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MICROBIAL PRODUCTION OF USEFUL FATTY ACIDS FROM USED SUNFLOWER OIL

Madiha A. Hassan and Saleh H. El-Sharkawy

Department of Pharmacognosy , Faculty of Pharmacy , University of Mansoura, Mansoura , Egypt.

ABSTRACT

Ninteen microorganisms have been evaluated for biotransformation of used sunflower oil to produce useful chemicals. Plamitic and stearic acids were produced by six microbes namely ; *Streptomycis paucisporogenes* ATCC 12596 *Streptomycis griseus var . cricensis* NRRL 3242, *Hansenula anomala* ATCC 20144, *Candida intermedia* ATCC, 12087, *Candida intermedia* ATCC, 5159 and *Nocardia restrictus* ATCC 14887.

Best yield was obtained with *Streptomycis paucisporogenes*. The produced fatty acids were isolated , purified by column chromatography and identified as their methyl esters by TLC, GC and by comparing their MS and NMR with authentic palmitic and stearic acids.

INTRODUCTION

There is a considerable interest in utilizing available natural resources such as cooking oils in the preparation of useful chemicals [1,2] . Plant oils are rich sources of many types of naturally occuring compounds, including mixtures of glycerides, fatty acids , glycerol, tocopherols and various sterols. About 80% of the world oil and fat production (70 million tons) is being used as food stuffs and 6% in animals feed [3]. However, of the overall production only 14% (about 10 million tons) is being consumed by the oleo- chemical industry as raw materials for lubricants, waxes and detergants [3,4] . Microorganisms have been used as catalytic reagents to oxidize various classes of hydrocarbons [5,6] .

Sunflower oil contains palmitic, stearic, arachidic, lignoseric, oleic and linoleic acids [7]. Experimental mutagenesis has succeeded in altering the plant genes for production of oleic acid rather than linoleic acid [3] . A number of vegetable oils and animal fats including olive, castor , corn , peanut, sesame, sperm oils and lard were used as a carbon source for the production of streptomycin, penicillin, glocerol, hydroxy fatty acids [5,6] and glucopyranoside[2]. *Rhizopus*, *Mycobacterium* and *Pseudomonus* species were used for the production of glycerol and fatty acids by lipolysis of differnt fats and oils. Cis- unsaturated fatty acids were stereospecifically hydrolysed by *Geotricum candidum* to the corresponding saturated fatty acids [8].

Oleic acid double bond was selectively hydrated by two *Nocardia* species and a *Mycobacterium* into 10-hydroxy, and 10-oxo-stearate (important components of grease and lubricant industries) in a high yield [1,9].

Many microorganisms grow on hydrocarbons as the only carbon source, and the hydrocarbon has to be transformed to the cell through the outer hydrophilic cell wall. This process is limited by the hydrophobic character of the oil or the hydrocarbon. Microorganisms such as *Torulopsis* and *Pseudomonus aerugenosa* have been utilized for the production of sphorolipids [10] and *rhamnolipids* [11] by emulsifying the hydrocarbon in the growth medium. Lipopolysaccharides (non- ionic surfactants) were also produced by *Candida*, *Rhodococcus*, *Mycobacterium* and *Arthrobacter species* [12]. Cellobiolipids (anionic extracellular surfactants) were produced by *Mystilago mydis* and *M. zeau*. Surfactin, subtilisin, lipoprotein and other surfactants were obtained from *Bacillus subtilis*, *Steptomycis sioyaensis* and *Pseudomonus aerugenosa* [13] . This report deals with the use of microorganisms to grow on the abundantly available , valueless , used (cooked) sunflower oil for the production of useful chemicals and pharmaceuticals .

EXPERIMENTAL

Chemicals - Nutrient agar , Nutrient broth (Winlab, 13% in dist. H_2O) , Silica gel GF Art. 7730 (Merck) for TLC , Silica gel (BDH) 60-120 mesh for column chromatography. All solvents used for extraction and purifcation were of analytical grade (El-Nasr Co).

Instuments - Melting points were determined on a Fisher- Johns melting point apparatus ^1H and ^{13}C NMR spectra were run on a Varian VXR - 300 FT spectrometer operating at 300 MHz, and 65 MHz, respectively. The mass spectra were determined using VG 707E-HF mass spectrometer.

Microorganisms - These were obtained from the culture collection at the University of Iowa , College of Pharmacy, and were originally obtained from North Regional Research Laboratories (NRRL), or purchased from American Type Culture Collection (ATCC).

Ninteen microorganisms have been screened including *Streptomycis paucisporogenes ATCC* 12596, *Strept. Griseus var. crizensis NRRL* 3242, Strept. *flocculus ATCC* 2543, *Hasenula amonala ATCC* 20144, *Candida intermedia* ATCC 12087, *C. itermedia* ATCC 5159, *C. lipolytica NRRL* 3242, *Nocardia restrictus* ATCC 14887, *N. aurantia* ATCC 12674, *Torulopsis* ATCC 20225, *Aspergillus niger* ATCC 16888. *A. ochraceus* ATCC 1008, *A. species* UM 111391, *Helicodudandron trighterinse* ATCC 16770, *Bacillus cereus* ATCC 13824, *B. subtilus* ATCC 2458, *Saccharomycis lipolytica* ATCC 11617, *Penicillium* species ATCC 12556, *Mycobacterium* species NRRL 3824-B.

