

## **RHODOTORULA SPP. A POTENTIAL CANDIDATE FOR BIODIESEL PRODUCTION**

By

M.A. OTERO-RAMBLA and O.A. ALMAZAN-DEL OLMO

*Biotechnology Division, Cuban Institute for Research on Sugarcane By-Products (ICIDCA)*

*Vía Blanca # 804 and Carretera Central, 11000 Havana, Cuba*

[miguel.otero@icidca.edu.cu](mailto:miguel.otero@icidca.edu.cu)

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### **Abstract**

THIS PAPER assesses the production of lipids in *Rhodotorula* spp. yeast biomass under certain propagation conditions. For lipid accumulation, a double-stage culture was arranged with a full balanced medium for cell propagation and afterwards, under nitrogen deficiency, to initiate the conversion of organic matter into triglycerides. This mode was compared with single-stage culture in terms of cell concentration, lipid productivity, protein content in final biomass, and lipid and biomass yields. Significant differences were found in respect to all parameters tested favouring single-stage culture except for cell concentration that was higher in the double-stage mode. Lipid productivity, the most important parameter, was 0.22 and 0.33 kg/m<sup>3</sup>/h for double and single-stage cultures respectively, whereas lipid content was 28.60% for double stage and 29.84% for single stage. Protein in final biomass was 29.98 and 30.46% for double and single-stage respectively. This latter is important, since the resulting biomass after lipid extraction can be used as animal feed. Taking into account that yeast production is far more intensive than with vegetal crops, oil-yeast production could be an attractive alternative to oil seeds for biodiesel production.

### **Introduction**

One of the major challenges facing mankind at present is diminishing fossil fuel stocks. The burning of these materials is one of the key factors for global warming due to carbon dioxide and other greenhouse gas emissions. In addition, with the rapid rise in the price of crude oil and projected decrease in oil supplies, there has been increasing interest in biofuels in scientific and entrepreneurial communities (Pahl, 2005; Soriano *et al.*, 2006; Kemp, 2006). To deal with these problems, alternative renewable biofuels are receiving considerable attention (Hill *et al.*, 2006). One of the most prominent renewable energy resources is biodiesel, which is produced from renewable biomass by transesterification of triacylglycerols, yielding monoalkyl esters of long-chain fatty acids with short-chain alcohols: for example, fatty acid methyl esters (FAMES) and fatty acid ethyl esters (FAEEs).

Biodiesel contributes no net carbon dioxide or sulfur to the atmosphere and emits fewer pollutants than normal diesel (Lang *et al.*, 2001; Vicente *et al.*, 2004). In the production of biodiesel, various renewable lipids have been chosen, including vegetable oils, animal fats and wasting oils (Aggelis *et al.*, 1995).

Palm oil, rapeseed oil, and soybeans have been used to produce biodiesel. However, these require energy and land area for sufficient production. In spite of the favourable impacts that its commercialisation could provide, the economic aspect of biodiesel production has been restricted by the cost of oil raw materials (Antolin *et al.*, 2002). If plant oil was used for biodiesel production, the cost of feedstock accounts for 70–85% of the whole production cost. Therefore, reduction of the high cost of biodiesel is of much interest.

Microorganisms have often been considered for the production of oils and fats as an alternative to agricultural and animal sources. However, a substitute for a fossil fuel should have not only superior environmental benefits or be economically competitive but also must provide a net energy gain over the energy sources used to produce it. Biodiesel production using microbial lipids has attracted increasing attention since it is expected to accomplish these requirements.

Yeasts and fungi (especially moulds) have been considered as favourable oleaginous microorganisms since the 1970s (Hall and Ratledge, 1977). Some yeast strains, such as *Rhodospiridium* sp., *Rhodotorula* sp. and *Lipomyces* sp. can accumulate intracellular lipids as high as 70% of their biomass dry weight.

