



## Optimization of Media and Reaction Conditions for Production of Polyol Oils from Soybean Oil by *Pseudomonas aeruginosa* E03-12 NRRL B-59991<sup>☆</sup>

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### ABSTRACT

Soy-polyol oils (oxygenated acylglycerols) are important starting materials for the manufacture of polymers such as polyurethane. We reported methods for microbial screening and production of polyol oils from soybean oil through bioprocessing (Hou and Lin, 2013). Using this screening method, we screened 650 cultures isolated and found 50 cultures were positive for converting soybean oil to polyol oil. The two most active of these positive cultures are *Acinetobacter haemolyticus* A01-35 and *Pseudomonas aeruginosa* E03-12 (Hou et al., 2015). The polyol oil produced by strain A01-35 was a mixture of hydroxy fatty acids-containing DAG. Strain E03-12 produced more polyol oil than strain A01-35 and its product contains both hydroxy fatty acids-containing DAG and TAG. We employed one-factor-at-a-time method to develop an optimized culture medium composition and reaction conditions for polyol oils production from soybean oil by strain E03-12. We found that among the different carbon sources studied, glucose at 12.5 g/L is the best. A combination of both tryptone at 12.5 g/L and yeast extract at 10 g/L serves as the best nitrogen source. Both iron and magnesium were studied for the effect of metal on the production of polyol oils. MgSO<sub>4</sub> 7H<sub>2</sub>O at 0.75 g/L and FeSO<sub>4</sub> 7H<sub>2</sub>O at 0.30 g/L produced the best result. Using this optimum medium composition, the best pH for the polyol oils production was found at 6.8. The optimum temperature for polyol oil production is 28 C. The optimum substrate concentration is 183 mg/30 mL. In the time course studies with these optimum reaction conditions, the best yield is 18.5 mg/30 mL at 48 h. Longer incubation time leads to the decrease in product polyol oils and increase in DAGs. The information obtained from this study is important for developing scale up production of polyol oils directly from soybean oil.

### 1. Introduction

Triacylglycerols (TAG) containing hydroxy fatty acids (FA), such as the ricinoleic acid, have many industrial uses (e.g., manufacture of lubricants, polymers, and coatings), because of the presence of the hydroxyl groups provides a functional group for performing a variety of chemical reactions. Castor oil is the only commercial source of ricinoleic acid and the presence of toxic component ricin presents a challenge with production scale-up. Polyol oils from soy oil have been used in the manufacture of polymers such as polyurethane, but this first requires a two step chemical process involving epoxidation and the subsequent opening of the oxirane ring (Hamdy, 2006; Demosthenes, 2009).

Previously, we have established that microbial systems can convert FA to ricinoleic acid-type oxygenated FA, including many bioactive FA such as monohydroxy-, dihydroxy- and trihydroxy-unsaturated FA,

tetrahydrofuranly unsaturated FA, and diepoxy bicyclic unsaturated FA (Hou, 1994, 1995; Hou et al., 1998, 2001; Gardner et al., 2000; Iwasaki et al., 2002; Hosokawa et al., 2003a, 2003b, 2003c; Hou and Hosokawa, 2005; Chang et al., 2007; Su et al., 2011; Bae et al., 2010). However, the biobased polymer industry requires acylglycerol (soybean oil) polyol oils and not polyol FAs. Recently we developed a new method to screen microorganisms for the direct production of polyol oils from soybean oil (Hou and Lin, 2013). Using TLC- and HPLC-based screening method, we screened 650 isolated cultures and found 50 cultures could convert soybean oil to polyol oils (Hou and Lin, 2013). The two most active strains were identified as *Acinetobacter haemolyticus* A01-35 and *Pseudomonas aeruginosa* E03-12 (Hou et al., 2014). The polyol oil produced by *Acinetobacter haemolyticus* A01-35 (NRRL B-59985) was a mixture of 57 molecular species of DAG containing mono-, di-,tri-hydroxy FA and normal FA (Hou and Lin, 2013).

