > (hazelnut = cotton seed) > grape seed > (shark = safflower = coconut). In media containing various oils, the hyphae of *S. erythraea* were longer and remained in a vegetative form after 8 days, while in the control medium, spores were formed and hyphae were lysed.

J. Hamedi · F. Malekzadeh (✉)
Department of Biology (Microbiology),
Faculty of Science, University of Tehran,
Tehran, Iran
E-mail: Falmero@yahoo.com
Tel.: +98-1-8088328
Fax: +98-1-8088328

A. E. Saghafi-nia
Shafa-e-Sari Co., Antibiotic Producing Co.,
Tehran, Iran

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Introduction

The composition of fermentation media plays an important role in the titer and productivity of secondary metabolites and the cost of raw materials/product. Also, fermentation parameters can influence the kinetics of the process. For example, carbon and phosphate limitation provided the classic "secondary metabolite" type of kinetics, with the peak in erythromycin production rate following that of the specific growth rate of *Saccharopolyspora erythraea*. But, growth-linked product formation was observed in a nitrate-limited medium [20]. In contrast, it was shown that erythromycin production was dependent on the growth of the strain in a medium deficient in oxygen and glucose, although vancomycin production by *Amycolatopsis orientalis* did show such a dependency [8].

Oils are among the essential components of industrial fermentation media and have been routinely supplemented into media for the production of secondary metabolites. They have been used as antifoams [2], sole carbon sources [24], auxiliary carbon sources [9], to provide precursors for antibiotic synthesis [6] and to remove the antibiotic from bacterial access and reduce its suppressive effect on antibiotic production [10]. Oils can serve as sources of precursors for the biosynthesis of 6-erythronolide B [7, 13, 14]. Recently, the efficiency of rapeseed oil in the production of erythromycin by *S. erythraea* was shown [9, 22]. However, few studies have been directed to evaluate the comparative effect of different oils on the production of erythromycin and the correlation of growth and product [11].

In this study, we compared the effect of different concentrations of usual and unusual oils on the growth and erythromycin production of *S. erythraea* NUR001.

Materials and methods

Bacterial strains and media

S. erythraea NUR001 was provided by the Shafa-e-Sari Antibiotic Producing Co., Tehran, Iran. *Micrococcus luteus* ATCC 9341 was employed for the microbiological assay of the antibiotic produced. The sporulation medium used was oatmeal agar [26]. The composition of the seed medium used was (per liter): 30 g soybean meal, 10 g glucose, 10 g glycerol, 1 g $(NH_4)_2HPO_4$, 3.5 g $(NH_4)_2SO_4$, 5 g $CaCO_3$, pH 7.0 ± 0.1. The composition of the fermentation medium used was (per liter): 35 g soybean meal, 70 g dextrin, 3 g $(NH_4)_2SO_4$, 0.5 g $(NH_4)_2HPO_4$, 12 g $CaCO_3$, pH 6.8 ± 0.1 [11].

Selected oils as a part of the carbon source

Unusual oils were extracted from plant seeds by the Folch method [12]. Various concentrations of each oil (30–60 g/l) were added to each 1,000-ml Erlenmeyer flask containing 150 ml of fermentation medium. The kinds and sources of the oils used are shown in Table 1.

Cultural method

A volume of 1 ml of a spore suspension (ca. 10^7–10^8 spores/ml) of *S. erythraea* NUR001 was inoculated in a1,000-ml Erlenmeyer flask containing 100 ml of seed medium and 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 1,000-ml Erlenmeyer flask containing 150 ml of fermentation medium and incubated at 30°C for 11 days on a rotary shaker at 240 rpm. All experiments were performed in triplicate in three batches.

