Wastes Recycling Via Their Utilization as Carbon Source for Single Cell Oil Production

Aouad Linda[1], and Saidi Mohamed

***Abstract*—**Waste represent unwanted or undesired material left over after the completion of a process, it can exist as a solid, liquid or gas. In the recent decade a new concept of waste treatment appeared, which is based on the utilization of these materials as carbon or nitrogen sources required for growth of microorganisms for Single Cell Oil (SCO) production. Twenty organisms were isolated from different sites and stained with Sudan Black B to select the lipid accumulating organisms. Those which took the stain were retained for their ability to accumulate lipid. Best results were obtained with two soil actinomycetes. The two tested organisms were cultivated on different wastes from food and dairy industries, oil refineries and petroleum wastes. Maximum lipid accumulation was obtained after 2-5 days. High lipid contents (about 80.49% of Cell Dry Matter (CDM) was obtained using molasses, carob, orange wastes, apple pomace and sweet potatoes. Different fatty acids compositions were obtained when the two tested organisms were cultivated on pure hydrocarbons, oils and agricultural wastes.

***Keywords*----**Single Cell Oil. Wastes recycling. *Gordonia*, *Rhodococcus opacus*. Central composite design.

I. Introduction

INDUSTRIES produce large quantities of wastes yearly, resulting from preparation and production processing. These wastes pose disposal and severe pollution problems and represent a loss of valuable nutrients. Man has practiced the recovery of human, animals, agricultural and industrial wastes for centuries in order to minimize pollution and to reuse these materials for the benefit of living organism. The bulk of the wastes from agricultural or food processing are not suitable for food or animal feed because they are too fibrous to be digested by monogastric animals (Birch et al., 1981), however, a significant proportion (30-70%) of the dry weight of these wastes are carbohydrates other than cellulose and are therefore readily assailable by microorganisms (Forage, 1979). Microorganisms are capable of utilizing the organic matter in wastes both as a source of energy for growth and as carbon for synthesis of cell biomass (Birch et al., 1981 and Worgan, 1976).

In the recent decade, a new concept of wastes treatment was developed, which is based on the utilization of these materials as carbon source required for growth of microorganisms that promote lipid accumulation. The lipid composition of microorganisms was found to be very responsive to changes in the chemical and physical properties of the environment. It was reported that lipid accumulation is a dynamic process, which depends on the microorganism, the growth conditions like pH, temperature, nutrients and aeration, and the growth phase. Therefore, the proper selection of the organism, process optimization and timing of harvest are essential for efficient SCO production (Kessel, 1968). A wide range of carbon sources are utilized by bacteria for biosynthesis of TAG. These carbon sources include sugars, organic acids, alcohols, n-alkanes, branched alkanes, phenylalkanes, oils and coal (Alvarez et al. 1996, 1997a, 1997b, 2001, 2002; Füchtenbusch and Steinbüchel 1999; Makula et al. 1975; Olukoshi and Packter 1994; Singer et al. 1985). The use of these traditional carbon sources, make lipid production by microorganisms very expensive.

In this work, different wastes derived from agro-industrial processes were surveyed to be used as cheap and available substrates to reduce the cost of the medium, for SCO production by two actinomycetes belonging to *Rhodococcus* and *Gordonia* genera, with resultant protection of the environment.

II. Materials and Methods

Bacterial strains and culture conditions

Rhodococcus opacus and *Gordonia* sp were grown aerobically at 30 °C in mineral salts medium (MSM) according to Shlegel et al. (1961) with 1% (w/v) sodium gluconate as carbon source. The concentration of ammonium chloride was reduced to 0.01% (w/v) to promote the accumulation of triacylglycerides (TAG). Thin sections of *R. opacus* PD630 and *Gordonia* sp. DG were performed and examined by TEM as shown in (figure 1) which illustrates the occurrence of oil droplets of various sizes.