The most efficient oleaginous yeast, *Cryptococcus curvatus*, can accumulate storage lipids up to >60% on a dry weight basis, when it grows under N-limiting conditions, with a percentage of saturated fatty acids of about 44% which is similar to many plant seed oils (Ratledge, 1982). Other authors found similar fatty acid concentration in *Rhodotorula glutinis* grown on sugarcane molasses with N-limitation in the oil accumulation step (Almazan *et al.*, 1981; Alvarez *et al.*, 1992; Diaz *et al.*, 1993; Alvarez and Steinbüchel, 2002). These fatty acids can be extracted, saponified and esterified with low molecular weight alcohols to yield biodiesel. The main limitation of yeast lipids as a feedstock is that they show almost 30% saturated fatty acids with scarce or no nutritional properties. On the other hand, some of these fatty acids have odd carbon numbers which is an additional drawback, as they cannot be metabolised by animals or human beings (Almazan *et al.*, 1981; Alvarez *et al.*, 1992).

The first step in such a production is the efficient accumulation of as much lipid as possible in yeast biomass. It is well known that *Rhodotorula* spp. synthesise yeast when nitrogen or any other important nutrient is scarce in growth medium (Enebo and Iwamoto, 1966; Coccuci *et al.*, 1975).

The propagation step can be arranged in many modes, batch or continuous. The comparison between double-stage or simple-stage continuous culture is the subject of the present paper.

## Materials and methods

### Microorganism

*Rhodotorula glutinis* from the ICIDCA collection was propagated in all experiments. Inocula were prepared from agar-malt slants, grown overnight in a medium containing sugarcane molasses at 20 mg/mL of total reducing sugars concentration and nutrient salts (diammonium phosphate and sulfate) to cover cell nutritional requirements in a rotary shaker at 32°C. A 2.5 L Marubishi MD5 fermentor was used to start batch propagation with the same culture medium at  $\mu(=D) = 0.20\text{h}^{-1}$ .

The exhausted medium with yeast cells was discharged in equal volumes to commence the second stage and was fed with fresh medium without nitrogen containing the same concentration of sugars as the first stage.

For single-stage experiments, the growth medium was the same except that nitrogen was fixed at 70% of nutritional requirements.

Dilution rate ( $=\mu$ ) was set at  $0.1\text{h}^{-1}$ . Other fermentation parameters were temperature 32°C, pH 4.0 and aeration one volume of air for volume of medium per minute (vvm). All experiments were run for 96 hours.

### Chemical analysis

Nitrogen was determined according to Kjeldahl (Anon., 1983) in a 1030 Kjeltac Auto System (Tecator AB, Hoganas, Sweden). Reducing sugars were estimated by copper reduction (Greenfield and Geronimus, 1985).

Dry matter was desiccated at 105°C overnight and lipids by extraction with ethyl ether and desiccation until constant weight in a vacuum oven at 60°C.

## Results

### *Batch culture*

The basic physiology of lipid accumulation in microorganisms has been understood for many years (Enebo and Iwamoto 1966; Hall and Ratledge 1977).

Normally, the organism is cultivated in a medium consisting of an excess of the carbon source and a limited quantity of an inorganic nutrient, usually nitrogen. Other limiting components of the growth medium can also be used to promote lipid accumulation such as P, Mg, etc., but they lead to lower biomass production and hence lower productivity.

When one of the nutrients is depleted, the organism can no longer grow and divide and it starts to synthesise lipids as nutritional stock. Figure 1 shows the behaviour of biomass and lipid concentration under nitrogen limitation.