Table 1 Selected oils as a part of the carbon source

Oil	Source
Sunflower	Behshahr Industrial Group, Behshahr, Iran
Pistachio	Extracted by the Folch method in our laboratory
Cottonseed	Omid Oil Pressing, Tehran, Iran
Melon seed	Extracted by the Folch method in our laboratory
Watermelon seed	Extracted by the Folch method in our laboratory
Lard	Coelo Co., Germany
Corn	Afia Co., Jedda, Saudi Arabia
Olive	Afarin Co., Manjil, Iran
Soybean	Omid Oil Pressing, Tehran, Iran
Hazelnut	Extracted by the Folch method in our laboratory
Rapeseed	Creol Co., Moscow, Iran
Sesame	Extracted by the Folch method in our laboratory
Shark	Iranian Fisheries Co., Bandar Abbas, Iran
Walnut	Omid Oil Pressing, Tehran, Iran
Safflower	Extracted by the Folch method in our laboratory
Coconut	Sime Derby, Singapore
Black cherry kernel	Extracted by the Folch method in our laboratory
Grape nut	Coelo Co., Germany

Assays

Samples, 5 ml, were removed on a daily basis. They were kept at −70°C for further analysis, after measuring the pH and biomass.

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.

Dextrin

The concentration of total sugars was measured by reaction with sulfuric phenol [4].

Erythromycin

The concentration of total erythromycin produced was measured by the modified colorimetric method, after removing the biomass and insoluble ingredients [25]. 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 and its absorbance was measured at 415 nm with a spectrophotometer. In order to confirm the production of biologically active erythromycin, 8-day fermentation broth samples were bioassayed against *M. luteus* ATCC9341, using the cylinder plate assay method [27]. Also, in these samples, the concentration of erythromycin A was determined by HPLC. A HPLC system (model K-2500; Knauer, Germany) was equipped with a UV detector (model K-2001; Knauer) at 205 nm. Other conditions were: C18 column, acetonitrile:methanol:0.2 M ammonium acetate:water (45:10:10:35) mobile phase at 1.0 ml/min, column temperature 40°C, sample injection volume 50 µl [28].

Residual oil

The weight of oil was measured after extraction by *n*-hexane and evaporation of the solvent [16].

Fatty acid profile of the oils

The kinds and frequencies of fatty acids in oil samples were determined by gas–liquid chromatography. Fatty

acids of the oil samples were prepared by alcoholic potassium hydroxide and the ester derivatives were prepared by sulfuric acid:methanol:toluene reagent [12]. Fatty acid methyl esters were analyzed using a gas chromatograph (model GC-16A; Shimadzu, Japan) equipped with a capillary column (50 m long, 0.25 mm i.d., model OV-17; Shimadzu), using N_2 (2 ml/min flow rate) and a flame ionization detector. The operating temperature was 210°C.

Data analysis

One-way analysis of variance and Tukey HSD test were done with SPSS ver. 10 software (Microsoft, USA).

Results

Effect of oils on the productivity of erythromycin

The effect of various oils on the production of erythromycin is presented in Fig. 1. The titer of erythromycin was very low in all media up to 2 days; and the addition of different oils had no effect on the onset of erythromycin production. However, in the oil-containing media, a higher productivity of erythromycin was obtained ($P < 0.01$). The maximum concentration of erythromycin was obtained after 8 days; and there was no difference in the time taken to reach the highest titer of erythromycin among the oils used.

Table 2 compares the effect of the oils on erythromycin production. As shown, the production of erythromycin under optimized conditions was increased. Also, the order and arrangement of the oils used differed. In any condition, the maximum and minimum erythromycin production levels were detected in black cherry kernel oil and grape seed oil, respectively.

Effect of oils on dextrin consumption

As shown in Fig. 1, the dextrin consumption rate was lower in the oil-containing media than in the control medium. In the media containing shark, hazelnut and coconut oils, the minimum concentrations of residual dextrin were obtained: 7.88, 12.27 and 13.76 g/l, respectively. In contrast, in the media containing walnut, rapeseed and olive oils the maximum residual dextrin were obtained: 51.12, 37.74 and 36.54 g/l, respectively. However, in all cases, the dextrin in the medium was not used completely.

Utilization of oils

When the concentration of residual oil were measured in the oil-containing media, it was found that watermelon seed oil was used the most (4.32 g/l of residual oil) and that shark oil was used the least (21.05 g/l of residual oil).