Cell dry matter (CDM) and total lipid extraction

In order to increase lipid content in cells of organisms under test and to reduce the cost of production, different agro-industrial wastes were investigated as carbon sources. The following wastes were used: potato infusion, barley seeds, wheat bran, tomato peel, orange, artichoke, carob, apple pomace, sweet potato, olive mill, date wastes, protomylasse, kitchen wastes, molasses, sweet whey and oils wastes. Sodium gluconate was used as reference carbon source. Pure hydrocarbons (tetradcane, pentadecane, hexadecane and dodecane) and commercially available oils like (olive, sesame,

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castor, cotton, pea-nuts, maize, sunflower and rape seed oil) were also tested.

This experiment was performed in 250 ml capacity Erlenmeyer flasks containing 100ml production medium with 50% (v/v) from the extract of the above-mentioned wastes except for hydrocarbons, oils and molasses, which were added at 1-2% (v/v) to the production medium. The pH value of media was adjusted to 7 and flasks were incubated at 30°C and 120 rpm for 2-5 days according to the organism and the carbon source under test. At the end of the incubation period, cells were analyzed for dry weight, total lipid content and for their fatty acids composition.

Analysis of fatty acids and thin layer chromatography (TLC)

Fatty acids content was determined by methanolysis followed by GC according to Brandl et al. (1988) and Timm et al. (1990). In this assay approximately 5-10 mg of lyophilized whole cells or the Triacylglyceride fraction (TAG)separated by TLC were subjected to methanolysis in a small teflon screw-capped glass test tube, with (1:1 v/v) chloroform and 15% methylated sulphuric acid. The tubes were then closed tightly and heated at 100ºC in a water bath for 3h to convert the constituent fatty acids to their methylesters. Thereafter the test tubes were left to cool down followed by the addition of 1 ml distilled water. The tubes were then shaked vigorously for 30-60 seconds in a vortex (VIBROFIX VF1, Janke & Hunkel, IKA Labortechnik) to separate the water phase from the chloroform phase. After phase separation the chloroform phase containing the lipids was aspirated, washed to neutrality and transferred to a small penicillin screw-capped glass vial. The fatty acid methylesters were then analyzed by a HP-Hewlett Packard 6890 gas chromatograph equipped with an HP-5 (5% dimethyl-95% diphenyl polysiloxane) non polar capillary column (30m length, 0.32mm ID, 0.25 μm layer thickness) equipped with a flame ionization detector (FID). A 1μl portion of the organic phase was analyzed using a splitless mode and nitrogen flow rate (0.8ml/min) as carrier gas. The temperatures of the injector and detector were 220°C and 250°C respectively. A temperature programmed mode was used for the separation of methlyesters (150°C for 1min; temperature increase of 5°C/min; 200°C for 10 min; 250 for 5 min) on the column. The fatty acids were identified by comparison of their relative retention times with those of standard mixture of fatty acid methylesters (Merck).

III. Experimental design

After optimizing the values of physical and nutritional factors by a 'one at a time method' three factors, namely carbon, nitrogen and magnesium sulfate concentrations were selected for further optimization in one experiment using response surface methodology according to the central composite design. The statistical software package 'Design-Expert 6.0.6, Stat-Ease, Inc. was used to analyze the experimental design.

IV. Results and discussion

Growth characteristics and lipid production by R. opacus PD630 and Gordonia sp. DG

The transmission electron microscope (TEM) in this study revealed the presence of lipid inclusions in the cytoplasm of the two organisms under investigation; the cells appear longer, wider, and full with granules (Fig 1). Alvarez et al. (1996) also reported that the microscopic observation by the electron micrographs of *Rhodococcus opacus* PD630 revealed large numbers of electron-transparent inclusions with a sphere like structure.

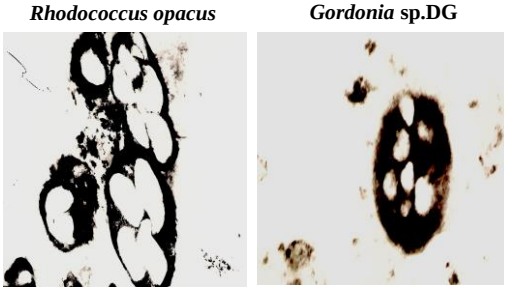

Fig. 1: Occurrence of oil droplets in cells of *Gordonia* sp. DG and in cells of *R. opacus* PD630 as seen by TEM.