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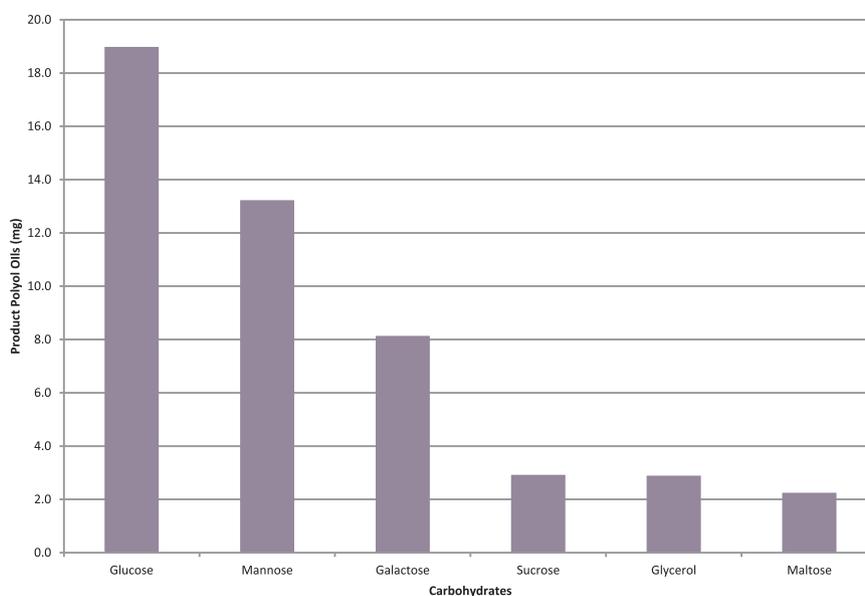


Fig. 1. Effect of carbon sources on polyol oils production from soybean oil by *Pseudomonas aeruginosa* E03-12.

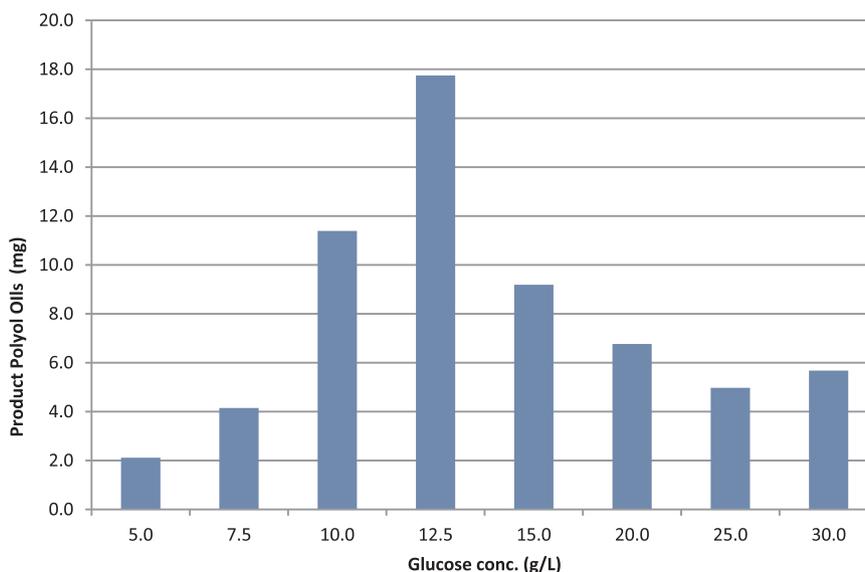


Fig. 2. Effect of glucose concentration on polyol oils production from soybean oil by *Pseudomonas aeruginosa* E03-12.

*Pseudomonas aeruginosa* E03-12 (NRRL B-59991) produced more polyol oil than strain A01-35 and its product contained both hydroxy fatty acids-containing DAG and TAG. Both hydroxy fatty acids containing DAG or TAG polyol oils can be used as starting materials for polymer industry. In the polyol oils produced by strain E03-12, we identified 41 molecular species of DAG, among them 32 molecular species containing one hydroxy FA and one normal FA, 8 molecular species containing two hydroxy FA without normal FA, and one molecular species containing two normal FA without hydroxy FA. The hydroxy FA included mono-, di- and tri-hydroxy FA. Eight molecular species of DAG contained one trihydroxy FA and 14 molecular species of DAG contained one dihydroxy FA. In addition, we also identified 64 molecular species of TAG, among them 13 molecular species containing two hydroxy FA, 42 molecular species containing one hydroxy FA and 9 molecular species containing no hydroxy FA (Hou et al., 2015).

