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Candida Freyschussii: an Oleaginous Yeast Producing Lipids from Glycerol

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A surplus of glycerol is obtained from biodiesel manufacturing and represents a waste product whose applications are lacking. Thus, the use of glycerol as substrate for fermentation processes yielding valuable products is very attractive. In this study, the utilization of glycerol as a growth substrate for the cultivation of oleaginous yeasts was explored with the aim to produce microbial oils. Forty strains of environmental non-conventional yeasts belonging to 19 different species were screened for the ability to grow on glycerol and produce intracellular lipids in a medium containing an excess of this carbon source (C:N = 48:1). Three strains, belonging to the species *Candida freyschussii*, *Pichia farinosa*, and *Saccharomyces spencerorum*, depleted 40 g/L glycerol within 120 h and produced intracellular lipids. *C. freyschussii* yielded the highest amounts, lipids accounting for the 33 % of biomass on dry basis. 1H-NMR analysis revealed that the lipid extract did not contain detectable free fatty acids and was composed mostly of triacylglycerols. Lipid composition, determined by GC-MS analysis, was similar to plant oils, and may be optimal feedstock for biodiesel production, being dominated by monounsaturated C16 and C18. As in other oleaginous yeasts, lipid production by *C. freyschussii* sp. increased with the increase of the C:N ratio of the medium, but growth was inhibited at glycerol concentrations higher than 40 g/L. As a result, lipid production was the highest with 40 g/L glycerol, yielding 4.7 g/L lipids, with a mean volumetric productivity of 0.15 g/L/h. In order to prevent growth inhibition over 40 g/L glycerol and extend the lipogenic phase, different fed-batch strategies were tried. The best performing processes took advantage from the feeding with concentrated media exhibiting the same C:N ratio of the basal medium, leading to very productive high cell density cultures. With the continuous feeding of 20X-concentrated medium, 29 g/L lipids (i.e. the 32 % of biomass) were obtained in 100 h of cultivation, with a mean volumetric productivity of 0.30 g/L/h. The values herein reported are among the highest yield and productivity values ever obtained for fermentative processes exploiting oleaginous fungi to produce lipids from glycerol. Therefore, *C. freyschussii* could be considered as an interesting microorganism to convert glycerol into microbial oils for biofuel industry.

1. Introduction

Biodiesel is a mixture of fatty acids (FAs) methyl esters derived from triacylglycerols (TAGs) and it is regarded as a major resource to face high energy prices and potential depletion of fossil oils reservoirs. Plant oils are still the major feedstock for biodiesel production, but encounter limitations regarding their availability at competitive price, that decrease the attractiveness of biodiesel as a competitive alternative to petroleum-based fuel. Therefore, other oil sources have been increasingly explored in order to meet the increasing demand of biodiesel production. During the past decade, heterotrophic oleaginous microorganisms have triggered significant attention and the utilization of this oleaginous biomass has been successfully exploited as a source of TAGs for the production of biodiesel (Azocar

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et al., 2010; Liu and Zongbao, 2007; Zhu et al., 2008). In fact, the biomass of oleaginous microorganisms is an optimal and abundant source of TAGs which exhibit similar FAs composition and energy value to plant oils. Microbial lipids have also many advantages (short life cycle, low affection by venue, season and climate, easy to scale-up) that promise to overcome many limitations of plant oils, but have necessarily to be produced from inexpensive substrates with high yield and productivity (Azocar et al., 2010; Liu and Zongbao, 2007; Rossi et al., 2011).
Among heterotrophic microorganisms, oleaginous yeasts have been reported as good producers of lipids, mostly consisting of TAGs. Oleaginous yeasts are capable to synthesize and accumulate high amounts of neutral lipids (up to the 70 % of biomass weight), which are stored in specialized intracellular compartments known as lipids bodies (Czabany et al., 2007). Appropriate process conditions are required by these organisms to induce lipogenesis and to produce such amounts of storage lipids, the most efficient condition being the occurrence of nitrogen limitation in presence of an excess of carbon source (Granger et al., 1993; Ratledge and Wynn, 2002). A few species of oleaginous yeasts have been identified within the genera *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporodium*, *Cryptococcus*, and *Lypomyces* and have investigated in depth (Ageitos et al., 2011; Li et al., 2008; Amaretti et al. 2010; Rossi et al., 2011). Nonetheless, an increasing number of yeasts have been recently recognized as TAGs-producers (Rossi et al., 2009) and environmental yeasts still represent an understudied source of potentially oleaginous strains. In this study, 40 environmental yeasts were screened for the ability to grow and produce intracellular lipids using glycerol, in order to develop a microbial process for TAGs production. In fact, glycerol represents an optimal costless carbon source for heterotrophic oleaginous microorganisms, since it is the main waste-product of biodiesel manufacturing and is getting available in increasing amounts (da Silva et al., 2009).