Survey of different wastes and pure carbon sources for lipid accumulation by R. opacus PD630 and Gordonia sp. DG

The two strains under test reach their maximum lipid accumulation after 1-5 days according to the carbon sources used. In case of sweet potatoes and apple pomace, maximum lipid content was obtained after one day. On the other hand, prolonged incubation time was required, when oils and hydrocarbons fractions were used as carbon sources (2 days).

Rhodococcus opacus PD630 and *Gordonia* sp. DG grew and accumulated lipids differently with the different carbon sources used. The two organisms gave better results concerning lipid accumulation reaching 93% for *R. opacus* PD630, 96% for *Gordonia* sp. DG with molasses as carbon source. High lipid content 85% (w/w of CDM) was obtained when *R. opacus* grown on orange waste, apple pomace or sweet whey. Olive mill waste and potato infusion gave the lowest lipid content (20-30%) by *R. opacus* PD630. on the other hand; potato infusion promotes lipid accumulation (70%) by *Gordonia* sp. DG.

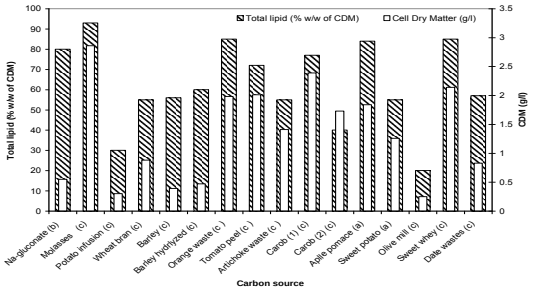

Fig 2: Effect of different agro-industrial wastes on cell dry matter (g/l) and lipid accumulation (% w/w of CDM) in cells of *R. opacus* PD630, (a) after 24h, (b) after 36 h and (c) after 96 h incubation.

Gordonia sp. DG also accumulated high lipid content (70%) when grown on orange waste, carob waste (1) and apple

pomace. It was also observed that the amount of TAGs was affected by the carbon source. Maximum TAGs (196 mg/l) was obtained by R. opacus PD630 with sweet potato as carbon source, followed by 88.9 mg/l with carob waste (1), then 73.5 mg/l with potato infusion. On the other hand, the maximum TAGs (77.6 mg/l) produced by *Gordonia* sp. DG was obtained with apple pomace as carbon source, followed by 57.7 mg/l with sweet whey, then 53.8 mg/l with orange waste.

Concerning the growth, it was observed that molasses, orange waste, tomato peel, carob wastes, apple pomace, sweet potato and sweet whey supported good growth by the two tested organisms with a maximum value (2.86 g/l) with molasses for *R. opacus* PD630 and with carob waste(2) for *Gordonia* sp. DG.

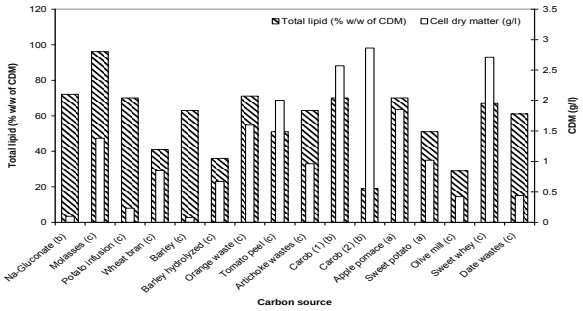

Fig 3: Effect of different agro-industrial wastes on cell dry matter (g/l) and lipid accumulation (% w/w of CDM) in cells of *Gordonia* sp. DG, (a) after 24h, (b) after 72 h and (c) after 96 h incubation.

