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Culture of Oleaginous Yeasts in Dairy Industry Wastewaters to Obtain Lipids Suitable for the Production of II-Generation Biodiesel

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***Abstract*—**The oleaginous yeasts *Lipomyces starkey* were grown in the presence of dairy industry wastewaters (DIW). The yeasts were able to degrade the organic components of DIW and to produce a significant fraction of their biomass as triglycerides.

When using DIW from the Ricotta cheese production or residual whey as growth medium, the *L. starkey* could be cultured without dilution nor external organic supplement. On the contrary, the yeasts could only partially degrade the DIW from the Mozzarella cheese production, due to the accumulation of a metabolic product beyond the threshold of toxicity. In this case, a dilution of the DIW was required to obtain a more efficient degradation of the carbon compounds and an higher yield in oleaginous biomass.

The fatty acid distribution of the microbial oils obtained showed a prevalence of oleic acid, and is compatible with the production of a II generation biodiesel offering a good resistance to oxidation as well as an excellent cold-performance.

***Keywords*—**Yeasts, Lipids, Biodiesel, Dairy industry wastewaters.

I. Introduction

BIODIESEL offers an attracting alternative to the petroleum-based diesel, as it is biodegradable, non-toxic, clean, and can be obtained from renewable sources.

The biodiesel synthesis is traditionally carried out using vegetable oils and animal fats as feedstock. Unfortunately, these sources of triglycerides cannot satisfy the demand for biodiesel at the current rate of consumption [1]-[2]. In addition, the cost of the biodiesel, mainly due to the vegetable oils used as feedstocks, still exceeds that of the mineral diesel. In addition, the diffusion of biodiesel is leading to the deforestation of large areas, and to social problems in some developing countries due to the increased price of edible oils. Alternative sources of triacylglycerols (TAG) are consequently required.

Oleaginous microorganisms are a potential source of TAGs, as they have the ability to produce aerobically more than 20% of their weight in the form of lipids under nitrogen-limiting conditions [3]-[8]. In particular, they can produce lipids from a wide range of residual organic matters, reducing the cost of the process and increasing its environmental benefits.

In this study we demonstrate that the oleaginous yeast *Lipomyces starkeyi* can be grown using DIW as culture medium, producing microbial lipids that are suitable as alternative feedstock for the synthesis of biodiesel.

The disposal of wastes generated from dairy industries is a main environmental problem. The treatment of 10 kg of milk produces about 1-2 kg of dairy products and 8-9 kg of wastes. A small dairy, producing an average of 20 m^3 of wastewater per day, may produce pollution comparable to that of a population of 10,000 inhabitants.

II. Materials and Methods

A. Microorganisms and Culture Medium

The oleaginous yeasts used within the present work (*Lipomyces starkeyi*, *Cryptococcus curvatus*, *Rhodotorula glutinis*, *Rhodosporidium toruloides*) were obtained by the collection of the Dipartimento di Biologia Vegetale of the Perugia University (Italy). The microorganisms were kept on potato dextrose agar (Sigma) at T = 5 ± 1 °C and cultivated in a synthetic N-limiting medium, containing (g/L): KH_2PO_4 (Serva), 1.0; $MgSO_4$ $7H_2O$ (BDH), 0.5; $(NH_4)_2SO_4$ (Carlo Erba), 2.0; yeast extract (Fluka) 0.5; glucose 70.0. The growth was carried out under aerobic conditions at 30°C on a rotary shaker at 160 rpm (Minitron, Infors HT, Switzerland).

B. Dairy Industry Wastewaters (DIW)

The wastewaters used in this study were obtained from a cheese factory (Caseificio Amodio, S. Anastasia-NA, Italy) during the production of two typical dairy products, namely Mozzarella cheese and Ricotta cheese. The flowchart of the process is shown in the Fig. 1: the fresh milk is inoculated with whey starter, then rennet is added for a partial coagulation of the milk proteins (mostly casein, whereas albumin and casein remain in solution). Subsequently, the curd (containing albumin) is used to produce Mozzarella cheese, whereas the whey (containing albumin and casein) is skimmed and used to produce Ricotta cheese.

Three wastes of the process were tested as nutrient for the oleaginous yeasts, namely: 1) the whey obtained after the curd

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separation, 2) the wastewaters of Mozzarella production (WW1), 3) the wastewaters of Ricotta production (WW2).