Since strain E03-12 produces more polyol oils than strain A01-35, and unlike strain A1-35, it produced both DAG and TAG polyol oils, we selected strain E03-12 for further studies. The objective of this study is to find an optimum medium composition and reaction conditions for

the production of polyol oils (including hydroxy fatty acids-containing DAG and TAG) directly from soybean oil by strain E03-12. We employed a one-factor-at-a-time and statistical methods in this study. We found that among the different carbon sources studied, glucose at 12.5 g/L is the best. A combination of both trypton at 12.5 g/L and yeast extract at 10 g/L serves as the best nitrogen source.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.75 g/L and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  at 0.30 g/L produced the best result. The best pH for the polyol oils production was 6.8 and the best temperature was 28°C. The optimum substrate concentration was 183 mg/30 mL. In the time course studies, the best yield 18.5 mg/30 mL was found at 48 h. Longer incubation time leads to the decrease in product polyol oils and increase in DAGs due to the action of lipase. This information is important for developing scale up production of polyol oils directly from soybean oil.

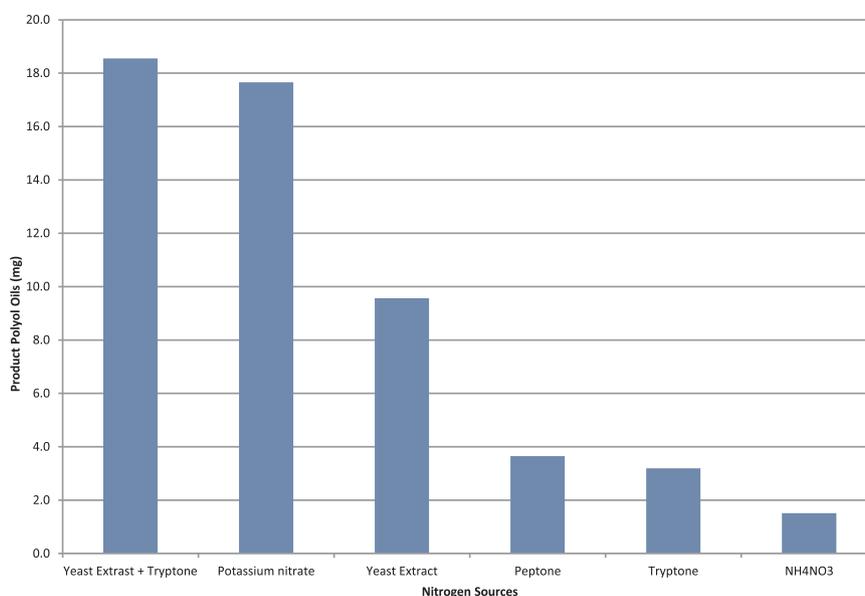


Fig. 3. Effect of nitrogen sources on polyol oils production from soybean oil by *Pseudomonas aeruginosa* E03-12.

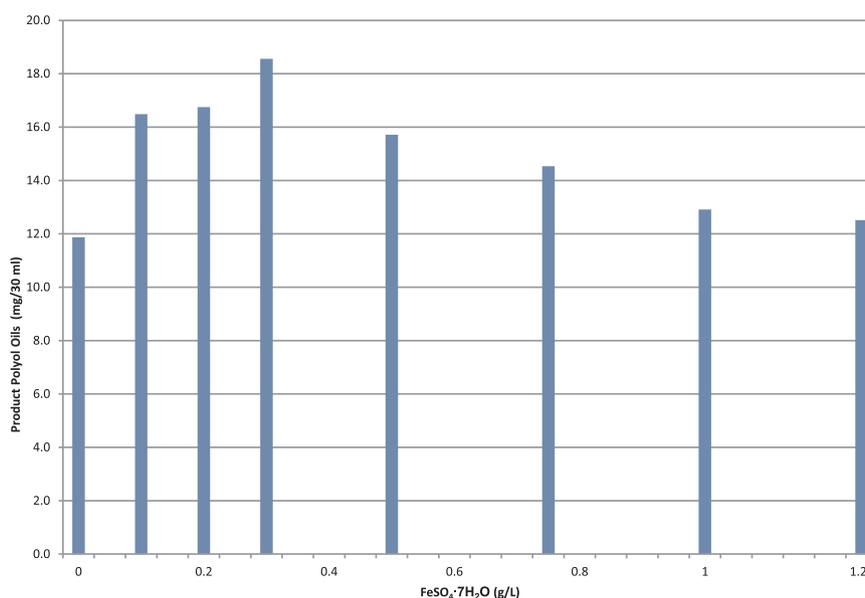


Fig. 4. Effect of iron concentration on polyol oils production from soybean oil by *Pseudomonas aeruginosa* E03-12.

