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Production of Useful Chemicals from Sunflower Oil by Microorganisms

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Abstract—A number of microorganisms have been screened for growth on sunflower oil as a sole carbon source for production of useful chemicals. *Rhizopus stolonifer* NRRL 1478 was found to transform the lipid contents of sunflower oil into dodecyl β-D-glucopyranoside and dodecanedioic acid in 15 and 25% yield respectively. The produced compounds were isolated and purified by column chromatography and its chemical identity were established using MS, IR, ^{1}H and ^{13}C NMR spectroscopy.

Keywords—Sunflower oil · microoganisms · *Rhizopus stolonifer*

The world annual production of oils and fats by agricultural techniques (plant oils, animal fats) is about 70 million tons in 1993 (Keckwer, 1994) and 68.2 million tons in 1985 (Baumann, *et al.*, 1988). Of the overall production only about 10 million tons (14%) are being consumed by the oleochemical industry for preparation of raw materials for lubricants, waxes, and detergents (Keckwer, 1994). About 80% of the world production was used in foodstuffs and 6% in animal feeds (Baumann *et al.*, 1988).

The production of unique lipids by hydrocarbon-utilizing microorganisms has gained scientists attention recently. Novel metabolic capabilities associated with microorganisms resulting from their ability to oxidize various classes of hydrocarbons attracted many investigators to use them as catalytic reagents (Perlman, and Wagman, 1952; Pan S.C.*et al.*, 1959; Wang *et al.*, 198; Yamamoto and Fujiwara, 1988).

Vegetable oils like Castor oil contain about 90% of ricinoleic acid which has been chemically modified for the manufacture of detergents, paints, and lubricants (Achay, 1971). Sunflower oil contains 80% linoleic acid (Baumann *et al.*, 1988). Experimental mutagenesis has succeded in altering the plant genes for production of oleic rather than linoleic acid. Sunflower and other vegetable oils were used for production of higher quality oils and products (Princen and Rothfus, 1984). A number of vegetable oils and animal fats including: oilve (Wang *et al.*, 1988), castor (Yamamoto and Fujiwara, 1988), corn, peanut, sesame, sperm oils and lard and mutton tallow (Perlman and Wagman, 1952) were used as a carbon source for the production of streptomycin (Perlman and Wagman, 1952), penicillin (Pan *et al.*, 1959), glycerol (Wang *et al.*, 1988) and hydroxy fatty acids (Yamamoto and Fujiwara, 1988).

Rhizopus, Mycobacterium and *Psuedomonas* species were used for production of glycerol and fatty acids by lipolysis of different fats and oils (Linfield *et al.*, 1984). About one million tones/year of glycerol is being produced and is considered as a world surplus due to its underutilization (Keckwer, 1994). Cis-unsaturated fatty acids were stereospecifically hydrolysed by *Geotrichum candidum* to the corresponding saturated fatty acids (Baillageon, 1990). Oleic acid double bond was selectively hydrated by

two *Nocardia* spp. and a *Mycobacterium* into 10-hydroxy and 10-oxo-stearate (important components in grease and lubricant industry) in high yield (El-Sharkawy and Rosazza, 1994; El-Sharkawy *et al.*, 1992). Many microorganisms are able to grow on hydrocarbons as the only carbon source and the hydrocarbon has to be transported through the outer hydrophilic cell wall into the cell. This process is limited by the hydrophobic character of the oil or the hydrocarbon Microorganisms such *Torulopsis* and *Pseudomonas auregenosa* have been utilized for production of sophorolipids (Cooper and Paddock, 1982, 1984) and rhamnolipids (Linhardt *et al.*, 1989; Syldatk *et al.*, 1985), respectively, by emulsifying the hydrocarbon in the growth medium. Lipopolysaccharides (nonionic surfactant) are also produced by *Candida, Rhodococcus, Mycobacterium* and *Arthrobacter* species (Syldatk and Wgner, 1987). Cellobiolipids (anionic extracellular surfactant was also produced by *Ustilago maydis* and *U. zeae* (Frautz and Wagner, 1984). Sur-factin, subtilisin, lipoprotein and other surfactants were obtained from *Bacillus subtilis, Streptomyces sioyaensis* and *P. aurogenosa* (Syldatk and Wagner, 1987).