The wastewaters were immediately frozen at -20° C until further use. Before each experimental test, DIW samples were de-frozen, and the solids were removed by centrifugation (4000 rpm, 30 min, 20° C) in a thermostatic centrifuge (Rotanta 460R, Hettich, USA). The composition of the DIW is given in the Table I.

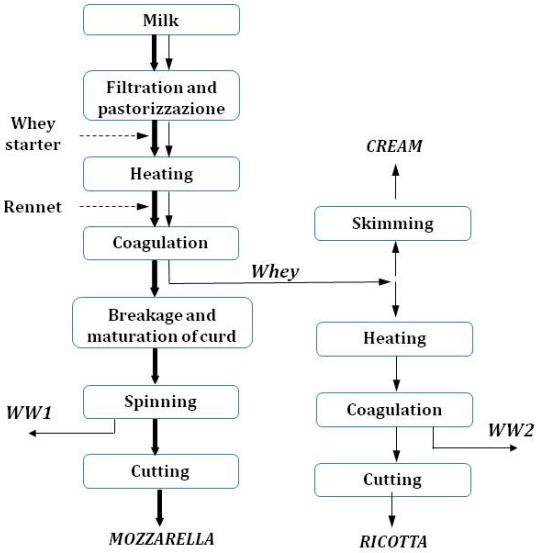

Fig. 1 Flow chart of the production of Mozzarella and Ricotta
WW1 = wastewaters of the Mozzarella production
WW2 = wastewaters of Ricotta production

C. Growth of Oleaginous Yeasts in DIW

In a typical fermentation test, a fixed volume (150mL) of DIW was inserted, without external organic supplement, in a conical flask of 500mL. A preliminary centrifugation of DIW (2000 rpm, 10 min) was carried out before each test. The liquid medium was inoculated by 2mL of microorganism suspension, obtained dissolving 5 loops of solid culture in 8mL of physiological solution. The flasks were incubated in a rotary shaker at an agitation rate of 160 ± 5 rpm and an incubation temperature T = 30 ± 1 °C.

D. Extraction and Measurement of Lipids

Methanol (5.0mL) and chloroform (2.5mL) were added to 200 mg of dry biomass and vortexed 5 seconds. Subsequently the cells were disrupted for 12 min in an Ultrasonic Homogenizer (Omni Ruptor 250, USA) at 50% power and 90% pulser. The cells were then filtered off with Whatman no.1 filter paper and the solvent-lipid mixture was placed in a 50mL centrifuge tube. The layers were separated by centrifugation for 10 min at 2000 rpm in a thermostatic centrifuge (Rotanta 460R, Hettich, USA) at 20°C. The lower layer was then transferred to a pear-shape flask with Pasteur pipette. Again, 10mL of 10% (v/v) methanol in chloroform were added to the residue, a new centrifugation was carried out, and the lower phase was added to that from the first extraction. The solvent in the pear-shape flask was evaporated to dryness (BÜCHI Rotavapor R-200, Switzerland) and extracted weight was finally recorded after drying at 105°C for 1 h.

TABLE I
COMPOSITION OF THE WASTEWATERS (in g/L)

Parameter	Whey	WW1	WW2
lactose	46	46	46
lactic acid	0,5	0,5	0,5
proteins	8	8	8
fats	5	5	5
Vitamins	12	12	12
ashes	5	5	5
total solids	64	64	64
BOD_5	21,1	21,1	21,1
COD	50,8	50,8	50,8

TABLE II
COMPARISON OF FOUR OLEAGINOUS YEASTS AS REGARDS THE BIOMASS AND THE TRIGLYCERIDE YIELD AFTER 120 H OF CULTURE
T = 30°C, 160 RPM

Microorganism	Biomass concentration, g/L	Triglyceride fraction, %
C. curvatus	10,2	16,6
R. glutinis	10,8	17,1
R. toruloides	9,8	18,4
L. starkeyi	12,5	18,5

E. Analysis of Biomass

The biomass concentration in the synthetic medium was measured by OD determination at 600 nm. When culturing microorganisms in the OMW, OD measurement could not be carried out due to the darkness of the medium. Consequently, the total count of microorganisms was carried out by sequential dilution and insemination in plate count agar medium (Difco Laboratories, Detroit, MI, USA). The colonies were counted after 24 h of culture on agar medium.