Voβ and Steinbüchel (2001) attempted to develop high-cell-density cultivation processes for *R. opacus* PD630 in order to produce large amounts of "high-value single-cell oil". They reported that beet molasses led to relatively good growth and to high amounts of accumulated TAGs in the cells, in comparison with a complex medium and a N-limited mineral salt medium (MSM) with Na-gluconate as sole carbon source.

The present work revealed that the use of different hydrocarbon fractions as carbon source also affected the lipid accumulation as well as the growth of the tested organisms. The results showed that the hydrocarbon promote the lipid accumulation by the two organism with a maximum value (84 and 69%) for *R. opacus* PD630 and *Gordonia* sp. DG in the presence of dodecane as carbon source. Maximum TAGs (142 mg/l) was observed by *R. opacus* PD630 with dodecane, while the maximum (161 mg/l) by *Gordonia* sp. DG was obtained in presence of tetradecane as carbon source. Tetradecane and hexadecane gave the maximum growth for the two organisms.

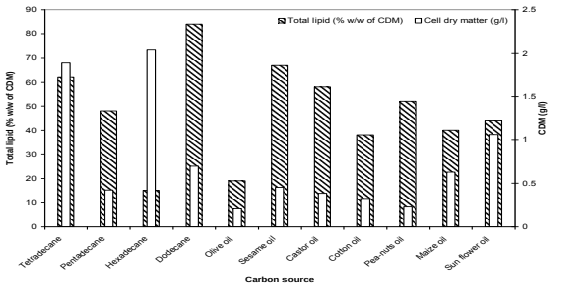

Fig4: Effect of pure hydrocarbons and oils on cell dry matter (g/l) and lipid accumulation (% w/w of CDM) in cells of *R. opacus* PD630 after 24 h incubation.

The tested plant oils also affected the lipid accumulation and the growth of the two tested organisms. The results presented in Fig (4 &5) revealed that all tested oils except olive oil promote the lipid accumulation by the tested organisms. All tested oils, except castor oil in case of *Gordonia* sp. DG (65.5 mg/l), observed low amount (1-18.8 mg/l) of TAGs. Maximum growth of *R. opacus* PD630 was obtained with sunflower oil and of *Gordonia* sp. DG with sesame oil.

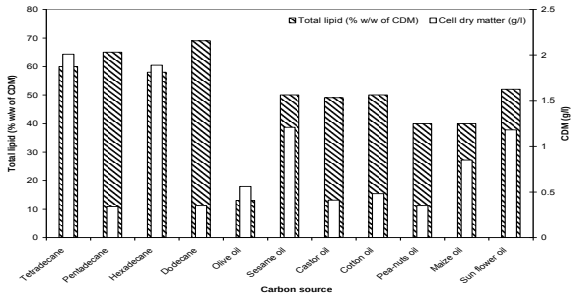

Fig 5: Effect of pure hydrocarbons and oils on cell dry matter (g/l) and lipid accumulation (% w/w of CDM) in cells of *Gordonia* sp. DG after 48 h incubation.

Alvarez et al. (1996) deduced that *R. opacus* was able to accumulate TAGs, which contributed up to 76% (w/w) or even more of CDM when cultivated in a nitrogen-free mineral medium with different carbon sources such as: gluconate, olive oil, alkanes, and phenylalkanes. They reported that *R. opacus* PD630 accumulate only 38-39% lipid in presence of hexadecane and pentadecane as carbon sources. They also found that olive oil was a good carbon source for lipid accumulation by this organism.

Fatty acid composition of some selected agro-industrial wastes and pure carbon sources- grown cells of R. opacus PD630 and Gordonia sp. DG

The lipid accumulated by the two tested strains consisted of saturated and unsaturated straight long chain fatty acids; the presence of such fatty acid with various compositions and different concentrations was highly dependent on the carbon source, and this was confirmed by Gas Liquid Chromatography (GLC).

In this study, the saturated fatty acids were predominantly accumulated during growth of *R. opacus* PD630 on molasses, barely seeds extract, tomato peel, carob waste and sweet whey. On the other hand, the unsaturated were dominant in cells grown on potato infusion, orange waste, artichoke waste, apple pomace, sweet potato and date waste. In case of *Gordonia* sp. DG, the unsaturated fatty acids were dominant only in cells grown on the extract of wheat bran, sweet potato and date waste.