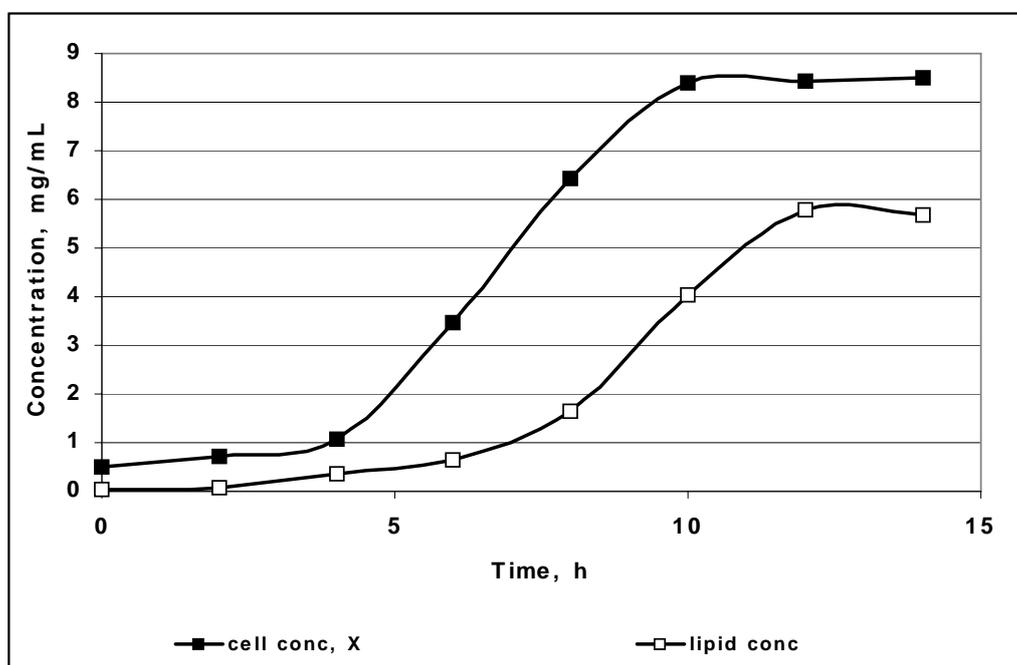


Fig. 1—Batch culture of *Rhodotorula glutinis* under 30% nitrogen deficiency. Other parameters: Molasses (TRS) = 30 mg/mL, T = 32°C, pH 4.5, aeration 1 vvm.

The concentration of the limiting nutrient defines the amount of biomass in which lipid can be accumulated, and therefore the amount of lipids in the culture.

However, in terms of lipid concentration with respect to biomass unit, lipids increase as the limiting nutrient decreases, since accumulation begins in earlier stages of culture development.

With a 100% limitation in required nitrogen, yeast cells accumulated up to 67% of lipids in batch culture with a production rate of 0.35 mg/mL/h in a 14 hour interval (Figure 1).

### *Continuous culture*

Continuous culture has several advantages when compared with batch mode. Productivity increases several times and the whole process is more homogeneous once steady state is achieved.

Lipid accumulation is a typical double-stage process in which cells are propagated under full-requirement conditions and, in a second stage, cells are left to accumulate lipids through nitrogen limitation (Almazan *et al.*, 1981).

A typical fermentation arrangement for double-stage culture can be seen in Figure 2.

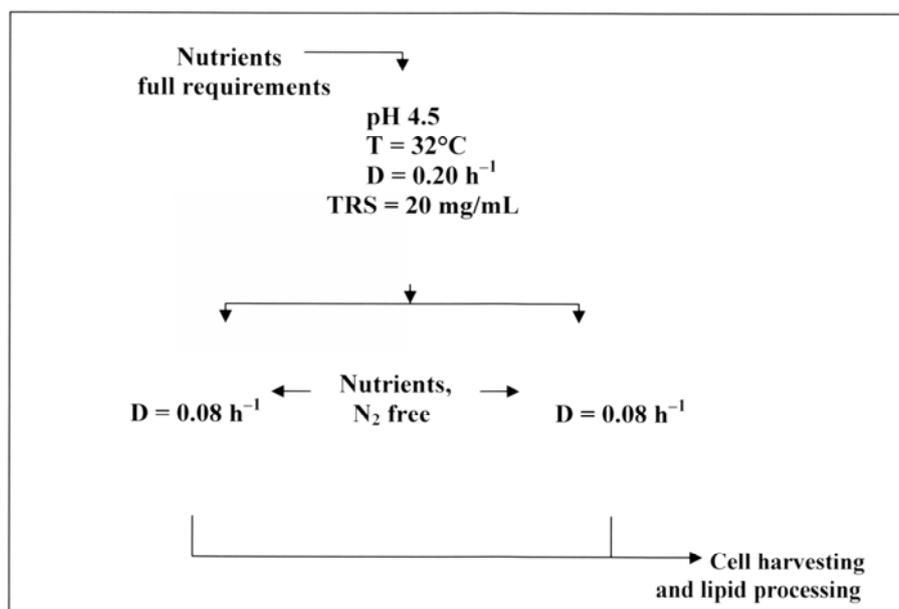


Fig. 2—Fermenter arrangement for a continuous double-stage culture.