This report deals with the use and screening microorganisms to grow on the abundantly available, low priced and underutilized sunflower oil for production of useful chemicals and pharmaceuticals.

Experimental

Chemicals - aniline phthalate, 2,7'- dichlorofluorescene (Aldrich Chemical Co, Milwaukee, WI). Chromatographic grade solvents (Fisher Scientific, USA) were used for TLC and column chromatography analyses. Extractions were done using analytical grade solvents (Fisher).

Maintenence of microorganisms - All microorganisms were obtained from the culture collection at the University of Iowa, College of Pharmacy, and were originaly obtained from North Regional Research Laboratories (NRRL) or purchased from American Type Culture Collection (ATCC). *Pseudomonas aurogenosa* UI 60690 was isolated from a sample of contaminated corn oil, and were maintained on agar slants and stored at 4°C. Microorganisms representing 9 genera (18 species) were used including: *Candida* (5), *Hansenula* (1), *Mycobacterium* (1), *Nocardia* (2), *Pseudomonas* (2), *Saccharomyces* (2), *Schizosaccharomyces* (1), *Rhizopus* (2) and *Streptomyces* (2).

Preparation of Inocula - For inocula unadapted to growth on fat, three 500 ml flasks each containing 100 ml nutrient broth inoculated with 100, 200, 400 ul of spore/cell suspension from slant in 10 ml distilled water. The flasks were incubated at room temperature at 150 rpm for 24 hours. Cultures entered the stationary phase had optical density at 550 nm (OD_{550}) of 2.5 (Tam and Gill, 1985). Cells are harvested by centrifugation, washed with 0.1 M phosphate buffer, pH 7 and resuspended in the same buffer for use as inocula.

Inocula adapted to growth on sunflower oil were obtained from cultures on oil medium (as described below), inoculated with cells and incubated overnight at 3000 g for 10 minutes and resuspended in phosphate buffer.

Screening and fermentation procedure - Biotransformation experiments were performed using shake culture technique using the medium described elsewhere ((Linhardt *et al.*, 1989). The media was adjusted to pH 6.8 before addition of purified sunflower oil (5 g) and sterilization by autoclaving for 15 minutes. Each of the 50 mL of the medium contained in 250 mL flask was inoculated with the adapted microorganisms and incubated at 28°C, 250 rpm. Cultural controls consisted of sterile media containing the same amount of substrate and incubated under the same conditions. Culture controls consisted of fermenta-

tion blanks in which the organism was grown under identical conditions without substrate.

Analytical methods - The fermentations were sampled (2 mL after 3, 7, 10, and 15 days) by acidification to pH 2 (6N HCl) and extraction with equal volume of EtOAc/EtOH (8:2, v/v). After evaporation of the organic solvent the residue was dried under nitrogen stream and weighed. The obtained residue was reconstituted in CH_3OH and chromatogra-phed on 0.25 mm silica gel TLC plates and developed in petroleum ether:acetone: Formic acid (9:1:0.1, v/v) and the spots made visible under UV light by spraying with 0.2% dichlorofluorescene in methanol and/or spraying with 0.5% para-anisaldehyde reagent and heating for 5 minutes at 105°C. The dry cell weight of the sample was determined by centrifugation and the pelleted cells was washed with water and transfered quantitatively to a preweighed vial and dried in an oven at 100°C and kept in a dessicator to constant weight.

Production of Metabolite 1 (Dodecyl β-D-Glucopyranoside) by *Rhizopus stolonifer* NRRL 1478 - A total 4 g of sunflower oil were added to each of *R. stolonifer* 25 mL cultures contained in 250 mL flasks. The cultures were incubated for 12 days. Each flask was seperatly extracted with 3 x 200 mL of EtOAc-C_2H_5OH (8:2, v/v), dried over anhydrous Na_2SO_4, concentrated to a viscous solution (1.75 g) which was loaded in the top of silica gel column (80 g, 70-230 mesh, Merck, Darmstadt, Germany). The column (2.5 x 60 cm was eluted using a linear gradient of petroleum ether/acetone/methanol with increasing polarity and 10 ml fractions were collected. Fractions showed the same R_f value (TLC) were pooled, concentrated and evaporated to dryness under reduced pressure to provide 400 mg and 750 mg of metabolite 1 and 2, respectively.