## 2. Materials and methods

### 2.1. Materials

The FA and tricapyrylin used in this study were purchased from NU-Check-Prep Inc. (Elysian, MN, USA). Soybean oil, castor oil, yeast extract, all solvents and chemicals were purchased from Sigma (St. Louis, MO).

### 2.2. Microorganisms

*Pseudomonas aeruginosa* E03-12 NRRL B-59991 was isolated from soil and water samples collected from the vicinities of a biodiesel manufacturing plant (Rooney et al., 2009) in Ralston, Iowa, U.S.A. The culture was identified and deposited in the USDA Culture Collection, Peoria, IL (Hou et al., 2014). The culture was aerobically grown at 28 °C with orbital shaking at 200 rpm in culture medium used in our previous research (Hou and Lin, 2013).

### 2.3. Bioconversion

Culture was grown in 10 mL medium (Hou and Lin, 2013) and incubated at 28 °C with orbital shaking at 200 rpm for 24 h as seed culture. A 0.3 mL of this seed culture was inoculated into 125 mL flasks with a working volume of 30 mL culture medium and cells were allowed to grow for 24 h before 183 mg substrate soybean oil was added. The flasks were incubated for an additional 2 days for the bioconversion reaction. At the end of incubation, the culture was acidified to pH 2 by adding 1200 µL 6 N HCl. 100 µL of a 100 mg/mL solution of tricapyrylin as internal standard was added and the culture was extracted twice with 75 mL ethyl acetate and the solvent was dried.

### 2.4. HPLC Analysis

The crude extract was dried under vacuum and transferred to a vial after dissolving in 4 mL chloroform/methanol (2:1). A 800 µL aliquot of this solution was removed, and then placed in an HPLC vial, dried

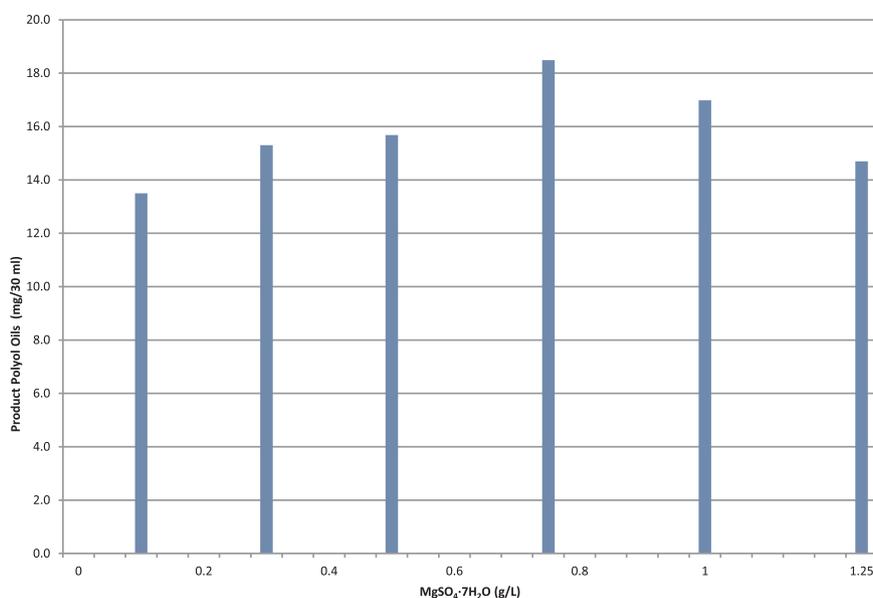


Fig. 5. Effect of magnesium concentration on polyol oils production from soybean oil by *Pseudomonas aeruginosa* E03-12.