Effect of oils on growth and morphology

The highest concentration of biomass was obtained in the shark oil-containing medium and the minimum concentration of biomass was seen in the cottonseed oil-containing medium. The biomass concentration in melon seed oil-containing medium was almost constant during 2–8 days of fermentation period. Considering the concentration of biomass after 8 days of fermentation in shark and black cherry oil-containing media, (48 g/l, 41.5 g/l, respectively), it seemed there was little difference in biomass production in many desirable and undesirable oils. The morphology and life-span of *S. erythraea* NUR001 were affected by the oils used. As shown in Fig. 2, in media containing various oils, hyphae were longer and remained in a vegetative form after 8 days (Fig. 2d–f), while in the control medium, spores were formed and hyphae were lysed (Fig. 2a–c). In the former case, more antibiotic was produced. The alteration in the morphology of the strain in various oil-containing media was not significant (Fig. 2g–i), except for shark oil, where short hyphae were formed after 4 days (Fig. 2j–l).

Effect of oils on fermentation medium pH

The pH variation in the control medium was greater than that in the oil-containing media. As shown in Fig. 1, the final pH in the control and shark oil-containing media increased significantly when compared with that of other oil-containing media ($P < 0.01$), while the pH change in the other oil-containing media was not significant ($P < 0.5$).

Relationship between growth and erythromycin production

The production of erythromycin and biomass were dependent on the medium composition, as shown in Fig. 1. In the media containing rapeseed, soybean, cotton seed, shark, safflower, coconut and sesame oils, there was a correlation between growth and the production of erythromycin, but this correlation was less evident in the control medium and media containing other oils.

Fatty acid profiles of the oils studied

The major fatty acid compositions of the oils studied are presented in Table 3. In the plant oils studied, unsaturated fatty acids were prevalent. The exception was coconut, which contained 84% saturated fatty acids