Figure 3 shows the pattern of the second stage for lipid accumulation in terms of cell biomass and lipid production. As lower D values favour lipid accumulation, this parameter has to be set up like a batch system to get as much lipid in the final biomass as possible. Under such conditions, lipid productivity ranged from 0.28 to 0.34 mg/mL/h.

The kinetic behaviour of lipid accumulation seems to be as if it were growth associated. This is a consequence of steady state propagation under continuous culture, even at a low dilution rate.

This pattern suggests that it is possible to achieve such a steady state in a unique stage provided that two premises were accomplished: a) nitrogen limitation has to be sufficient to sustain growth, so total absence of this nutrient is not possible, and b) dilution rate must be higher than those values corresponding to maintenance, otherwise growth will not occur.

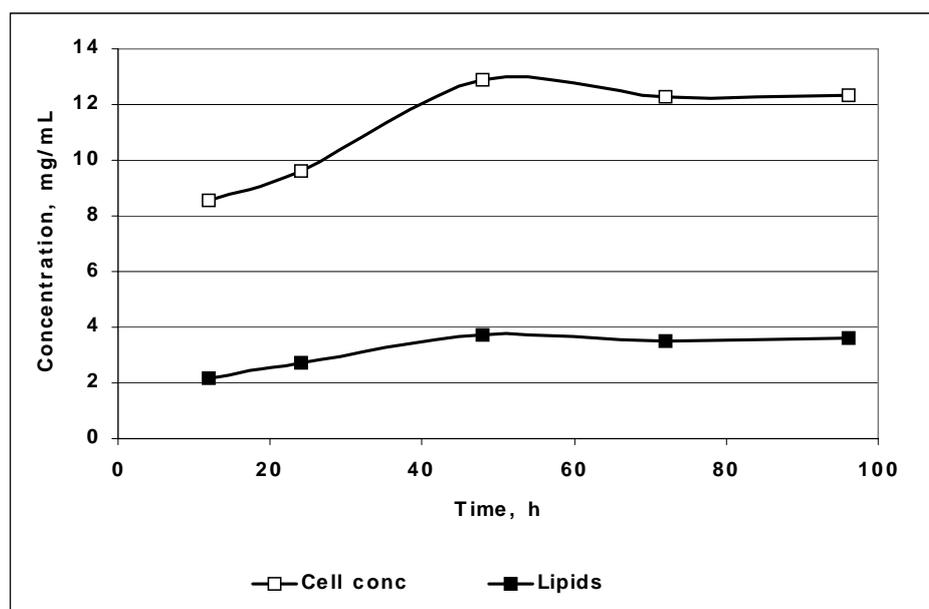


Fig. 3—Pattern of cell and lipid concentration in lipid-accumulation step in continuous double-stage culture of *Rhodotorula glutinis*.

Given these conditions, single-stage culture was set up at the same temperature and pH as above for double-stage. Total reducing substances were fed at 30 mg/mL and nitrogen with 70% of sufficiency. Finally,  $D$  was established at constant value of  $0.1\text{h}^{-1}$ .

Single-stage culture was set up according to parameters given in Material and Methods section. Figure 4 shows the behaviour of yeast culture under this arrangement.

Nitrogen limitation up to 30% of cell requirements guarantees similar results as in the double-stage culture in a unique reactor. Lipid productivity, the main interest in this technology, ranges from 0.31 to 0.36 mg/L/h, 6% higher than that obtained in a double-stage arrangement.