After each fermentation test, the biomass was recovered by centrifugation (3500 rpm for 10 min) and lyophilised (LYOBETA- 50, Spain), to enable the determination of the dry biomass and the lipid concentration measurement.

III. RESULTS AND DISCUSSION

A. Growth of Oleaginous Yeasts in Synthetic Medium

Four oleaginous yeasts *(Lipomyces starkeyi, Cryptococcus curvatus, Rhodotorula glutinis* and *Rhodosporidium toruloides*) were cultured in a synthetic medium (described in the Materials and Methods paragraph) with a C/N ratio = 58. As a matter of facts, a N-limiting medium is required to promote the accumulation of lipids as storage material within the microbial cells [7]-[8].

The growth profiles reported in the Table II demonstrate that the highest biomass yield after a 120 h growth period was obtained with *L. starkeyi* (12,5 g/L). The triglyceride fraction of *L. starkeyi* (18,5 %) was higher as compared to these pertaining to the other microorganisms. *L. starkeyi* were selected for the subsequent tests, also because their

reutilisation of the stored lipids is minimal [9].

B. Growth of Lipomyces starkeyi in the Presence of Wastewaters of Mozzarella Cheese Production (WW1)

In a first series of experimental tests, WW1 was used as culture medium without any additions or treatments. Some physicochemical properties of WW1 are reported in the Table I. The profiles of CFU (Colony Forming Units) and TOC as a function of the culture period are reported in the Fig. 2. During the first 96 hours, the TOC (full circles in the Fig. 2b) progressively decreased from about 20000 g/L to about 12000 g/L. In the same period, the biomass concentration (full circles in the Fig. 2a) reached a maximum of $1.8 \cdot 10^7$ CFU/ml. Subsequently, the gradual extinction of the yeasts was observed, without significant changes in the TOC level. Evidently, the consumption of organic substances (TOC) was strictly associated to the survival of microorganisms.

The TOC degradation was not complete, possibly due to the exhaustion of a nutrient or to the accumulation of a metabolic product. In order to investigate the causes of the incomplete degradation of the organic components, the medium obtained after a 96-hr growth was diluted adding 3 different media:

1) a first sample was diluted to 50% with saline solution (full squares in the Figs. 2a-b), to reduce by 50% the concentration of all the solutes, including potentially toxic products;
2) a second sample was diluted to 25% with WW1 (full rhombus in the Figs. 2a-b), therefore adding fresh nutrients;
3) in a third sample (data not shown), nitrogen source $(NH_4)_2SO_4$ was added to achieve the same amount of N concentration initially present in the sample.

The experimental data in Fig. 2 show that the addition of fresh WW1 after a culture period of 96 h (full rhombus in the Figs. 2a-b) did not produced appreciable changes in CFU and TOC profiles. Therefore, blocking the growth of yeasts was not due to the exhaustion of nutrients contained in the wastewaters.

Similar results were obtained by adding $(NH_4)_2SO_4$ (data not shown) demonstrating that nitrogen is not lacking in the culture medium after 96 hr.

Nevertheless, the addition of saline solution (full squares in the Figs. 2a-b) produced a recovery of microbial growth and of organic substances degradation. This result indicates that the block the growth of microorganisms was due to the accumulation of a metabolic product beyond the threshold of toxicity.

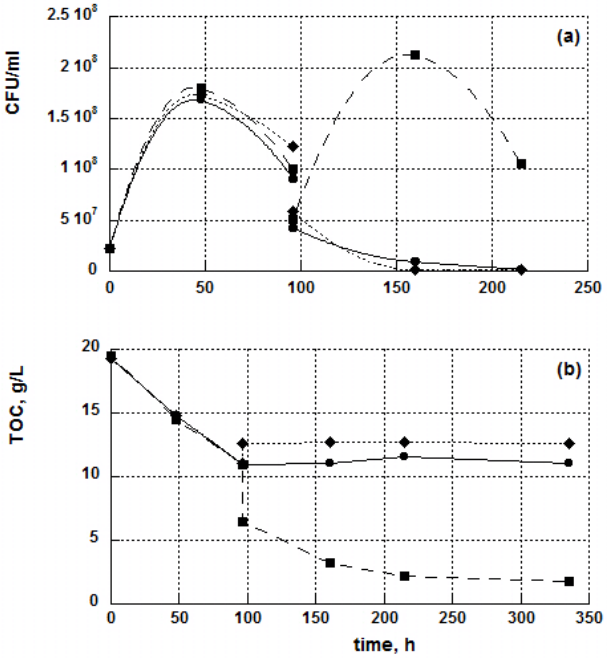

Fig. 2 Cell concentration (a) and TOC (b) during the growth of *L. starkeyi* in the presence of WW1
T=30°C, 160 RPM. Effect of additions after 96 h: undiluted wastewaters (●), 50% dilution with saline solution (■), 25% dilution with WW1 (◆)