Screening and fermentation procedure :

Subculture of the microorganisms was carried out on nutrient agar in test tubes. Nutrient broth (50 ml) was added in each of 250 ml conical flasks (20 flasks) and the flasks were sterilised by autoclaving at 121°C for 15 min. The microorganisms were separately transferred to 19 of the sterile flasks from the subculutre slants and incubated at room temp. using the shake culture technique at 200 rpm for 72 h (stage I). Five ml of each of stage I was transferred to another sterile 50 ml nutrient broth in 250 ml conical flasks and the flasks were incubated under the same conditons for 24h (stage II). About 5g (2 ml) of sterile used sunflower oil was separately added to each of stage II flasks and the flasks were incubated under the same conditions and sampled after 7, 10, and 15 days. Each sample was extracted with 2ml of a mixture of EtOAc: MeOH (9: 1) and monitored with TLC developed in light petr: Et_2O: AcOH (8 : 2 : 0.1). The spots were visualized with dichlorofluorescene spray reagent (0.5% in MeOH).

Six microorganisms showed transformation after two-week incubation as represented by good emulsifying properties and the appearance of several spots on the TLC chromatograms. These microorganisms are *Strept. paucisporogenes ATCC* 12596, *Strept. griseus var. crizensis NRRL* 3242, *Hansenula anomala ATCC* 20144 C. *intermedia ATCC* 12087, *C. intermedia ATCC* 5159 and *Nocardia restrictus* ATCC 14887.

The experiments were carried out in triplicate, using suitable controls (i.e using oil and medium only, using organism and medium only or using organism, medium and oil). The best results were obtained with *Strept . pauciporogenes ATCC* 12596.

Large scale production of *Streptomycis* metabolites :

Five new slants of *Srept. paucisporogenes* were made on nutrient agar . The organism from each slant was subcultured on sterile, nutrient broth (100 ml contained in 250 ml conical flask and incubated for 72h using the shake flask technique at 200 rpm (stage I). Five ml of stage I was transferred to fresh sterile nutrient broth (75 ml) contained in 250 ml conical flasks (50 flasks) and incubated for 24h under the same conditions (200 rpm) . About 5 ml of sterile used sunflower oil was then added to each flask and the flasks were incubated for two weeks using the same technique and under the same incubation conditions. The flasks were then pooled together and extracted with EtOAc: MeOH (9 : 1) . The extract was dried with anhyd. Na_2SO_4 and concentrated under vacuum. Oily extract was obtained (30 g), and about 3g of the extract were chromatographed onto 300 g of silica gel column using light petr. (b.r. 40 - 60°C) : Et_2O: AcOH (8 : 2: 0.1) , then light petr. : EtOAc (7: 3) as the eluting solvents. Two pure substances were obtained, without optimization , A (about 600 mg R_f 0.51) and B (about 300 mg , R_f 0.49) in light petr.: Et_2O : AcOH (6 : 4 : 0.1) .

RESULTS AND DISCUSSION

Methods commonly used in research laboratories for screening of microorganisms were employed to identify cultures capable of biotransforming used sunflower oil into useful products. Screening experiments were conducted with TLC as the method of analysis and no attempts were made to optimize used oil - metbolite production A total of 19 microorganisms were examined based on procedure reported (1) . TLC analysis revealed that six microorganisms namely; *Streptomycis paucisporogenes ATCC* 12596,*Strept. griseus var. crizensis NRRL 3242, Hansenula anomala ATCC 20144 , Candida intermedia ATCC* 12087, *C. intermedia ATCC* 5159 *and Nocardia restrictus ATCC* 14887 were capable of forming either palmitic and / or stearic acid . In general , preparative scale fermentation gave reaction profiles and relative yields of metabolites similar to those observed during screening . However, preparative - scale - reactions were allowed to proceed until substances were consumed before culture harvest, extraction and isolation of metabolites.

A total of 250 grams of used oil were distributed among 50 flasks , each containing 250 ml medium and shaken for 15 days at 200 rpm at 28 °C. At the end of the incubation period, when all the substrate has been consumed (indicated by TLC) the combined cultures were extracted with ethyl acetate / methanol (9:1) mixture. The combined extracts were washed with water, dried over anhydrous sodium sulfate and concentrated under vaccum to give 30 g of brown oily residue . Three grams of this residue were dissolved in methanol, adsorbed on 2 g of silica gel and loaded on a silica gel column (300 g 2.5 /120 cm) . The column was eluted with light petr. : Et_2O: Ac OH mixture then light

petr. : EtOAc mixture. Similar fractions (TLC) were pooled together and concentrated .