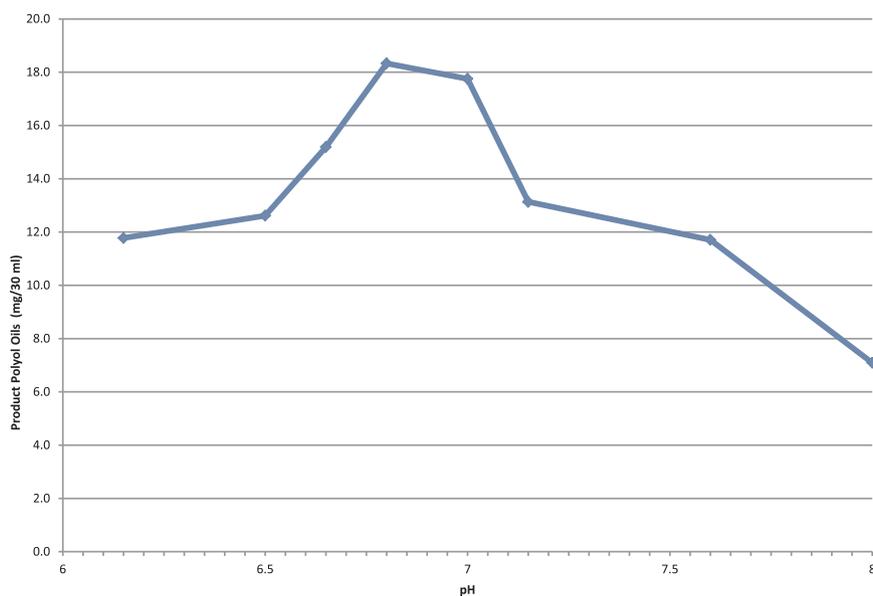


Fig. 6. Effect of pH on polyol oils production from soybean oil by *Pseudomonas aeruginosa* E03-12.

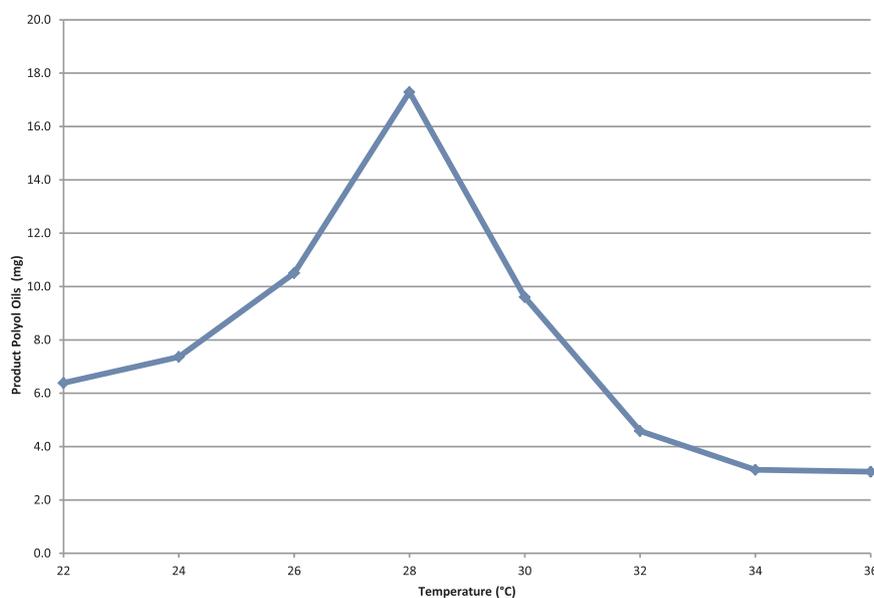
down, and then dissolved in 1 mL of a 1:1 methanol: 2-propanol solution. A 1  $\mu$ L of sample was injected. All samples, including 10 mg/mL soybean oil as standard at the beginning and the end (to check if the HPLC functions correctly), were run on a Shimadzu model SCL-10A HPLC equipped with a SPD-M10A Diode Array Detector and a SIL-10AF Auto Injector (Columbia MO). A linear gradient starting with 100% methanol going to 100% 2-propanol over 35 min at 1 mL/min flow rate was used with a Phenomenex 25 cm x 4.5 mm, 5  $\mu$  C18 reversed phase column. Detection was monitored using evaporative light scattering detector (ELSD) (MK III, Alltech Associates, Deerfield, IL, USA). The drift tube temperature of the ELSD was set at 75  $^{\circ}$ C. The nitrogen gas flow of the nebulizer of the ELSD was set at 1.0 L/min. The nitrogen pressure on the regulator of the nitrogen tank was set at 65 p.s.i. The hydroxy DAGs and hydroxy TAGs can only be identified by HPLC/MS, therefore the product polyol oils reported here by HPLC analysis includes both hydroxy fatty acids-containing DAG and TAG.

