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Vegetable oils in fermentation: beneficial effects of low-level supplementation

AM Jones and MA Porter

Central Research, Cargill Inc, Minneapolis, MN 55440, USA

To evaluate their potential to enhance fermentation performance, vegetable oils were investigated in a model tetracycline fermentation. With sucrose as the carbon source, the fermentation efficiency of *Streptomyces aureofaciens* (ATCC 10762) was enhanced by the inclusion in the medium of low levels of vegetable oil. Soybean and sunflower oils significantly improved the rate of sucrose consumption and tetracycline production suggesting that oil is an excellent adjuvant for improving fermentation productivity. For optimum benefit, the dosage level was critical. Little difference was observed between crude and refined oils. These data contribute to the assessment of industrially available fermentation feedstocks, and to the development of new feedstock products for specific fermentation applications.

Keywords: fermentation; vegetable oils; tetracycline; *Streptomyces aureofaciens*

Introduction

For the development of an effective and efficient fermentation process, design of the fermentation medium is critical. This is because the fermentation medium affects the product yield and the volumetric productivity, as well as the process economics. Microorganisms require carbon, nitrogen, and minerals for growth and metabolism. Lipids and oils are considered essential components of many fermentation media since they possess defoaming properties as well as serve as a supplemental nutrient source for growth and maintenance of the microbial cells. Numerous examples of the successful use of vegetable oils as carbon sources are cited in the literature [1–4,6–13,15]. Early work showed that lard and soybean oils increased production of penicillin nearly 50% when used to replace or supplement standard sugars. Similar observations have since been reported for other antibiotic fermentations. Important to achieving such results are the timing of oil addition and the dosage level. Too much oil or oil supplied at the wrong time could have negative effects. For example, Anderson *et al* [1] reported optimum results when oil was added frequently during fermentation, but was not allowed to accumulate in the medium. Conversely, as illustrated by the study of Ohta *et al* [8], other fermentations benefit from using oil as a sole carbon source.

However, few studies have directly compared oil types. For the production of cephamycin C, Park *et al* [11] reported that while a variety of vegetable oils were suitable as sole sources of carbon, soybean oil supported 16% higher productivity than did methyl oleate, or cottonseed or corn oils. Olive, rapeseed, and peanut oils supported nearly one third less antibiotic production. Sesame [12], soybean, lard, peanut, and linseed oils and methyl oleate [9] were all effective as carbon sources for the production of cephalosporin C.

In addition to its role as an energy-containing nutrient, oil added to a fermentation medium can provide other benefits. For instance, oil-soluble products such as josamycin [6] can partition into the oil phase during fermentation. This effectively removes the product from the aqueous medium thereby reducing repression of its synthesis, and increasing product yield. A second, incidental benefit is the suppression of foaming. There is a long history of oils and oil-like materials being used to suppress foam formation in fermentation processes.

Since industrial fermentations use a complex mix of feedstocks, the current study was undertaken to assess the ability of various vegetable oils to enhance fermentation productivity. For this purpose, the production of tetracycline by *Streptomyces aureofaciens* was chosen as a model fermentation, and the effect of adding a variety of vegetable oils as a feedstock supplement on fermentation productivity and yield was studied.

Materials and methods

Oils

Vegetable oil samples were obtained through Cargill Refined Oils (Minneapolis, MN, USA). These included crude and refined oils derived from soybean, canola, and sunflower.

Model tetracycline fermentation

Streptomyces aureofaciens (ATCC 10762) was obtained from the American Type Culture Collection, Rockville, MD, USA. It was allowed to sporulate on a sporulation medium consisting of (g L^{-1}): yeast extract 1.0, beef extract 1.0, tryptose 2.0, $FeSO_4$ 0.01, glucose 10.0, agar 15.0, pH 7.2. Agar plates were incubated at 30°C for 14 days at which point the culture was well sporulated. To prepare a vegetative mycelium suspension, a loopful of the sporulated culture was transferred into 50 ml of sporulation broth in

Correspondence: Dr AM Jones, Cargill Inc, 1 Cargill Drive, Eddyville, IA 52553–5000, USA
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a 250-ml Erlenmeyer flask, and incubated at 30°C (230 rpm) for 2–4 days. An aliquot (35 ml) of this culture was centrifuged (2000 × *g*; 10 min), and the pellet was resuspended in 30 ml of fresh sporulation broth containing 20% (v/v) glycerol. The resulting mycelium/glycerol suspension was dispensed in 1.3-ml volumes into sterile culture vials, and stored frozen at –70°C. To provide a standard inoculum, one vial was used to inoculate each seed culture flask as described below. Composition of the seed and fermentation media developed for the model tetracycline fermentation are detailed in Table 1.

