Biocatalysis and Agricultural Biotechnology 2 (2013) 1–6

Contents lists available at SciVerse ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Methods for microbial screening and production of polyol oils from soybean oil through bioprocess ☆

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ARTICLE INFO

Article history:
Received 2 August 2012
Received in revised form
17 September 2012
Accepted 18 September 2012
Available online 23 October 2012

Keywords:
Soypolyol oils
Soybean oil
Bioconversion
Hydroxy diacylglycerols
Hydroxy fatty acids
Oils separation

ABSTRACT

Soypolyol oils (oxygenated acylglycerols) are important starting materials for the manufacture of polymers such as polyurethane. Currently, they are produced by a two-step chemical process involving epoxidation and the subsequent opening of the oxirane ring. The objective of this study is to develop a bioprocess to produce polyol oils directly from soybean oil. For product separation, we found that TLC with a two step development solvent systems could separate the polyol products from substrate soybean oil. The products and substrates were separated in the following order: substrate triacylglycerols (R_f 0.8), free fatty acids (FA, R_f 0.7), product dihydroxy TAG (R_f 0.4), trihydroxy TAG (R_f 0.3), monohydroxy FA (R_f 0.1) and dihydroxy FA (R_f 0.05). We also found that HPLC with a C18 reverse phase column and a linear gradient of 100% methanol to 100% 2-propanol over 60 min at 1 mL/min flow rate was able to separate product polyol oils and substrate soybean oil. Free FA and polyol oils (diacylglycerols (DAG) containing hydroxyl FA) were eluted between 5 min and 15 min. DAG containing two normal FA was eluted between 15 min and 28 min and the substrate soybean oil was eluted between 36 min and 45 min. A total of 400 microbial cultures were screened and we identified 25 hits. Polyol oils' products were purified through a silica gel column chromatography, fractionated by HPLC and then analyzed by MS. A total of 57 molecular species of DAG containing tri-, di-, monohydroxy FA and normal FA were identified by MS. HPLC chromatogram of evaporative light scattering detector was used for semi-quantification of these DAG in the purified polyol oils. The total content of the DAG containing two normal FA was about 25% and the total content of DAG containing hydroxy FA might be about 75%. The yield of bioconversion by culture A01-35 from soybean oil to polyol oil products (DAG containing hydroxyl FA) plus DAG containing normal FA was 31% by weight. Bioconversion of soybean oil to polyol oils is a new research area without available methodology to follow. Here we report a new bioprocess for the production of soypolyol oils directly from soybean oil.

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1. Introduction

In the 2002–2003 growing season, 30.6 million tons of soybean oil were produced worldwide, constituting about half of worldwide edible vegetable oil production, and 30% of all fats and oils produced, including animal fats and oils derived from tropical plants (United States Department of Agriculture, 2004). The major use of this oil is for food products such as shortenings, salad and cooking oils, and margarines. Despite the growth in such markets as biodiesel and epoxidized oils, other markets continue to be challenged by more cost-competitive petroleum products. 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 constituents. 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. DAG is the intermediate molecules 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 a two-step 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 bioprocesses. Our previous research established that microbial systems can convert FA to ricinoleic acid-type oxygenated FA, including many bioactive FA such as

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1878-8181/$ - see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.bcab.2012.09.002

monohydroxy-, dihydroxy- and trihydroxy-unsaturated FA, tetrahydrofuranyl unsaturated FA, and diepoxy bicyclic unsaturated FA (Hou, 1994, 1995, 1997; Gardner et al., 2000; Hou et al., 1998, 2001; Iwasaki et al., 2002; Hosokawa et al., 2003a, 2003b, 2003c; Hou and Hosokawa, 2005; Chang et al., 2007; Suh et al., 2011; Bae et al., 2010). However the biobased polymer industry requires acylglycerol (soybean oil) polyols and not FA polyols. The objective of this study is to develop a new bioprocess for the production of polyol oils directly from the substrate soybean oil. Bioconversion of soybean oil to polyol oils is a new research area without available methodology to follow. Here we report a new microbial screening and product separation method, as well as a bioprocess for the bioconversion of soybean oil directly to polyol oils.