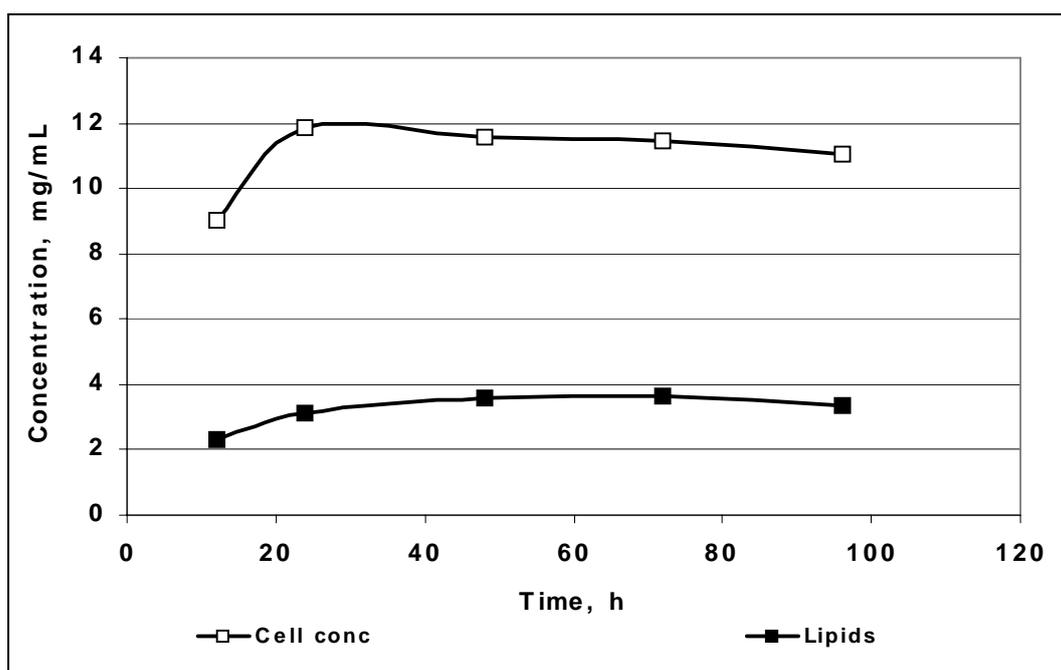


Fig. 4—Pattern of cell and lipid concentration in the lipid-accumulation step in continuous single-stage culture of *Rhodotorula glutinis*.

## Discussion

Biodiesel production from microalgae has been suggested by different research groups. These microalgae have the advantage that they are sunlight-driven cell factories that convert carbon dioxide to potential biofuels (Metting and Pyne, 1996; Spolaore *et al.*, 2006) and can provide several different types of renewable biofuels, including methane produced by anaerobic digestion of the algal biomass (Banerjee *et al.*, 2002, Gavrilescu and Chisti, 2005), and they are high lipid microbes. On the other hand, they need larger acreages for culture and a longer fermentation period than bacteria or yeasts.

Bacteria such as *Arthrobacter sp.* and *Acinetobacter calcoaceticus* can accumulate about 20–40% of lipids per dry biomass but propagate at pH values close to neutrality; thus, the probability of foreign contaminants to compete for nutrients is a real drawback.

Yeasts are able to propagate in an ample range of pH, are significantly larger than bacteria, which means lower  $g$  values in industrial separators, and yeast culture is widely known everywhere. In addition, in an area slightly larger than two hectares, a 30 t/day factory can be implemented with an output of about 15 t/ha, a yield not achieved by any known oleaginous seed. Therefore, yeast seems to be the most promising candidate for microbial biodiesel production.

Single-stage culture has several advantages over the double-stage arrangement. In addition to a slight increase in lipids and biomass productivity, we have to take into account the capital

investment savings incurred in connection with the number of reactors needed in both arrangements which make single-stage culture less expensive than double-stage.

Some liquid wastes such as distillery vinasse or black liquors from paper industry could be tested to assess its utilisation in this process, which would add another environmental advantage.

In the course of fatty acid production, there are usually some other by-products useable by industry. In addition to oils, oleaginous microorganisms contain significant quantities of proteins, carbohydrates and other nutrient contents (Sanchez *et al.* 2003); therefore, how to make use of these by-products and improve their industrial value is another way to reduce the production cost of biodiesel.

For example, the residual biomass from biodiesel production can be used potentially as animal feed (Chisti, 2007), and also to produce methane by anaerobic digestion. The waste glycerol from the biodiesel industry could, therefore, be easily transformed into highly valuable chemicals (Hirschmann *et al.*, 2005). *Rhodotorula* spp. produces  $\beta$ -carotenes (provitamin A) and important proteins useful for animal feeding.