Typically, seed cultures were prepared in 250-ml shake flasks each containing 50 ml of seed medium and inoculated with a *S. aureofaciens* glycerol stock culture. The flasks were incubated at 30°C on a rotary shaker (230 rpm) for 24–72 h, or until the pH dropped to 4.5–5.0. In this pH range the seed cultures were harvested [5]. Fermentations were conducted in 500-ml baffled shake flasks each containing 95 ml of fermentation medium, and inoculated with 5.0 ml (5% v/v) of washed seed culture. The fermentation flasks were incubated at 30°C on a rotary shaker at 230 rpm, and sampled daily for sucrose, biomass, pH, and tetracycline as described below.

Analytical

Sucrose was estimated using a YSI Select Biochemistry Analyzer (Model 2700) (Midwest Scientific Inc, Minneapolis, MN, USA). Clear supernatants filtered through 0.45-μm nylon filters were analyzed in 1-ml volumes using 5.0 g L^{-1} or 25.0 g L^{-1} sucrose (Sigma Chemical Co, St Louis, MO, USA) solutions as calibration standards.

Because the fermentation medium used for tetracycline production contained particulate matter in the form of soybean meal, the mycelial mass could not be determined accurately. Growth of *S. aureofaciens* was therefore *estimated* at each sampling time during the tetracycline fermentations. For this, a 5.0-ml aliquot of culture fluid was removed from each flask to a graduated centrifuge tube, and the pH recorded. To extract the tetracycline, the broth was acidified to pH 1.7 with concentrated HCl. All tubes were centrifuged (2000 × *g*; 10 min); after transferring the resulting supernatant broth to a fresh tube for tetracycline determination, the volume of the pellet was noted. Mycelium biomass was estimated by the packed bed volume (ml pellet ml^{-1} whole culture), and expressed as % v/v.

Tetracycline was measured by reverse phase HPLC [14]. Clear supernatants (through 0.45-μm filter) collected as above were diluted as appropriate with 0.01 M phosphate buffer (pH 2.2). Samples were injected into a Microsorb MV C_{18} column (Rainin Instruments Co, Woburn, MA, USA) maintained at room temperature. Tetracycline was eluted from the column with a mixture of methanol: acetonitrile:H_2O:0.2 M phosphate buffer (50:20:20:10) at pH 2.5, and a flow rate of 0.6 ml min^{-1}. Tetracycline was detected at 280 nm using a Model 486 variable UV wavelength detector (Waters Chromatography, Milford, MA, USA).

Within each experiment, tetracycline hydrochloride (Sigma) was used to generate a standard curve (0–100 μg ml^{-1}). Using the standard curve, sample peak areas were then converted to tetracycline concentrations, taking into account the appropriate dilution factor. *Streptomyces aureofaciens* is also capable of producing chlorotetracycline if Cl^- ions are present in the fermentation medium. Therefore, to verify that tetracycline was indeed the sole product of this fermentation, a chlorotetracycline (Sigma) reference standard was also run (data not shown). Data shown represent the average of at least duplicate flasks.

Medium costs

The cost of the simple medium described above was approximated using current (1996) price estimates. Table 2 describes the assumptions and calculated values. Based on these estimates, the base price of the medium used in these experiments was $0.022 per liter. This value can be used to compare the cost of the 'control' medium to that of any of the experimental media supplemented with vegetable oil. For these purposes, the oil prices as detailed in Table 3 can be used.

Table 1 Composition of seed and fermentation media for tetracycline production

Seed medium[a]		Fermentation medium[b]	
Component	g L^{-1}	Component	g L^{-1}
Sucrose	30	Sucrose	27
Soybean meal	5	$ZnSO_4 \cdot 7H_2O$	0.03
Sodium citrate	1	$(NH_4)_2SO_4$	4
$(NH_4)_2SO_4$	3.3	$CaCO_3$[c]	4
$MgSO_4 \cdot 7H_2O$	0.25	Soybean meal	7
KH_2PO_4	0.1	Oil[d]	–
K_2HPO_4	0.1		
$CaCO_3$	1		
$MnSO_4 \cdot 7H_2O$	0.0001		
$ZnSO_4 \cdot 7H_2O$	0.04		
K_2CrO_7	0.016 mg		
Acetic acid	0.4 ml		

[a]Initial pH = 7.8. From Darken *et al* [5].
[b]Prepared with tap water. The pH was adjusted to 6.0 with H_2SO_4.
[c]Dried at 150°C for 2 h.
[d]Oil added on a weight-by-volume basis to some final concentration as appropriate.