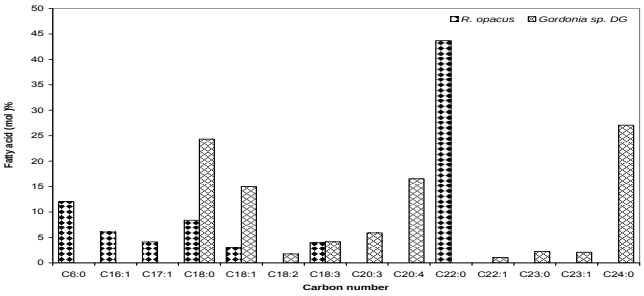

Fig 6: Fatty acid composition (TAGs) of *R. opacus* PD630 and *Gordonia* sp. DG after growth on Molasses.

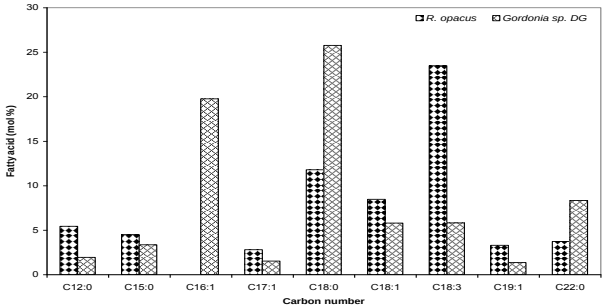

Fig 7: Fatty acid composition (TAGs) of *R. opacus* PD630 and *Gordonia* sp. DG after growth on Apple pomace.

The fatty acids of cells grown on hydrocarbons fractions were also analyzed. When *R. opacus* PD630 and *Gordonia* sp. DG were cultivated on tetradecane, the odd-numbered fatty acids represented about 4-7% for the two organisms. In contrast, when cells were cultivated on pentadecane, hexadecane and dodecane, the fatty acids with an odd number of carbon atoms, such as C5:0, C15:0, C17:1, C19:1 and C23:0 accounted to about 11-48% of the total identified fatty acids produced by the two organisms.

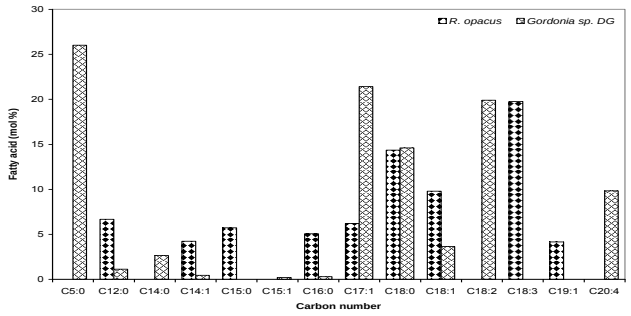

Fig 8: Fatty acid composition (TAGs) of *R. opacus* PD630 and *Gordonia* sp. DG after growth on Pentadecane.

It was also observed that the tested organisms predominantly accumulated saturated fatty acids in presence of hydrocarbons as carbon source, except in case of *R. opacus* PD630 on pentadecane and hexadecane, where the unsaturated were dominant. The use of plant oils as carbon source also affect the fatty acid patterns of the two organisms. It was found that odd-numbered fatty acids were absent from *R. opacus* PD630 cells grown on sesame and pea-nuts oil and that unsaturated fatty acids were dominant in cells grown on cotton, maize and sunflower oils. On the other hand, the saturated fatty acids were predominantly accumulated in cells of *Gordonia* sp. DG grown on all tested oils.