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## **RHODOTORULA SPP. UN CANDIDAT POTENTIEL POUR LA PRODUCTION DE BIODIESEL**

Par

M.A. OTERO-RAMBLA et O.A. ALMAZAN-DEL OLMO

*Biotechnology Division, Cuban Institute for Research on Sugarcane By-Products (ICIDCA)*

*Vía Blanca # 804 and Carretera Central, 11000, La Havane, Cuba*

[miguel.otero@icidca.edu.cu](mailto:miguel.otero@icidca.edu.cu)

**MOTS-CLÉS: Biodiesel, Huile Microbienne, Levure Oléagineuse, Protéine, d'Alimentation,  $\beta$ -Carotène.**

### **Résumé**

CE DOCUMENT évalue la production de lipides dans la levure *Rhodotorula* spp. sous certaines conditions de propagation. Pour l'accumulation des lipides, une culture en deux étapes a été réalisée avec un support équilibré pour la propagation de la cellule et par la suite sous carence azotée pour initier la conversion de la matière organique en triglycérides. Ce mode a été comparé avec une culture en une étape en termes de concentration cellulaire, de productivité des lipides, de teneur en protéines dans la biomasse finale, de rendement en biomasse ainsi qu'en lipides. Des différences significatives ont été trouvées en fonction des paramètres évalués favorisant la culture en une seule étape à l'exception de la concentration de cellule qui a été plus élevée en deux étapes. La productivité en lipides, le paramètre le plus important, était 0.22 et 0.33 kg/m<sup>3</sup>/h pour les cultures en

deux étapes et une étape respectivement, considérant que la teneur en lipides était 28.60 % pour la phase double et 29.84% pour la seule étape. Les protéines dans la biomasse finale étaient de 29.98 et 30.46% pour les doubles et simple étape respectivement. Ce dernier point est important, car la biomasse obtenue après l'extraction de lipides peut être utilisée comme aliments pour bétail. Étant donné que la production de levure est beaucoup plus intensive que les cultures végétales, la production debiodiesel à base de levure pourrait être une alternative intéressante aux espèces d'oléagineuses.

## **RHODOTORULA SSP. UN CANDIDATO POTENCIAL PARA LA PRODUCCIÓN DE BIODIESEL**

Por

M.A. OTERO RAMBLA y O.A. ALMAZÁN DEL OLMO

*División de Biotecnología, Instituto Cubano de Investigaciones de los Derivados  
de la Caña de Azúcar (ICIDCA)*

*Via Blanca 804 y Carretera Central, 11 000 Habana, Cuba*

[miguel.otero@icidca.edu.cu](mailto:miguel.otero@icidca.edu.cu)

**PALABRAS CLAVE: Biodiesel, Aceite Microbial,  
Levaduras Oleaginosas, Proteínas Comestibles,  $\beta$ -Caroteno.**

### **Resumen**

ESTE ARTÍCULO describe la producción de lípidos en la biomasa de la levadura *Rhodotorula ssp.* Bajo ciertas condiciones de propagación. Para la acumulación de lípidos se estableció un cultivo en doble etapa, con una primera etapa de propagación celular a partir de medio de cultivo totalmente balanceado, seguido de la segunda etapa bajo condiciones de deficiencia de nitrógeno para inducir la conversión de la materia orgánica en triglicéridos. Este esquema se comparó con el cultivo en simple etapa en términos de concentración celular, productividad de lípidos, contenido de proteína en la biomasa final, así como rendimiento de lípidos y biomasa. Se encontraron diferencias significativas con respecto a todos los parámetros estudiados, favoreciendo el cultivo en simple etapa, excepto en lo referido a concentración de biomasa que fue mayor en el esquema de doble etapa. La productividad de lípidos, el parámetro más importante, resultó de 0.22 y 0.33 kg/m<sup>3</sup>/hr para doble y simple etapa respectivamente, mientras que el contenido de lípidos fue de 28.60% para la doble etapa y 29.84% para simple etapa. Los contenidos de proteína en la biomasa final fueron de 29.98% y 30.46% para simple y doble etapa respectivamente. Esto último es importante, en cuanto la biomasa resultante después de la extracción de los lípidos puede ser empleada como alimento animal. Tomando en consideración que la producción de levadura es mucho más intensa que los cultivos vegetales, la producción de levaduras ricas en grasas resultaría una alternativa atractiva en comparación con el cultivo de semillas de oleaginosas para la producción de biodiesel.