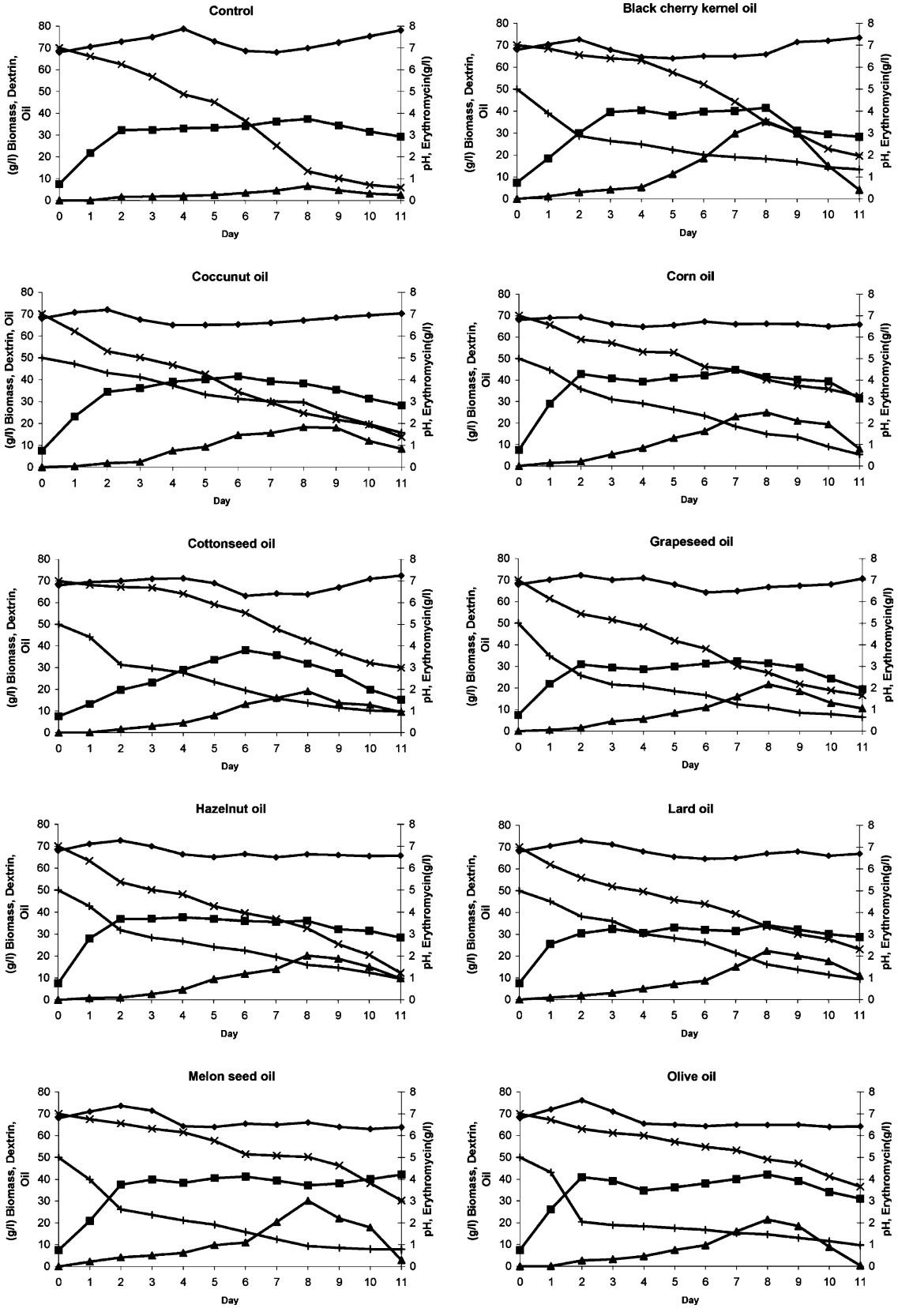

Fig. 1 Effect of various oils on the production of erythromycin by *S. erythraea* NUR001 in basal medium containing 50 g/l of various oils. A control fermentation conducted in the absence of any oil supplement was also included. *Filled triangles* Erythromycin concentration, *filled squares* biomass, × dextrin concentration, + oil concentration, *filled diamonds* pH

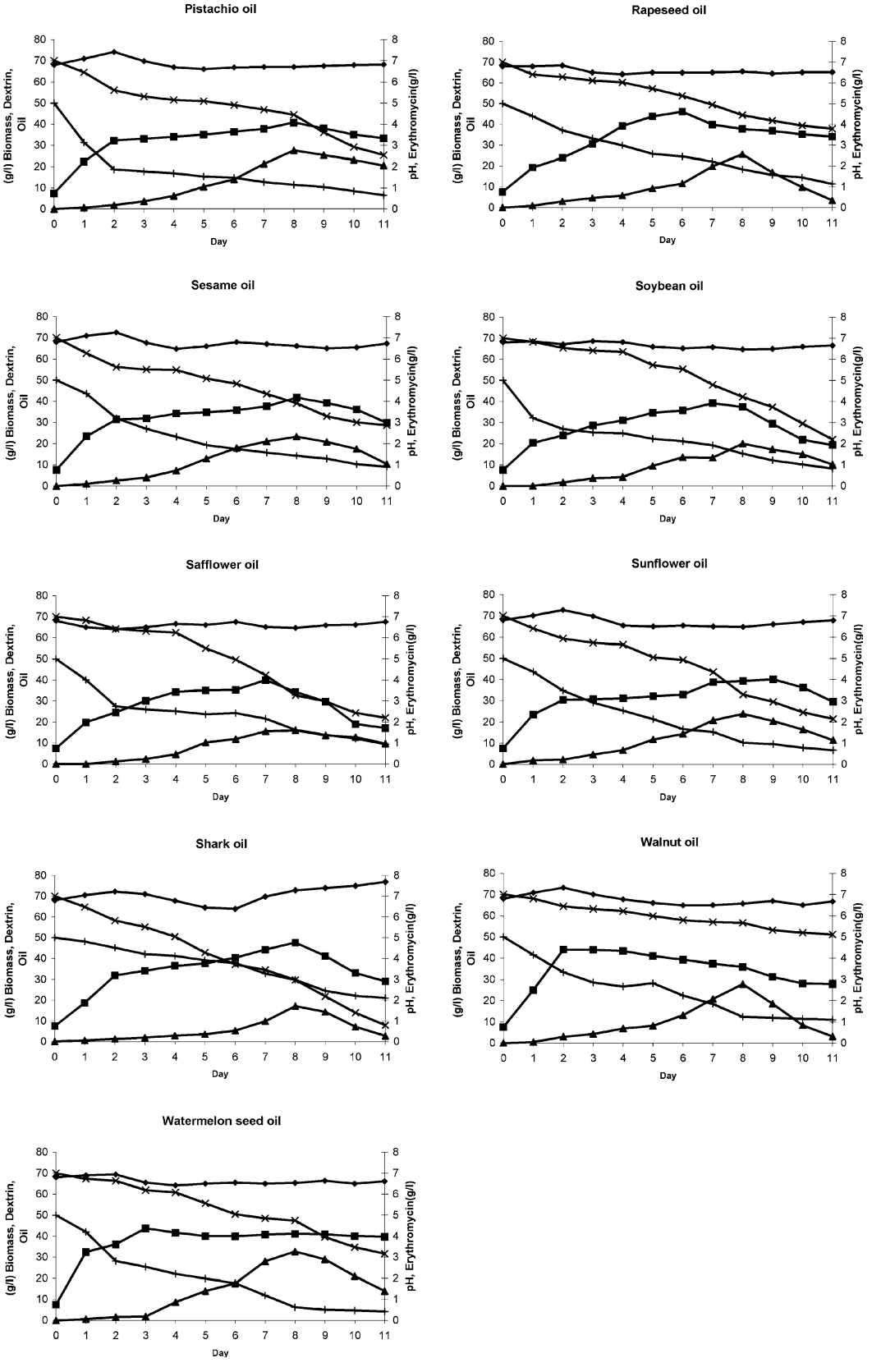
Pistachio oil
Rapeseed oil
Sesame oil
Soybean oil
Safflower oil
Sunflower oil
Shark oil
Walnut oil
Watermelon seed oil
(g/l) Biomass, Dextrin, Oil
pH, Erythromycin(g/l)
Day