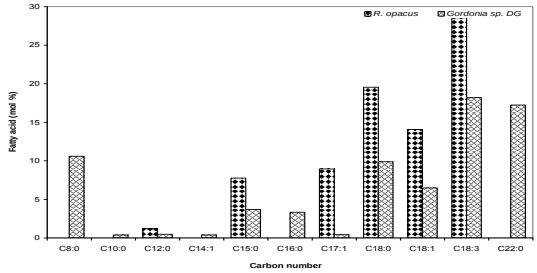

Fig 9: Fatty acid composition (TAGs) of *R. opacus* PD630 and *Gordonia* sp. DG after growth on Maize oil.

The results are in agreement with that obtained by Alvarez et al. (1997b) who reported that when olive oil, hexadecane and heptadecane were utilized as sole carbon source, the chain length of the predominant fatty acid occurring in the inclusion correlated to that of the substrate. In addition, the occurrence of unsaturated fatty acid corresponded to the chain length of the compounds used as carbon source, such as hexadecane or heptadecane, and suggested the presence of a putative fatty acid desaturase system in this microorganism during growth on these substrates.

Optimization using central composite design

The statistical design approach using response surface methodology (central composite design) was used to study the interactive effects of three independent factors (orange waste extract, ammonium chloride and magnesium sulphate) on growth, lipid accumulation and TAGs content of *Gordonia* sp. DG. The results indicate that using 60% (v/v) of orange waste extract, 0.05 g/l ammonium chloride and 0.2 g/l magnesium sulphate gave the maximum lipid content of 80/% (w/w) of CDM, TAGs of 45.3 mg/l and maximum cell dry mass of 1.88 g/l. lowering the carbon source concentration and/or increasing the nitrogen sources gave the lowest lipid content. Also, increasing the carbon concentration in presence of low nitrogen concentration decreased the lipid content. The magnesium sulphate concentration revealed low effect on lipid accumulation.

Response surface methodology used for medium optimization for lipid accumulation by *Gordonia* sp. DG indicated an interaction between the different tested factors. The results revealed that lipid accumulation was mainly affected by carbon and nitrogen concentration and to lesser extent by magnesium sulfate concentration. Maximum lipid 80% (w/w of CDM) was obtained at high C:N ratio and moderate sulfate concentration, which is in accordance with the results reported by Voβ and Steinbüchel (2001).

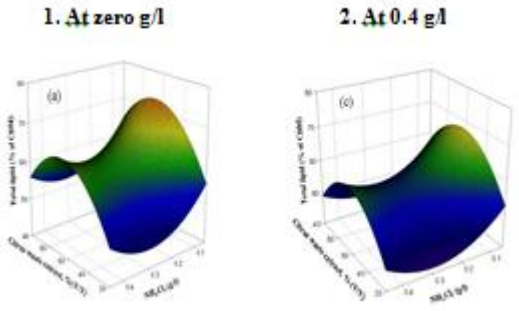

Fig 10: Response surface curve of total lipid accumulation (% of CDM) by *Gordonia* sp. DG showing interaction between orange waste extract and ammonium chloride concentration at different magnesium sulfate concentration: zero and 0.4g/l.

The three-dimensional curves are very helpful in visualizing the main effects and interactions of the factors. Thus, smaller and less time-consuming experimental designs could generally suffice for the optimization of many fermentation processes (Ellaiah, 2002).

V. Conclusion

The present study reported that different food wastes, especially orange waste, are suitable substrates for lipid accumulation by *Gordonia* sp. DG, which may reduce the cost of microbial lipid production and at the same time minimize the pollution problems resulted from this industry.

The study also allowed the identification of nutritional parameters affecting the lipid production by *Gordonia* sp. DG. In addition, the variability in fatty acid pattern with the different carbon sources gives a good chance to select the appropriate carbon source to produce lipid with certain properties.

Acknowledgement

I which to acknowledge the facilities offered by German Academic Exchange Service (DAAD, Bonn) The German Agency for technical corporation (GTZ, Eschborn), Deutsche Forchungsgemeinschaft (DFG), SUMED petroleum company, Pharco pharmaceuticals and last but not least to Prof. Alexander Steinbüchel, director of the Institute of Molecular Biology and Biotechnology, Munster, Germany.

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