Results and discussion

In this study, each fermentation trial was monitored for: tetracycline titer, residual sucrose in the medium, biomass,

Table 2 Calculated cost of the basal fermentation medium used in this study

Ingredient	g L^{-1}	lbs per 1000 L	$ per lb of ingredient[a]	Ingredient cost ($ per 1000 L)
Sucrose	27	59.4	0.32	19.01
Ammonium sulfate	4	8.8	0.02	0.18
Soybean meal	7	15.4	0.13	2.03
Calcium carbonate	4	8.8	0.05	0.39
Zinc sulfate	0.03	0.066	15.34	1.01
Total				22.62

[a]As obtained in 1996.

Table 3 Cost[a] of the various oils used in this study

Oil type	Cost ($ per lb)
Crude soybean	0.25
Refined soybean	0.30
Crude Trisun (sunflower)	0.50
IMC-130 (canola)	0.47

[a]As obtained in 1996.

and pH. Commercial industrial fermentations are concerned with the economics of titer, yield and production rate. Thus, an alternate feedstock is valuable if it: (i) increases the total product titer at similar cost; (ii) decreases the time required to obtain a certain titer; and/or (iii) decreases the cost of obtaining a certain titer. All experiments performed in this study were analyzed with the intent of finding one or more of these benefits. The other measures were used only to explain significant differences between treatments.

Effect of vegetable oils on fermentation

A typical profile for the model tetracycline fermentation using sucrose (30 g L^{-1}) as the sole carbon source is shown in Figure 1. The production of tetracycline by *S. aureofaciens* began after a short lag and continued as long as sucrose was present in significant amounts. Production effectively ended after approximately 100 h, at which point a tetracycline concentration of 3000 μg ml^{-1} was routinely observed (Figure 1). Supplementation of the sucrose with 0.05% crude soybean oil did not increase the ultimate tetracycline titer significantly, but appeared to decrease the time required to achieve the maximum level by 50 h (Figure 1). This represents a 40% improvement in productivity. When provided in the fermentation medium at a concentration of 30 g L^{-1}, the cost of the sucrose is about $0.021 per liter (30 g sucrose at $0.32 per lb). Likewise the cost of the soybean oil supplement would be $0.00027 per liter (0.5 g crude oil at $0.25 per lb). Therefore, supplementing the fermentation medium with 0.05% soybean oil results in a significant increase in productivity at a mere additional cost of less than 1.2% of the total medium cost.

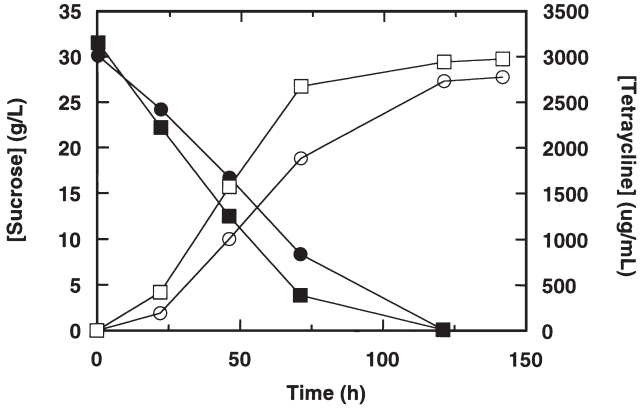

Figure 1 Fermentation of sucrose by *S. aureofaciens* in the absence (circles) or presence (squares) of 0.05% (w/v) crude soybean oil. Sucrose consumption (closed symbols) and tetracycline production (open symbols) were monitored.

Supplementation of the fermentation medium with this low level of vegetable oil did not decrease the rate of sucrose consumption, rather it stimulated it. As shown in Figure 1, at 75 h the oil-supplemented flasks contained only half the sucrose of those remaining unsupplemented. Stimulation of sucrose consumption by low levels of vegetable oil was consistently observed in these experiments, and suggests that oil is not replacing sucrose as a nutrient/energy source, but is functioning in some different role.

In this study, a soybean oil supplement of 0.05% had a strong positive effect on productivity, as measured by production rate—not final titer. This increased turnover is available at a cost of less than 2% of the sucrose cost. However, supplementation with a 10-fold higher concentration of soybean oil (0.5%) had a negative effect on both productivity and total production (data not shown). Moreover, in this model fermentation, a total replacement of the sucrose with soybean oil as the sole carbon and energy source led to even poorer results with only about 150 μg ml^{-1} tetracycline being produced. Similar results were obtained with other sources of oil, including canola and sunflower (data not shown).