Fig. 1b (Contd.)

Table 2 The effects of oils on the production of erythromycin before and after optimization. In each case, the oils are ranked in order of erythromycin production. Values are averages ± SD of three triplicate experiments

Before optimization		After optimization		
Oil (50 g/l)	Concentration of erythromycin (g/l)	Oil	Concentration of erythromycin (g/l)	Concentration of oil (g/l)
Black cherry kernel	3.55 ± 0.03	Black cherry kernel	4.5 ± 0.04	45
Watermelon seed	3.3 ± 0.03	Watermelon seed	4.19 ± 0.03	45
Melon seed	3.0 ± 0.02	Melon seed	3.92 ± 0.03	45
Walnut	2.78 ± 0.03	Walnut	3.5 ± 0.02	45
Pistachio	2.76 ± 0.01			
Rapeseed	2.6 ± 0.06	Rapeseed	3.4 ± 0.03	55
Corn	2.5 ± 0.01	Soybean	3.25 ± 0.02	45
Sunflower	2.4 ± 0.02			
Lard	2.2 ± 0.03	CornSesame	3.19 ± 0.03	45
Sesame	2.3 ± 0.01		3.15 ± 0.04	55
Olive	2.15 ± 0.03	Olive	2.75 ± 0.02	45
Grape nut	2.17 ± 0.01	PistachioLard	2.76 ± 0.01	50
		Sunflower	2.8 ± 0.01	40
			2.58 ± 0.03	55
Soybean	1.99 ± 0.05	Cottonseed	2.3 ± 0.04	55
Cottonseed	1.9 ± 0.04	Hazelnut	2.4 ± 0.03	45
Hazelnut	2.02 ± 0.03			
Shark	1.7 ± 0.01	Grape nut	2.17 ± 0.02	50
Safflower	1.6 ± 0.01			
Coconut	1.83 ± 0.01			
Control	0.66 ± 0.05	Control	0.66 ± 0.05	0

(mostly lauric acid). In the animal oils used, the concentration of saturated fatty acids were more than that of unsaturated fatty acids. The amount of unsaturated fatty acids in lard and shark oils were 31% and 41%, respectively. Shark oil was the liver oil of *Carcharhinus dussumieri*, the predominant species in the Persian Gulf and Oman Sea [1]. Frankly, it was difficult to find a precise correlation between erythromycin production and the fatty acid composition of the oils used. However, considering Table 3 and comparing the prevalence of erythromycin produced with the percentage of saturated and unsaturated fatty acids, it seemed that the best oil for erythromycin production must have a high content of unsaturated fatty acids.

