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Original Research Paper

Identification of molecular species of polyol oils produced from soybean oil by *Pseudomonas aeruginosa* E03-12 NRRL B-59991 ☆

Ching T. Hou [a,*], Jiann-Tsyh Lin [b], Karen Ray [a]

[a] Renewable Product Technology Research Unit, National Center for Agricultural Utilization Research, ARS, USDA, 1815 N. University Street, Peoria, IL 61604, USA

[b] Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, CA 94710, USA

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ABSTRACT

The objective of this study is to identify the chemical species of the polyol oil products produced from soybean oil by *Pseudomonas aeruginosa* E03-12 NRRL B-59991. We reported earlier the polyol products produced from soybean oil by *Acinetobacter haemolyticus* A01-35 (NRRL B-59985) (Hou and Lin, 2013). The polyol oil produced by strain A01-35 were a mixture of 57 molecular species of DAG containing tri-, di-, mono-hydroxy FA and normal FA. In this study, tricaprylin was selected as internal standard for HPLC quantitative estimation of products. A CombiFlash chromatographic method was established for separation of the polyol oil products. The molecular species of the polyol oils produced from soybean oil by strain E03-12 were identified with HPLC/MS. We identified 41 derivatives 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 specie containing two normal FA without hydroxylated FA. The hydroxy FA included mono-, di- and tri-hydroxy FA. Eight molecular species of DAG containing one trihydroxy FA and 14 molecular species of DAG containing one dihydroxy FA. We have 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 hydroxylated FA. This is different from our previous findings with *A. haemolyticus* A01-35 which produced only DAG polyol oils. E03-12 is a better strain for developing into an industrial bioprocess.

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1. Introduction

Triacylglycerols (TAG) containing hydroxy fatty acids (FA), e.g., castor oil, have many industrial uses such as the manufacture of aviation lubricant, plastic, paint, nylons and cosmetics, because of the hydroxyl groups on the FA of TAG. Castor oil is the only commercial source of TAG containing hydroxy FA. Diacylglycerols (DAG) containing hydroxy FA can also be used in the above mentioned industries. DAGs are intermediates in the biosynthesis of TAG and phospholipids. Soy-polyols (oxygenated TAG) are important starting materials for the manufacture of polymers such as polyurethane. Currently, they are produced by two steps chemical process involving epoxidation and then the subsequent opening of the oxirane ring (Hamdy, 2006; Demosthenes, 2009).

We have been working on finding new uses and new materials from soybean oil through bioprocessing. Our previous research 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, tetrahydrofuranyl unsaturated FA, and diepoxy bicyclic unsaturated FA (Hou, 1994, 1995, 1997; 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) polyols and not FA polyols. Recently we developed a new method to screen microorganisms for the direct production of polyol oils from soybean oil (Hou and Lin, 2013). We found that TLC with two step solvent system development or HPLC with a C18 reversed phase column and a linear gradient of 100% methanol to 100% 2-propanol could separate the polyol oils products from the soybean oil substrate. We discovered new DAGs with normal fatty acids among our soybean oil bioconversion products (Lin et al., 2013a). The top 11 cultures from our screen

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* Corresponding author.
E-mail address: Ching.hou@ars.usda.gov (C.T. Hou).

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were identified (Hou et al., 2014) as bacteria belonging to either Pseudomonades or Acinetobacters. The objective of this study is to identify the chemical species of the polyol oil products produced from soybean oil by *Pseudomonas aeruginosa* E03-12 NRRL B-59991 and compare them with the polyol oils products produced from our previous study *Acinetobacter* strain. We found that *Pseudomonas* strain E03-12 produced triacylglycerol polyol oils; this is different from those produced by the *Acinetobacter* strain A01-35.

2. Materials and methods

2.1. Materials

The FA and tricaprylin 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

P. 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, USA. The culture was identification and deposited in the USDA Culture Collection, Peoria, Illinois (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

Starter 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 cultures. 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 150 μL substrate soybean oil were 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, a 100 μL of a 100 mg/ml solution of tricaprylin as internal standard was added and was extracted twice with 75 ml ethyl acetate and the solvent was dried.