2. Materials and methods

2.1. Materials

The FA used in this study were purchased from NU-Check-Prep Inc. (Elysian, MN, USA). Soybean oil, castor oil, yeast extract, Silica gel 60 A 70–230 mesh, all solvents and chemicals were purchased from Sigma (St. Louis, MO). Thin-layer precoated Kieselgel 60F_{254} plates were obtained from EM Science (Cherry Hill, NJ).

2.2. Microorganisms

Microorganisms were isolated from soil and water samples collected from the vicinities of a biodiesel manufacturing plant (Rooney et al., 2009) in Ralston, Iowa, USA. Each culture was from a single colony on TGY agar plate and was pure from any contamination judging from microscopic observation. These cultures were aerobically grown at 28 °C with orbital shaking at 200 rpm in culture medium used in our previous research (Hou, 1995).

2.3. Bioconversion

Original flask cultures were grown in 3 mL medium (Hou, 1995) and incubated at 28 °C with orbital shaking at 200 rpm for 24 h as seed cultures. These seed (0.1 ml) cultures were inoculated into 50 ml flasks with a working volume of 10 ml culture medium and cells were allowed to grow for 24 h before 50 μL substrate soybean oil were added. The flasks were incubated for an additional 2–3 days for the bioconversion reaction. At the end of incubation, the culture was acidified to pH 2 by adding 400 μL 6 N HCl, and was extracted twice with 40 ml ethyl acetate and the solvent was dried.

2.4. TLC

TLC plates were developed with a two stage development procedure (Hou, 1997): (i) benzene/ether/ethyl acetate/acetic acid (80:10:10:1 v/v) was developed with the solvent was 8.5 cm above the origin and (ii) hexane/ether/formic acid (80:20:2 v/v) developed in the same direction to the top of the TLC plate. The plate was air-dried before the second development. After development, products on the plate were identified first by exposure to iodine vapor and then by spraying with 60% aqueous sulfuric acid and charring.

2.5. HPLC

The crude extract was dried under vacuum and transferred to a vial using a total of 2 ml chloroform/methanol (2:1). A 300 μL aliquot of this was removed, placed in an HPLC vial, dried, and redissolved in 1 ml of 2-propanol. A 5 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 SPD-M10A Diode Array Detector and 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 60 min at 1 mL/min flow rate was used for our operation with a Supelco 25 cm × 4.5 mm, 5 μ C18 reverse phase column. Detection was monitored at UV 205 nm.

2.6. HPLC fractionation of DAG in purified soybean oil bioconversion product

The fractionation of the molecular species of DAG in the purified soybean oil bioconversion product was as previously reported (Lin et al., 2003). 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 a 1 mL/min flow rate, and detected at 205 nm. Fractions were collected every 30 s and corresponding fractions were pooled from seven 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 half 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 utilized for MS analysis of the various molecular species of AG in HPLC fractions. The infusion at a 2.5 μL/min flow rate from a syringe (250 μL) pump produced stable singly charged lithiated parent ions which were subsequently fragmented for MS^2 and MS^3 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, 1.5*m*/*z*; normalized collision energy, 27–42%; scan ranges, 100–1500 *m*/*z*. Acquire time was 3 min.

2.8. LC–MS

Surveyor LC system (ThermoFisher Scientific) was used. Injection volume was 10 μL of ethanol solution containing 50 μg of the purified soybean oil bioconversion product for the autosampler. The LC conditions were the same as those of HPLC fractionation described. A flow splitter of 10:1 (Model 620, Analytical Scientific Instruments, El Sobrante, CA, USA) was used and 100 μL/min was introduced into the MS. Methanol solution of ammonium acetate (100 mM) was introduced to the LC flow through a T joint at 5.0 μL/min flow rate from a syringe (500 μL) pump. The MS of the combined flow was scanned without fragmentation (MS^1). ESI source conditions were as follows: sheath gas flow rate, 20 au; aux/sweep gas flow rate, 0 au; spray voltage, 4 kV; capillary temperature, 300 °C; capillary voltage, 5 V; tube lens offset, 15 V. By opening the result file of the LC–MS run in Qual Browser of Xcalibur software, chromatogram and MS spectrum (MS^1) were shown as two-cell arrangement on the computer screen. By selecting the peak (or any retention time) on chromatogram, MS^1 spectrum on that particular retention time was shown and the *m*/*z* values of the ammonium adducts of AG, $[M+NH_4]^+$, could be obtained.