Given that at 0.05% the rate of fermentation was enhanced but at a concentration of 0.5% it was inhibited, the effect of supplementing the sucrose-based fermentation medium with a variety of crude vegetable oils at an intermediate concentration, 0.2%, was investigated. The results are shown in Figure 2. Compared to fermentations conducted in the presence of sucrose alone, soybean oil at 0.2% provided a significant advantage in terms of both productivity and maximum titer. Similar results were obtained with crude high-oleic sunflower oil (Trisun). In contrast, however, a lesser advantage was observed when IMC-130 canola oil was used as the oil supplement. The reason for this is not obvious, but is further evidence that when selecting feedstock products it is always best to test a wide variety so as to ensure that the optimum is identified for a given fermentation application.

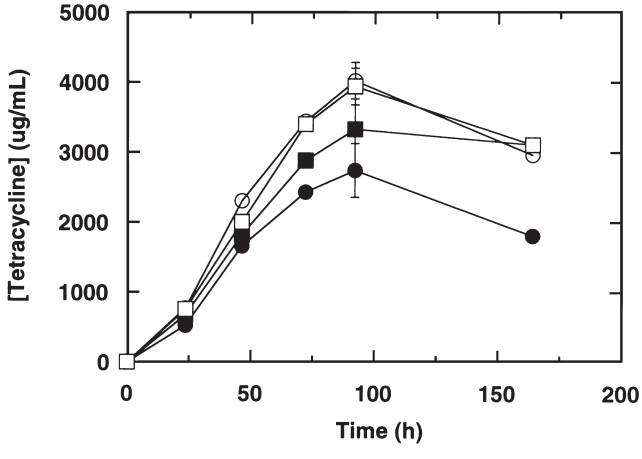

Figure 2 Effect of 0.2% (w/v) crude vegetable oil on the production of tetracycline from sucrose by *S. aureofaciens*. Various oils were tested including those derived from: sunflower (Trisun, open circles); canola (IMC-130, closed squares); and soybean (open squares). A control fermentation conducted in the absence of an oil supplement (closed circles) was also included.

In contrast to Figure 1 where only the time to maximum concentration increased with 0.05% oil addition, the data in Figure 2 show that 0.2% oil increased both the rate of production and the maximum concentration of tetracycline. Our experiments did not reveal a consistent relationship between oil type or concentration and improvement to titers or rates. Further experimentation is required to clarify this relationship.

In the presence of 0.2% soybean or sunflower oil, the maximum tetracycline titer was 33% higher than with sucrose alone. At this concentration, the additional cost of adding the oil to the fermentation medium represents \$0.0011 per liter for soybean oil, and \$0.0022 per liter for Trisun sunflower oil. Therefore, at face value, a 33% increase in antibiotic titer is achieved with an additional 5% (soybean) to 10% (Trisun) increase in medium costs.

To this point all experiments were performed by adding the oil supplement to the fermentation medium prior to inoculation. Another strategy is to supply the oil upon depletion of the sucrose in the hope of prolonging the productive phase of the fermentation. However, in fermentations where oil was added to the medium upon complete utilization of the sucrose no further tetracycline production was observed (data not shown). Thus oil addition at this late stage in the fermentation is not worthwhile.

Taken together, these results suggest that soybean oil is a poor sole source of carbon for tetracycline production by this strain of *Streptomyces aureofaciens*, but an excellent adjuvant for improving productivity. Not all oils appear to be equally capable of boosting fermentation productivity, and the dosage as well as at what time during the fermentation process the oil supplement is added may be critical to achieving the maximum benefit. Higher doses may not be better than lower doses. Overall, productivity gains, whether measured as maximum titer or time to maximum titer, seem disproportionately large compared to the additional costs of the oil.

As suggested above, the ability of soybean oil to enhance fermentation productivity was dependent on its concentration. When profiles of fermentations conducted in the presence of soybean oil at 0–1% were compared, it is clear that there was an optimal oil dose—about 0.2% —that maximized both the rate of sucrose consumption (Figure 3a) and the tetracycline titer (Figure 3b). Concentrations lower or higher than this optimum were far less effective.