Effect of oils as antifoam on the production of erythromycin

The comparative results of erythromycin production in media containing a silicon antifoam agent (Merck, Germany) or rapeseed oil (positive control) and basal medium (negative control) are shown in Table 4. The erythromycin concentration in the media containing antifoam agent (50 g/l) and rapeseed oil (50 g/l) were, respectively, 3.86 times and 1.86 times higher when compared with that of the basal medium. Although dissociation of the physico-chemical behavior of the oil as an antifoam or as a source of energy/carbon was difficult in a fermentation system, we speculated that the result obtained showed that the antifoam property of the oil may have a partial beneficial effect in the production of erythromycin.

Oil as a main carbon source

The results of erythromycin production in the medium containing rapeseed as a main carbon source, compared with that of the control medium (dextrin as main carbon source) and a medium containing dextrin plus rapeseed oil, are shown in Table 5. The results show that oils were not suitable as main carbon sources for erythromycin production by *S. erythraea*, while they may be auxiliary carbon sources, providing precursors for erythromycin production.

Discussion

Although there is a common consensus of opinion on the positive effects of oils on the production of antibiotics, the impacts of different oils vary greatly in the production of antibiotics [10, 24]. In this research, erythromycin production in the control medium (containing dextrin with soybean flour as the main substrates) was less than that in oil-supplemented media. Since dextrin was not depleted completely in the control medium at the end of the fermentation, it was not likely that the low rate of growth and poor erythromycin production were due to a lack of carbon source. The dextrin consumption rate in the oil-containing media was lower than that in the control medium, indicating that *S. erythraea* NUR001 consumed oil as an alternative energy/carbon source. However, our results show that oils were not suitable as a main carbon source for erythromycin production and a medium containing dextrin plus oil was more suitable. With taking into