C. Growth of Lipomyces starkeyi in the Presence of Wastewaters of Ricotta Cheese Production (WW2)

L. starkeyi were also grown using WW2 as culture medium (see Table II for physicochemical properties). The profiles of CFU and TOC as a function of the culture period are reported in the Fig. 3. The experimental data showed a progressive reduction of the TOC, until more than 85% of the total organic carbon is converted. A corresponding increase in the number of microorganisms was observed. As the TOC reduction was stopped (after about 120 h), the biomass concentration profile reached correspondingly a maximum, to decrease subsequently. This confirms that the consumption of organic substances (TOC) is associated to the biomass growth. On the basis of the results obtained, it can be said that the organic components of WW2 can be degraded by *L. starkeyi* without major difficulties.

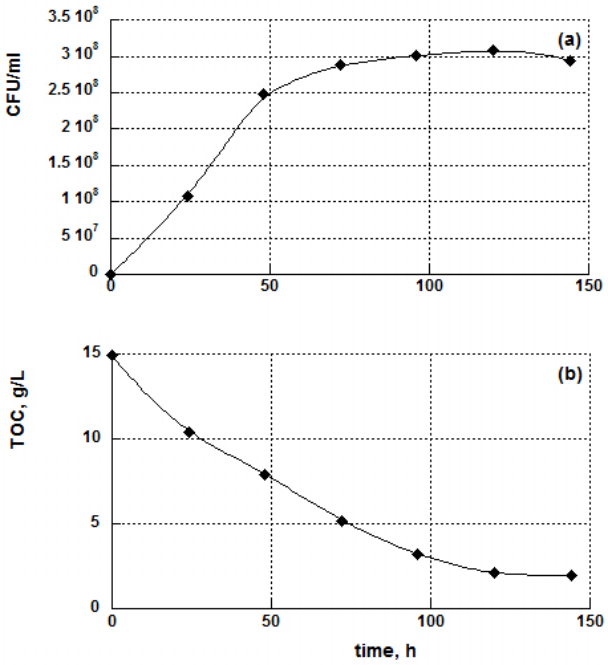

Fig. 3 Cell concentration (a) and TOC (b) during the growth of *L. starkeyi* in the presence of WW2
T=30°C, 160 RPM

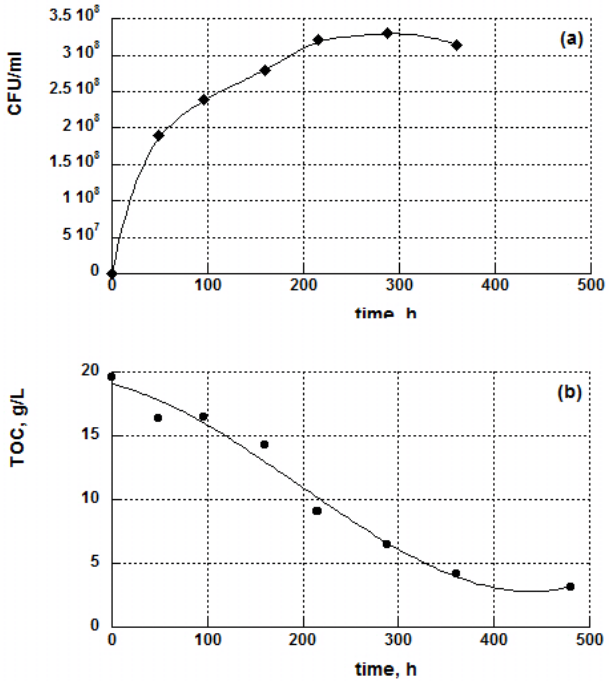

Fig. 4 Cell concentration (a) and TOC (b) during the growth of *L. starkeyi* in the presence of Wuey
T=30°C, 160 RPM

D.Growth of Lipomyces starkeyi in the Presence of Whey

L. starkeyi were also grown using WW2 in the the whey obtained after the curd separation (see Table II for physicochemical properties). The profiles of CFU and TOC, reported in the Fig. 4, showed a substantial reduction of the TOC, though slower in comparison to that observed in the test with WW2. A corresponding increase in the biomass concentration was observed, showing that most organic components of WW2 can be degraded by *L. starkeyi*.