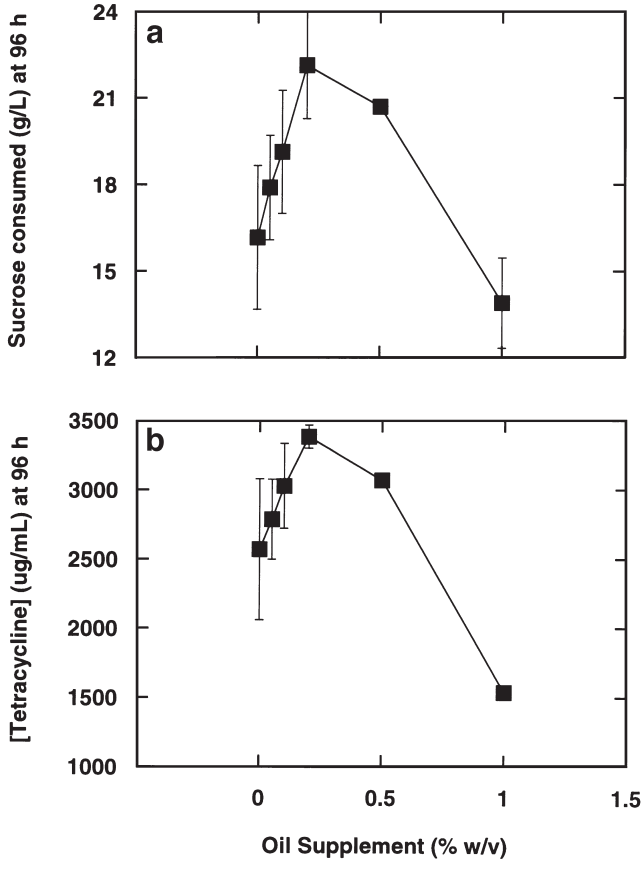

Figure 3 Effect of crude soybean oil dosage level on sucrose consumption (a) and tetracycline production (b) by *S. aureofaciens* at 96 h. The error bars indicate the 95% confidence interval around the mean.

Crude vs refined vegetable oil

The experiments described above were performed to evaluate the effect of small amounts of crude vegetable oils on fermentation performance. Some of the potential advantages of using crude oil as opposed to refined include the presence of: emulsifiers/surfactants (phospholipids); some nutrients (eg, iron, phosphorus, calcium); and antioxidants (tocopherols) in the crude oils. It is not clear how significant these compounds are to the fermenting microorganism. On the other hand, the biggest disadvantage to the use of crude oils is the presence of pesticides in the oil. Though present in low concentrations, pesticides could negatively affect microbial growth rates, especially in the critical early phase of cell growth. Therefore, despite their higher cost, refined oils are preferred to crude oils for some fermentation applications.

Using the model tetracycline fermentation, crude and refined soybean oils were compared to determine whether they could be distinguished in terms of their effect on either total tetracycline production or production rate. Both crude and refined oils performed similarly. Regardless of whether the soybean oil was refined or not, inclusion of a small amount in the fermentation medium resulted in a significantly higher tetracycline concentration than was obtained from sucrose alone. On average, the tetracycline titer at 100 h was 1.5-fold higher in the presence of oil than it was in its absence (data not shown).

Comparison of vegetable oils

While the above results serve to illustrate that small amounts of vegetable oil can significantly stimulate tetracycline production, there was not a direct focus on comparing oils from different sources. Therefore, a series of experiments was performed to compare crude and refined oils derived from various sources for their ability to enhance tetracycline production from sucrose by *S. aureofaciens*. A comparison of various crude oils from different origins is illustrated by Figures 1 and 2. Supplementing the basal fermentation medium with refined soybean, sunflower (Trisun), and canola (IMC-130) oils to a final concentration of 0.2% yielded similar tetracycline concentrations at 96 h

(data not shown). Although refined sunflower oil (Trisun) appeared to provide slightly greater benefits, its higher cost would require consideration in assessing its overall cost-effectiveness for improving fermentation yield.

Conclusions

Vegetable oils significantly improved the rates of sucrose consumption and tetracycline production by *S. aureofaciens* when provided at low levels in the fermentation medium. However, total replacement of the sucrose, or even significant substitution of the sucrose, did not seem to provide an equivalent or enhanced benefit. Rather, the oil supplement seemed to increase the rate of sucrose consumption. Vegetable oils thus appear to be excellent adjuvants for improving fermentation efficiency. Possible modes of action include: improved oxygen uptake by the medium through interfacial effects; increased tetracycline production by extracting the antibiotic into the oily phase of the culture broth thereby removing it from the aqueous phase; and decreased damage to microbial cells by foam formation. That the oils provide additional nutrients to the fermentation broth appears to be a less likely explanation. Finally, experiments using a tetracycline model fermentation suggested little direct benefit to using refined over crude oil, or to using any particular oil source. It is not known which, if any, of the possible mechanisms of action proposed for the oils in this application is responsible for the beneficial effects observed. However, none would likely depend on the type or source of oil employed.

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