2.4. HPLC

To establish internal standard for HPLC, we searched many potential HPLC internal standards including triacylglycerols with shorter acyl chain length, diacylglycerols with longer chain length, and hydroxy fatty acids, to avoid overlapping with substrate soybean oil and products polyol oils. we found that tricarprylin served well as an internal standard on HPLC analysis.

The crude extract after dried down on a Rotovap was transferred to vial using a total of 2 ml chloroform/methanol (2:1). An 800 μL aliquot was removed, and then placed in an HPLC vial, dried down, and then brought up in 1 ml 2-propanol. A 1 μL of sample was injected. All samples, including 10 mg/ml soybean oil as standard at the beginning and the end, were run on a Shimadzu model SCL-10A HPLC equipped with a SIL-10AF Auto Injector (Colombia MO). HPLC method was modified from our previous reports (Hou 1997; Lin et al., 2003). A linear gradient starting with 100% methanol going to 100% 2-propanol over 35 min at 1 mL/min flow rate was used for our operation with a Phenomenex 25 cm × 4.5 mm, 5 μ 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 °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 psi.

2.5. CombiFlash chromatography

Instead of using preparative TLC for products Purification as reported in our previous paper (Hou and Lin, 2013), in this study, we established a CombiFlash (Teledyne Lincoln, NE) chromatographic method for the Purification of many products. CombiFlash with a normal phase silica column was run with 100% hexane for 10 column volume (CV), rise slowly to 25% acetone in 40 CV, up to 80% acetone in 15 CV, hold for 15 CV, down to 0% acetone in 10 CV and then hold for 10 CV. the column was then rinsed with 1 L 2-propanol. ELSD was used for detection.

2.6. HPLC fractionation of the polyol oils fraction from CombiFlash

Chromatographic fractionation was performed using a Waters HPLC (Waters Associate, Milford, MA, USA) and a C_{18} analytical column (Gemini, 250 × 4.6 mm, 5 μ, C18, Phenomenex, Torrance, CA, USA). One milligram of the sample in ethanol (50 μL) was chromatographed at 22 °C (room temperature) with a linear gradient from 100% methanol to 100% 2-propanol in 40 min, at 1 mL/min flow rate, and detected at 205 nm. Fractions were collected every s and corresponding fractions were pooled from eight HPLC runs. HPLC fractions were used for MS studies. The final methanol solutions of samples were prepared for direct infusion into the mass spectrometer by combining one fourth of each HPLC fraction with 50 μL of a methanol solution of 100 mM lithium acetate and diluting to a total volume of 250 μL.

2.7. Electrospray ionization mass spectrometry (ESI-MS)

An LCQ Advantage ion-trap mass spectrometer (MS 2.0) with Xcalibur 2.0 SR2 software (ThermoFisher Scientific, San Jose, CA, USA) was used for MS analysis of the various molecular species of acylglycerols in HPLC fractions of the soybean oil bioconversion product. The infusion at 2.5 μL/min flow rate from a syringe (250 μL) pump produced stable singly charged lithiated parent ions (positive ions) which were subsequently fragmented for MS^2 analysis. ESI source conditions were as follows: sheath gas flow rate, 10 arbitrary units (au); aux/sweep gas flow rate, 0 au, spray voltage, 4 KV; capillary temperature, 200 °C; capillary voltage, 5 V; tube lens offset, 15 V. Scan conditions were as follows: isolation width, 1.0*m/z*, normalized collision energy, 27–42%; scan ranges, 100–2000*m/z*. Acquire time was 3 min.

3. Results and discussion

3.1. Production of products polyol oils from soybean oil by strain E03-12

1 mL of one-day old culture of *P. aeruginosa* E03-12 NRRL B-59991 was inoculated into 500 ml flasks each containing 100 ml of culture medium. After incubation for 24 h, a 0.5 mL of soybean oil was added and the incubation continued for additional two days at 28 °C 200 rpm. At the end of incubation, the flasks were combined and then acidified to pH 2 by adding 6 N HCl. The combined culture medium was extracted twice with equal volume of ethyl acetate and the solvent was dried with a rotary evaporator. A small sample of this crude extract was subjected to HPLC analysis. As shown in Fig. 1, polyol oils were produced and were separated nicely from internal standard and substrate soybean oil.