E. Characterization of the Microbial Triglycerides

The triglyceride fractions measured in the yeasts is shown in the Table III. The data indicate that about 18% of the total biomass was made of triglycerides, whatever the waste material used as growth medium. This value is compatible with a commercial application of the process.

TABLE III
COMPARISON OF THE BIOMASS AND THE TRIGLYCERIDE YIELDS OBTAINED IN THE PRESENCE OF DIFFERENT WASTES
T = 30°C, 160 RPM

Microorganism	Biomass concentration, g/L	Triglyceride fraction, %
WW1	8,7	17,4
WW2	9,2	18,2
Whey	9,5	18,5

The microbial lipids obtained from *L. Starkey* were then characterized as regards the distribution of fatty acids. The results are shown in the Table IV. In all instances, a balanced distribution between saturated and unsaturated fatty acids was observed. This means that the microbial triglycerides obtained can be used to obtain an excellent biodiesel, offering an excellent cold-performance, as well as a good oxidative stability.

TABLE IV
COMPARISON OF THE BIOMASS AND THE TRIGLYCERIDE YIELDS OBTAINED IN THE PRESENCE OF DIFFERENT WASTES
T = 30°C, 160 RPM

Microorganism	Whey	WW1	WW2
Palmitic acid (C16:0)	24,2	22,9	24,0
Stearic acid (C18:0)	14,6	14,8	13,9
Oleic acid (C18:1)	48,5	47,1	48,6
Linoleic acid (C18:2)	5,5	5,8	5,9

In conclusion, the results obtained demonstrate that the wastes obtained from the production of dairy products can be used for the II-generation biodiesel synthesis. Their exploitation allows a sustainable production of renewable fuels, and reduces the competition between energy and food crops for fertile lands.

REFERENCES

[1] Y. Christi, "Biodiesel production from microalgae", *Biotechnol. Adv.*, vol. 25, pp. 294-306, 2007.

[2] Y.D. You, J.L. Shie, C.Y. Chang, S.H. Huang, C.Y. Pai, Y.H. Yu, and C.J. Chang, "Economic cost analysis of biodiesel production: case in soybean oil", *Energy & Fuels*, vol. 22**,** pp. 182-189, 2008.
[3] B. Liu, and Z.K. Zhao, "Biodiesel production by direct methanolysis of oleaginous microbial biomass", *J. Chem. Technol. Biotechnol*., vol. 82(8), pp. 775-780, 2007.
[4] Y.H. Li, Z.B. Zhao, and F.W. Bai, "High-density cultivation of oleaginous yeasts *Rhodosporidium toruloides Y4* in fed-batch culture", *Enzyme Microb. Technol.*, vol. 41, pp. 312-317, 2007.
[5] C.C. Dai, J. Tao, F. Xie, Y.J. Dai, and M. Zhao, "Biodiesel generation from oleaginous yeast *Rhodotorula glutinis* with xylose assimilating capacity", *African J. Biotechnol*., vol. 6, pp. 2130-2134, 2007.
[6] C. Angerbauer, M. Siebenhofer, M. Mittelbach, and G.M. Guebitzn, "Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production", *Biores. Technol.*, vol. 99, pp. 3051-3056, 2008.
[7] S. Papanikolaou and G. Aggelis, "Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production", *Eur. J. Lipid Sci. Technol*. vol. 113, pp. 1031-1051, 2011.
[8] S. Papanikolaou and G. Aggelis, "Lipids of oleaginous yeasts. Part II: Technology and potential applications", *Eur. J. Lipid Sci. Technol.* vol. 113, pp. 1052–1073, 2011.
[9] J.E. Holdsworth, M. Veenhuis, and C. Ratledge, "Enzyme activities in oleaginous yeasts accumulating and utilizing exogenous or endogenous lipids", *J. Gen. Microbiol*., vol. 134, pp. 2907-2915, 1988.