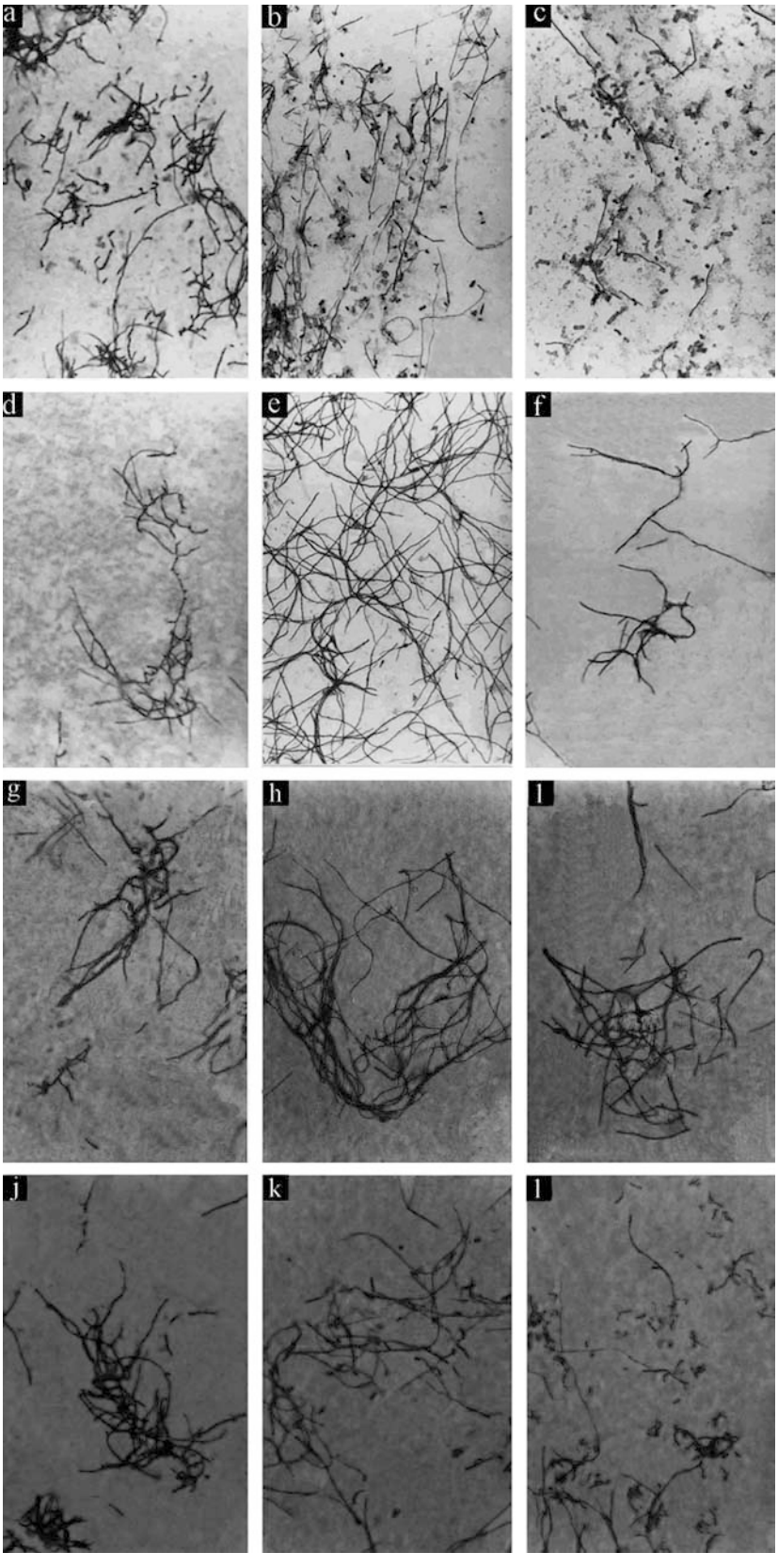

Fig. 2 Effect of the oils on the morphology of *S. erythraea* NUR001. **a–c** control medium. **d–f** Rapeseed oil-containing medium. **g–i** Black cherry oil-containing medium. **j–I** Shark oil-containing medium. **a,d,g,j** Hyphae after 2 days. **b,e,h,k** Hyphae after 8 days. **c,f,i,l** Hyphae after 11 days

consideration the chemical structure of erythromycin (lipid with carbohydrates), we concluded that dextrin and the oils used may play partial roles in providing the precursors for the production of erythromycin. *S. erythraea* NUR001 used the oils as supplements for carbon/energy sources rather than as sole carbon sources. The

Table 3 Major fatty acids composition of oils used (% total fatty acids)

Fatty acids	Black cherry kernel	Coconut	Corn	Cotton seed	Grape seed	Hazelnut	Lard	Melon seed	Olive	Pistachio	Rapeseed	Safflower	Sesame	Shark	Soybean	Sunflower	Walnut	Watermelon seed
Lauric acid	0	46	0	0	1	0	3	0	0	0	0	0	0	0	0	1	0	0
Myristic acid	0	13	0	1	0	1	0	0	0	0	0	0	0	7	0	0	0	0
Palmitic acid	7	6	10	24	5	5	18	10	10	11	5	7	8	24	11	0	7	10
Stearic acid	2	2	0	2	12	2	8	5	0	1	2	3	5	8	4	3	3	5
Palmitoleic acid	0	0	0	0	0	0	0	0	0	2	1	0	0	14	0	0	0	1
Oleic acid	0	6	25	12	11	77	0	29	85	50	55	12	21	0	44	25	11	12
Linoleic acid	77	3	58	55	70	12	50	53	2	33	27	73	57	18	40	55	67	66
Linolenic acid	11	0	1	0	0	0	7	0	0	1	9	3	0	0	0	0	10	0

oils used acted partially as antifoams and partially provided precursors for the biosynthesis of erythronolide B.

Our results show that the best oils for erythromycin production could maintain *S. erythraea* NUR001 in long-form hyphae, while short hyphae or spores were the predominant forms of the bacterium in the control or shark oil-containing media. Fragmentation of the mycelia caused the production of more viable units, thus helping the bacterium to escape from undesirable conditions. It seems that hyphal morphology was correlated with the physiological properties of the strain and the physical conditions of the medium. The relationship between hyphal morphology, size and antibiotic production was reported previously [5, 29]. However, in this research, it was shown for the first time that the oils used exhibited some effect on this event and that erythromycin production was correlated to biomass production in some oil-containing media.

S. erythraea produced a reddish-brown water-soluble pigment in medium containing shark, coconut and cottonseed oils and in the control medium after 3–4 days of growth. These media were not suitable for erythromycin production (Table 2). It seems that undesirable conditions such as some oils or media having a limited amount of oxygen [8] favored the production of this secondary metabolite. As reported, the reddish-brown pigment was a shunt to maintain the metabolic equilibrium in *S. erythraea* which had a negative effect on erythromycin production.