### 3. Results and discussion

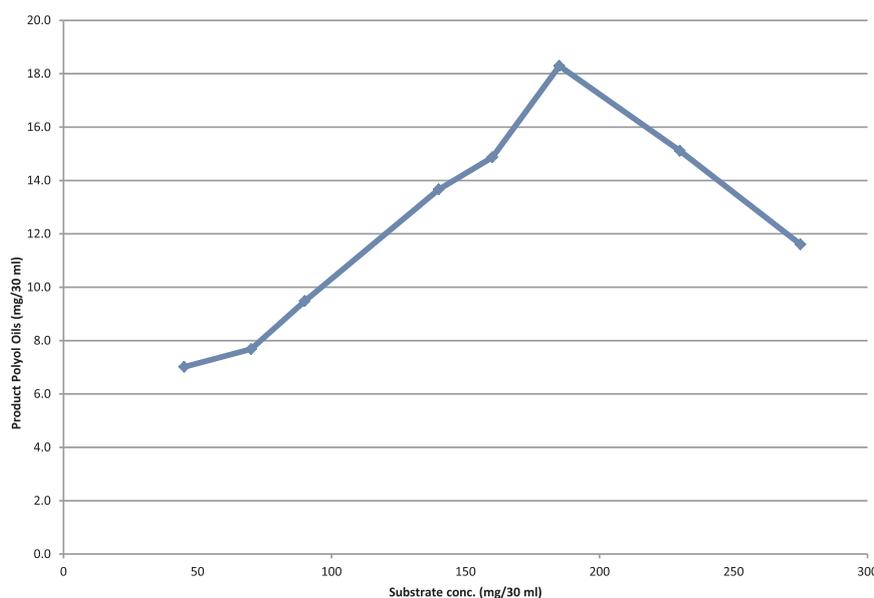
Since our screening medium composition (Hou and Lin, 2013) lead us to identify many positive polyol oils production strains, we used this medium as the starting culture medium to study optimum medium compositions for poly oil oils production. All experiments were performed in triplicates. The average of these triplicates was used. The product yield shown in Y axes in the figures is mg/30 mL.

#### 3.1. Culture medium optimization

Glucose (10.0 g/L), mannose (10.0 g/L), galactose (10.0 g/L), sucrose (10.0 g/L), glycerol (10.0 g/L) and maltose (10.0 g/L) were used to study the effect of carbon sources. The cell density after growth on different carbon sources was not adjusted. It was counted as advantage or disadvantage of that carbon sources that the cells grew on that specific carbon source. As shown in Fig. 1, glucose is the best carbon source for the production of polyol oils. Then we studied the optimum



**Fig. 7.** Effect of temperature on polyol oils production from soybean oil by *Pseudomonas aeruginosa* E03-12.



**Fig. 8.** Effect of substrate concentration on polyol oils production from soybean oil by *Pseudomonas aeruginosa* E03-12.

glucose concentrations for this reaction from 5.0 g/L to 30 g/L. Results are shown in Fig. 2. Glucose at 12.5 g/L is the best for polyol oil production. Higher than that concentration, the polyol oil product decreased and the amount of DAGs increased due to lipase action. Therefore 12.5 g/L of glucose was used for the following studies.

The effect of nitrogen sources was studied with 10 g/L each of ammonium nitrate, tryptone, peptone, yeast extract, potassium nitrate, or a combination of yeast extract (10 g/L) + tryptone (12.5 g/L). The polyol oil products produced from the use of either potassium nitrate or the combination of both tryptone and yeast extract were quite close, 5.5%. For convenience, we select to use the combination of yeast extract and tryptone for further studies (Fig. 3).

The effect of metals on the production of polyol oils was studied. The trace metals used in our screening medium did not result in a significant change in polyol oils production. However, since iron and magnesium are known to be involved in many enzyme reactions, we studied the effect of different concentration of these two metals on the production of polyol oils from soybean oil by strain E03-12. Fig. 4

shows the effect of iron concentration on polyol oils production from soybean oil by *Pseudomonas aeruginosa* E03-12. It was found that  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  at a range of 0.10–0.30 g/L concentration in culture medium produced around the same amount of product polyol oil. We selected the 0.30 g/L of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  for our further studies. The effect of different concentrations of magnesium in the culture medium on the production of polyol oils from soybean oil by strain E03-12 is shown in Fig. 5. The polyol oil production from either 0.75 g/L or 1.0 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  are about the same within the standard deviation of 5.9%. The 0.75 g/L of magnesium sulfate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) was selected for use in further studies.