Metabolite 1 :

A major spot at R_f 0.51 gave 0.60 g (2.4% yield) of colorless amorphous compound which gave the following physical properties : m . p. 59- 61°C , GC of methyl ester (Rt 2.89 min.), Ms; m/z (rel. abun. %): 257 [$M^+ + 1$] (7.4) , 256 (M^+) (6.1) 213 ($M^+ + 1 - CO_2$)] (6.1), and 101 [$M^+ - C_{11}H_{23}$] (100) , PMR ($CDCl_3$) δ 0.89 (t, 3H , CH_3) , 1.37 (m, 2H, C_{15}) , 1.71 (m,2H , C_{14}). 2.31 (m, 20H, methylene C_{2-11}) , 3.44 (m, 2H, C_3) and 4.37 (t, 2H, C_2) . ^{13}C-NMR ($CDCl_3$) , δ : 13.99 (C_{16}) , 23.65 (C_{15}), 34.90 (C_{14}), 29.79-30.51 (C_5 to C_{13}), 24.91 (C_4), 34.38 (C_3) , 49.98 (C_2) and 174.90 (C_1). The product was identified as palmitic acid by comparison of spectral data (mass, ^1H -and ^{13}C-NMR) and physical properties of the isolated compound with authentic plamitic acid. The metabolite methylester was also identical to authentic methyl palmitate by GC analysis (R_t 2.89 min).

Metabolite 2:

The same fermentation protocol was followed as described above. The fermentation reaction was terminated after 15 days and worked up as described above for the isolation of metabolite 1. Following extraction and crystallization from acetone 0.3 g of pure metabolite 2 (1.2% yield) was obtained as a white amorphous powder. Metabolite 2 was identified as stearic acid as follows : m. p. 69°-70° C ; TLC R_f 0.49, GC methyl ester R_t 4.63 min. MS; m/z (rel. abun. %): 285 [$M^+ + 1$] (8.5), 241 [$M^+ + 1 - CO_2$] (6.6), 256 [$M^+ - CO$] (12.4) , 68 [C_5H_8] (100) . PMR ($CDCl_3$), δ , 0.90 (t , 3H , CH_3), 1.29 (m, 2H, C_{17}) 1.70 (m, 2H, C_{10}), 2.01 - 2.42 (m, 22H, methylene C_{5-15}) , 2.05 (m,2H, C_4), 3.40 (m, 2H, C_3) 5.06 (t,2H C_2). ^{13}C-NMR ($CDCl_3$) δ : 14.56 (C_{18}) , 23.39 (C_{17}) , 49.70 (C_2) and 174.60 (C_1). The extracellular lipase of microorganisms released mainly the saturated fatty acids, plamitic and stearic, *Rhizopus stolonifer* was reported to utilize sunflower oil and produces dodecandioic acid (25% yield) and dodedecyl β - D - glucopyranoside (15% yield) [2] . Lard was tranformed by yeast lipases into pure palmitic and stearic acid [1] .

Used sunflower oil more abundantly available as a renewable lipid feedstock because of the expanding snack industry in Egypt. This may render this transformation economically feasable as it produces valuable and usable pure fatty acids that represent important chemicals in many phamraceutical, cosmetics, lubricants, paints and wax industries. Practical efforts to isolate and characterize the lipase (s) in these organisms are underway.

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إنتاج أحماض دهنية مفيدة من زيت عباد الشمس المستخدم بواسطة الميكروبات

مديحة أمين حسن ، صالح حسن الشرقاوى

قسم العقاقير - كلية الصيدلة - جامعة المنصورة - المنصورة - مصر

هذه دراسة لإعادة تدوير زيت عباد الشمس المستخدم لإنتاج مواد مفيدة حيث تم إختيار تسعة عشرة من الميكروبات لإنتاج مواد كيميائية مفيدة من زيت عباد الشمس المستخدم ونتج من الدراسة حمض الباليتيك والاستياريك بواسطة سته من هذه الميكروبات وهى :

ستربتوميسيس بوسيسبوروجينز ، ستربتوميسيس جريسيس صنف كريزنسيس هانسينيولا لا أنومالا ونوعان من الكانديدا انترميديا ونوكارديا ريستركتس

وقد وجد أن أحسن إنتاجية من الأحماض قد أنتجت من الزيت بإستخدام ميكروب ستربتوميسيس بوسيسبوروجينز وتم فصل وتنقية الأحماض المنتجة بواسطة كروماتوجرافيا العمود وتم التعرف عليها بواسطة كروماتوجرافيا الطبقة الرقيقة وكروماتوجرافيا الغاز ومقارنة تحاليل طيف الكتلة والرنين النووى المغناطيسى للمواد المفصولة بالتحاليل المماثلة للمواد القياسية .

تعتبر هذه الدراسة مفيدة للتخلص من الزيوت المستخدمة وعدم التلوث البيئى بها واعادة تدويرها لإنتاج مواد مفيدة مثل حمضى البالمتيك والاستياريك .