The pH variation of the control medium was greater than that of oil-containing media, with the exception of the medium containing shark oil. It was concluded that the addition of vegetable oils to the erythromycin fermentation medium maintained the pH at a suitable level. The optimum pH (ca. pH 7.0) in the oil-containing media could help to provide better growth of *S. erythraea* NUR001. The contradictory results obtained in the media containing vegetable and shark oils could be due to differences in the fatty acid contents of the oils used. As shown in Table 2, linoleic and oleic acids were the predominant fatty acids in the vegetable oils used, while palmitic and palmitoleic acids were the major fatty acids in the shark oil. Also, the percentage of saturated fatty acids in the shark oil was more than that in the vegetable oils used.

Melon seeds, besides possessing a medicinal application [3, 17, 30], are used as a food in northeast of Iran. The seed contains 25% oil, having linoleic acid, oleic, palmitic and stearic acids as the prevalent fatty acids (Table 2) and is rich in proteins (19.3–53.9%, depending on the variety) [21]. We showed that watermelon seed and melon seed oils were substantial materials for erythromycin production, while growth was limited. In these media, the increase in biomass production after 48 h was negligible. Because biomass is one the factors most affecting the fouling of membranes in the microfiltration process [9], a lower biomass production can cause a decrease in the cost of down-stream processing.

Table 4 Effect of oil as antifoam on erythromycin production by *S. erythraea* NUR001. Values are averages ± SD of three triplicate experiments

Treatment	pH	Biomass (g/l)	Erythromycin (g/l)
Basal medium + silicone antifoam (50 g/l)	6.5 ± 0.03	26.5 ± 0.02	1.27 ± 0.01
Negative control [basal medium]	6.7 ± 0.01	22.9 ± 0.03	0.72 ± 0.03
Positive control [basal medium + rapeseed oil (50 g/l)]	6.3 ± 0.02	30.5 ± 0.02	2.68 ± 0.02

Table 5 Effect of oil as a main carbon source on erythromycin production by *S. erythraea* NUR001. Values are averages ± SD of three triplicate experiments

Main carbon source	pH	Biomass (g/l)	Erythromycin (g/l)
Dextrin (70 g/l)	7.7 ± 0.2	23.4 ± 0.2	1.3 ± 0.03
Rapeseed oil (50 g/l)	7.2 ± 0.3	22.1 ± 0.1	2.7 ± 0.04
Rapeseed oil (75 g/l)	7.5 ± 0.1	19.5 ± 0.2	2.1 ± 0.02
Rapeseed oil (50 g/l) + dextrin (70 g/l)	7.2 ± 0.2	27.7 ± 0.2	3.7 ± 0.02

Rapeseed oil had a significant effect on the production of erythromycin, as indicated recently [9, 22], but its beneficial effect was not as high as that of melon seed oil. Growth of *S. erythraea* NUR001 was higher in rapeseed oil-containing medium and the utilization of this oil was the greatest, when compared with that of other oils, thus giving more erythromycin production. The consumption rate of rapeseed oil was the greatest, when compared with that of other oils used.

It was shown that soybean oil had a beneficial effect on the production of various antibiotics, such as tetracycline [15], cephamycin [24] and an immunotolerant [16]. We have shown that walnut and soybean oils were relatively acceptable for erythromycin production.

Black cherry kernel oil containing 77% linoleic acid is used in the preparation of salads and cosmetics in the United States [19] and, in Iran, it is used in the preparation of drugs in traditional medicine. So far, there has been no report of its biotechnological application. For the first time, it was shown that black cherry kernel oil as a waste product from fruit juice-preparing factories could be used as a stimulant in the production of erythromycin [11].

Although we recognized the role of the oils used as a supplement for energy/ carbon sources, this may not be the only factor governing erythromycin production. The results obtained were helpful in understanding the effects of various oils on erythromycin production, but with the data available at present, we are not able to pinpoint a specific component of the oils used as a main factor affecting antibiotic production. Further investigations are needed, using various combinations of the fatty acids and other components of the oils, in order to find out which factor (or factors) is the most effective and how it operates.

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