Metabolite 1 was obtained as white amorphous powder in 43% yield (6.9 g/l) and was identified as dodecyl β-D-glucopyranoside. It showed R_f=0.32, mass spectrum (EI/CH_4) m/e 349 (M^++1, 1%), 348 (M, 6%) while the FAB 697 ($2M^+$+1, 0.6%), 349 (M^++1, 87%), 348 (M^+, $C_{18}H_{36}O_6$, 0.2%), 185 (M^+-$C_6H_{13}O_6$, 100%), 163 ($C_6H_{11}O_5$, 71%), and 255 ($C_6H_{10}O_5$+$C_3H_8O_3$), 145, 116, 99, 85, 74, and 60. IR (KBr) 3425 (OH), 1725 (ester carbonyl) (Ourisson *et al.*, 1964). ^{1}H-NMR showed chemical shifts δ_H 0.89 (3H, t, CH_3), 1.20 (2H, m, H_{10}), 1.59 (14H, m, $(CH_2)_7$), 1.61 (2H, t, H_{11}), 3.16 (1H, m, $H_{2'}$), 3.28 (1H, m, $H_{4'}$), 3.30 (1H, m, $H_{5'}$), 3.67 (1H, d, $H_{3'}$), 3.88 (1H, m, $H_{6'}$), and 4.23 (1H, d, *J*=9 Hz, $H_{1'}$). ^{13}C-NMR δ_C 14.55 (C_{12}), 23.81 (C_{11}), 30.81 (C_4), 30.84 (C_3), 33.15 (C_2), 62 75 ($C_{6'}$), 70.95 ($C_{4'}$), 75.13 ($C_{2'}$), 77.89 ($C_{3'}$), 78.12 ($C_{5'}$), 104.34 ($C_{1'}$) and 71.56 (C_1).

Metabolite 2 (Dodecanedioic acid) was obtained in 15% yield (2.4 g/l) as a white amorphous powder and was ultimately identified as 1, 10 decanedicarboxylic acid. It displayed the following physical properties: mp 126-128°C, R_f=0.12. MS (CI/NH_3) showed m/e (% relative abundance) 248 (M^++1+NH_3), 347 (M^++NH_3, 2%), 259 (M^++NH_3-2CO_2, 1%), 185 (M^+-CO_2H, 2%) and 85 (100%, C_6H_{13}). EI (DIP) showed 230 (M^+), 187, 158, 85 and 69. ^{1}H-NMR δ_H 5.18 (4H, t, H_1, H_{10}), 3.48 (4H, t, H_2, H_8), 2.26 (10H, m, H_3-H_7). ^{13}C-NMR δ_C 26.83 (C_4-C_6), 30.94 (C_3, C_7), 31.26 (C_2, C_8), 36.01 (C_1, C_{10}), and 176.65 (two carboxyl carbonyls).

Hydrolysis of dodecyl β-D-glucopyranoside - (1) *Enzymatic hydrolysis*: A sample of 5 mg of the metabolite 1 was incubated seperately with β-glucuronidase and α-glucosidase (Sigma Chemical Co., St. Louis, MO.) in 5 ml of 0.1 M acetate buffer (pH 5) at 37°C (El-Sharkawy and Abul Hajj, 1987), and El-Sharkawy *et al.*, 1991). Control experiments were performed concurrently in the absence of enzyme. Complete hydrolysis was observed after 3 hours of incubation. The reaction mix-

ture was extracted with CH_3Cl, both the organic and the aqueous was then concentrated under nitrogen stream and used for thin layer chromatography analysis. Chloroformic extract showed a compound with a mp and R_f value similar to dodecanoic acid and D-glucose was detected in the aqueous phase at R_f value similar to glucose.