Therefore, the optimum medium composition for the production of polyol oils from soybean oil by strain E03-12 is (final volume 1 L): glucose 12.5 g/L; yeast extract 10 g/L; tryptone 12.5 g/L;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.30 g/L;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.75 g/L;  $\text{K}_2\text{HPO}_4$  1.5 g/L; and  $\text{KH}_2\text{PO}_4$  3.5 g/L, adjust pH to 6.8.

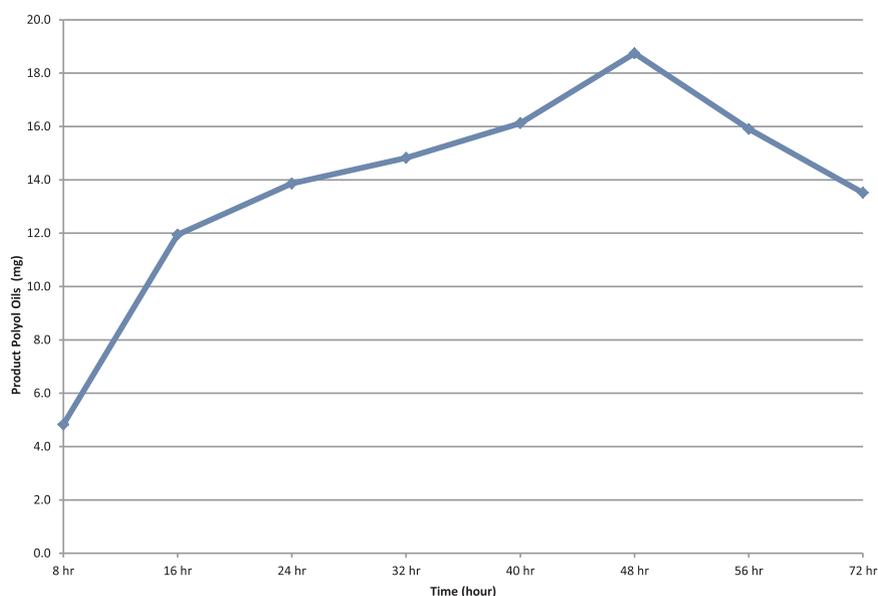


Fig. 9. Time course of polyol oils production from soybean oil by *Pseudomonas aeruginosa* E03-12.

### 3.2. Optimum reaction conditions

The best culture medium composition obtained above was used to study the optimum reaction conditions for the production of polyol oils.

### 3.3. Optimum pH

Eight cultures made with 8 different pHs from pH 6.17 to 7.96 (adjusted with 6N HCL or 6N NaOH to desired pH) in triplicates were studied for the effect of pH on the production of polyol oils by strain E03-12. Fig. 6 shows that the product polyol oil produced at either pH 6.81 or pH 6.99 are about the same within the standard deviation of 6.4%. We selected pH 6.8 to use in our further studies.

### 3.4. Optimum temperature

The cultures were grown at 28 C before adding the substrate. The reaction was conducted at different temperatures indicated. The effect of temperature on the production of polyol oils from soybean oil by E03-12 is shown in Fig. 7. The polyol oil yield among the three triplicates at each temperature point varied too much. We selected the average of the closest two points to prepare this figure. At higher temperature, strain E03-12 also produces more DAG in addition to the product polyol oil. However, the trend of the effect of temperature on the polyol oil production by strain E0-12 is clearly at around 28 C. Therefore, we select 28 C for our further studies.

### 3.5. Optimum substrate concentration

The effect of substrate concentrations was studied from 91 mg/30 mL to 550 mg/30 mL. The standard deviation was 5.9%. It was found that the best substrate concentration for this reaction was 183.4 mg/30 mL (Fig. 8).

### 3.6. Time course of the production

The best culture medium composition and the best reaction conditions obtained above were used to study the time course of the reaction. As shown in Fig. 9, the time course for the production of polyol oils from soybean oil by E03-12 was after 48 h of reaction. Longer incubation time lead to a decrease in the product polyol oils and an increase in DAGs due to the action of lipase. This indicates that lipase in strain E03-

12 further catalyzes the conversion of both TAGs and hydroxy fatty acids-containing TAGs to DAGs and hydroxy fatty acids-containing DAGs.

These optimum culture medium composition and optimum reaction conditions obtained will be used in the scale-up production of polyol oils from soybean oil by strain E03-12.

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