(2) *Acid hydrolysis*: Metabolite 1 (10 mg) was dissolved in 25 ml of 0.1 N HCl and refluxed in a round-bottomed flask for 2 hours (El-Sharkawy and Abul, 1987), and El-Sharkawy *et al.*, 1991). After cooling at room temperature, the hydrolysate was extracted with CH_3Cl. Both the organic and the aqueous layer were treated as described before.

Results and Discussion

Sunflower oil is readily available at low cost and its use as a carbon source for the growth of microorganisms is a poetntial for production of useful chemicals and pharmaceuticals. The gorwth of *R. stolonifer* on sunflower oil resulted in production of an extracellular lipase. A lag occurred when the washed sunflower oil-grown cells were inoculated, this was explained for the need to establish a cell substrate association (Tam and Gill, 1985). The microbial utilization of insoluble substarte is limted by its dispersion in the aqueous phase and also the produced lipase specificity towards the substrate. Some microorganisms are known to produces surfactant to disperse water soluble alkanes into oil/water emulsion. Emulsification of the alkane increases the surface area available for utilization by microorganisms (Tam and Gill, 1985). Figure 1 showed that the cells are slowly growing and a long lag period is required by the microbial cells for adaptation to the oily substrate after which the cells grew readily on the oil. It is asumed that the adaptation period is required (about 2 days) for lipase production and enhance the association of the bacterial cells with fat.

Rhizopus stolonifer produced an emulsifier

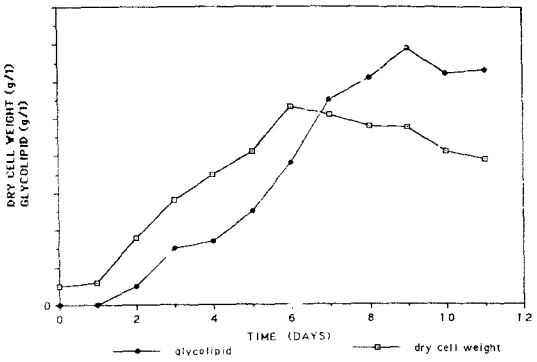

Fig. 1. Batch fermentation of *Rhizopus stolonifer* Time in days is plotted versus (⊡) dry cell weight and (•) glycolipid concentration.

when grown on a liquid culture. This emulsifier was recovered by ethylacetate/ethanol extraction of the cell-free spent medium. The maximum productivity of the glycolipid was obtained at 9 days of incubation (Fig. 1) and also coincided with increase number of cells reaching 4-5 g/l for days 6-9 after which the number of cells declined due to cell death and substrate depletion.

Metabolite 1 was identified as dodecyl β-D-glucopyranoside based on the mass spectral analysis which showed a molecular ion of 348 for $C_{18}H_{35}O_6$. Its lipid nature was indicated from the TLC analysis as it showed a fluorescent spot at 0.32 when sprayed with fluorescene reagent. The glycosidic character was shown by both acid and enzymatic hydrolysis. The ^{1}H-NMR showed signals in the area characteristic for the sugars (3.2-5.50 ppm) (Agrawal, 1992) in addition the ^{13}C-NMR analysis displayed six carbon signals of a hexose. The doublet at 4.23 (J=9 Hz) was assigned for an anomeric proton of a β-linked sugar (Agrawal, 1992). It was reported (Agrawal, 1992; Harborne and Mabry, 1982) that the β-glucosides anomeric proton resonates at higher upfield position than the α-glucosides and $^3J_{1,2}$ of 9 Hz are consistent with the β-anomeric configuration (for α J=1-4 Hz) (Agrawal, 1992; Harborne and Mabry, 1982). This was confirmed by enzymatic hydrolysis

where the sugar was hydrolysed by β-glucuronidase not by a glucosidase. The signals at δ_C 104.34, 78.12, 77.89, 75.13, 70.95, and 62.75 were assigned for $C_{1'}$, $C_{5'}$, $C_{3'}$, $C_{2'}$, $C_{4'}$ and $C_{6'}$, respectively, of a β-linked hexose. The glucopyranoside structure was confirmed from the $H_{2'}$ coupling constant at J=2Hz (Agrawal, 1992). Irradiation (9 Hz) of the proton signal at 4.23 ppm of the enomeric proton in selective INEPT experiment resulted in an enhancement of the carbon signals at 171.56 ppm indicating the glucosylation at the carboxyl end of the dodecanoyal moeity. This finding was supported by both acid and enzymatic hydrolysis of 5 mg of metabolite 1 which gave a sugar identified bv TLC as D-glucose gave yellowish green color with anisidine phthalate reagent at R_f 0.53 on silica gel GF_{254} developed in isopropanol- EtOAc-H_2O (7:2:1, v/v). The lipid portion was identified as dodecanoic acid (C_{12}-fatty acid) by cochromatography. The chemical shifts of the β-O-linked sugar is in full agreement with previously reported data (Agrawal. 1992; Harborne and Mabry, 1982).

Figure 2 shows a proposed pathway of the formation of metabolites 1 and 2 as they are formed from the hydrolysed fatty acids which undergoes β-oxidation ultimately to acetate. Gluconeogenesis leads to formation of metabolite 1 as proposed in the Fig. 2.

Conjugation of organic compounds by different microorganisms have been reproted by several investigators (El-Sharkawy and Abul, 1987; El-Sharkawy *et al.*, 1991; Cerniglia *et al.*, 1982; Kuo *et al.*, 1989).

Metabolite 2, was produces as 2.4 g/l and identified as dodecanedioic acid based on mass and NMR spectral data which showed the M^+ is 348 consistent with the proposed structure.

Long chain dicarboxylic acids are important chemical intermediates for the preparation of plasticizers, lubricants, polyurethans, polyamides and perfumes (Fukui and Tanaka, 1980). It has been well known that the production of long chain dicarboxylic acids by

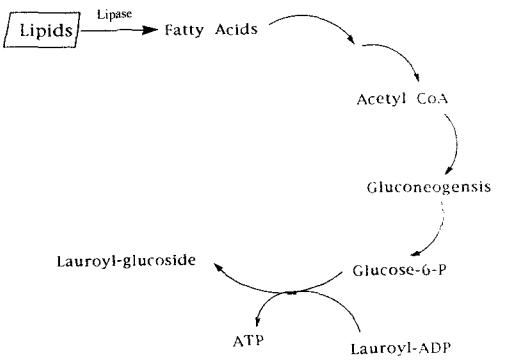

Fig. 2. A Proposed pathway of glycolipid production

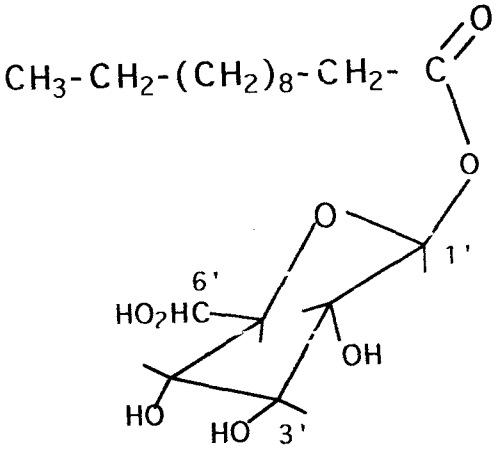

Fig. 3. Structure of Glycolipid isolated from *Rhizopus stolonifer* culture

chemical synthesis or by chemical oxidation is not easy and difficult, only short chains are available by these methods. Many research papers have been reported the use of n-alkanes for the production of dicarboxylic acids using microorganisms (Buhler and Schindler, 1984; Fukui and Tanaka, 1980).

This communication describes the isolation and characterization of two products namely, dodecyl β-D-glucopyranoside (Fig. 3) and 1, 10 decanedicarboxylic acid in high yield. The products were obtained when the microorganism was grown solely on sunflower oil as a carbon source. The obtained emulsifier and dicarboxylic acid make this approach viable for simi-

lar products of commercial value.

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