2. Materials and methods

2.1 Strains and culture conditions

Forty environmental yeast strains belonging to the species *Aureobasidium pullulans*, *Candida castellii*, *Candida freyschussii*, *Candida maltosa*, *Candida sake*, *Cryptococcus gilvescens*, *Kluyveromyces bacillosporus*, *Kluyveromyces lactis*, *Kluyveromyces lodderae*, *Kluyveromyces marxianus*, *Pichia farinosa*, *Rhodotorula laryngis*, *Saccharomyces castellii*, *Saccharomyces cerevisiae*, *Saccharomyces dairenensis*, *Saccharomyces exiguous*, *Saccharomyces spencerorum*, and *Zygosaccharomyces rouxii* were obtained from the Industrial Yeasts Collection DBVPG (University of Perugia, Italy, 2012), or from our own collection. The strains were aerobically cultured at 30 °C in YPD broth and maintained at 4 °C in slants of YPD agar (BD Difco, Sparks, MD, USA). Lipid production was evaluated in GMY that contained 40 g/L glycerol, 8 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 3 g/L yeast extract (BD Difco), and 0.1 ml PTM1 microelements solution (Rossi et al. 2009; Zhang et al. 2000). To investigate the effect of the C:N ratio, batch experiments were carried out in GMY medium containing 4, 8, 16, 24, 40, 80, and 160 g/L glycerol. All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany).

2.2 Batch and fed-batch experiments and bioreactor operation

Batch experiments were carried out in a benchtop bioreactor (Labfors, Infors, Bottmingen, Switzerland) with 2 L of GMY medium containing 40 g/L glycerol. The culture, that was inoculated 5 % v/v with a 24-h seed culture grown in GMY containing 4 g/L glycerol, was kept at 30 °C, sparged with 1 v/v/min filter-sterilized air, and stirred at 150 to 900 rpm to keep the DOT at 20 %. Samples were collected periodically to monitor the growth and to analyze glycerol and lipids. Cell counts were quantified in a Bürker chamber and biomass dry weight was determined gravimetrically using pre-weighed cellulose nitrate membrane filters. Glycerol was analyzed by HPLC-RID (Amaretti et al. 2010).
Fed-batch experiments were initiated batchwise in 2 L GMY medium containing 40 g/L glycerol. As the culture entered into the stationary phase, one of the following feeding modes was applied: i) pulses of 400 g/L glycerol, repeatedly given to reinstate 40 g/L glycerol whenever the carbon source was exhausted; ii) pulses of 10X-CMM medium (containing 400 g/L glycerol, 30 g/L yeast extract, 8 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.1 ml/L PTM1 solution), repeatedly given to reinstate 40 g/L glycerol; iii) continuous feeding 20X-CMM medium (10X-CMM containing 800 g/L glycerol and 60 g/L yeast extract), to provide 5.5 g/L/h glycerol.

2.3 Lipid analysis

Biomass from 50 mL culture samples was harvested, washed with distilled water, frozen at -80 °C and lyophilized. Lipids were extracted and TAG composition was determined according to Rossi et al. (2009). The unsaturation index (UI) was calculated as the number of the double bonds of FAs multiplied by their relative amount.

2.4 Statistical analysis

All values are means of three separate experiments. Differences in means were analyzed using ANOVA for independent measures, followed by Tukey post hoc comparisons. Differences were considered statistically significant for $P < 0.05$.

3. Results

3.1 Screening of unconventional yeasts for lipid production from glycerol

Forty yeast strains belonging to 18 species were screened for the ability to grow and produce intracellular lipids in the glycerol-rich GMY medium. Only *Candida freyschussii* DBVPG 6208, *Pichia farinosa* L19, and *Saccharomyces spencerorum* DBVPG 6746, depleted 40 g/L glycerol within 120 h of growth at 30 °C and gave rise to intracellular lipid bodies.

Table 1: Biomass and lipid production by120-h cultures of C. freyschussii DBVPG 6208, P. farinosa L19, and S. spencerorum DBVPG 6746 in GMY medium. Values are significantly different (P < 0.05, n = 3).

Strain	Total biomass g/L	Biomass lipids g/L	Lipids / biomass content %	Lipids / glycerol conversion %
C. freyschussii DBVPG 6208	11.9	3.2	26.4	7.9
P. farinosa L9	9.5	1.7	17.4	4.1
S. spencerorum DBVPG 6746	6.4	2.4	37.4	6.1

C. freyschussii DBVPG 6208 exhibited the highest ($P < 0.05$) biomass concentration (comprehensive of cellular lipids) and gave the highest lipid production, in terms of lipid concentration, lipid content of biomass, and glycerol conversion to lipids (Table 1). 1H-NMR analysis revealed that the lipid extract from *C. freyschussii* did not contain detectable free FAs and was composed mostly of TAGs. GC-MS analysis revealed that TAGs from *C. freyschussii* were similar to plant oils in terms of FAs composition. The FAs profile was dominated by C16 and C18 FAs (accounting for 36 and 64 %, respectively), bearing on the average one unsaturation (UI = 0.95).

3.2 Lipid production by *C. freyschussii*

Shake-flask batch cultures of *C. freyschussii* DBVPG 6208 were carried with different initial glycerol concentrations in the range from 4 and 160 g/L. Biomass was harvested and analyzed for the lipid content as soon as glycerol run out, always within 30 h for cultures grown with 4 to 40 g/L glycerol. 80 and 160 g/L negatively affected both growth, that become slower (30 and 130 g/L glycerol remained unutilized after 72 h, respectively), and lipid production. Based on final cell counts, the cultures were nitrogen limited with 16 g/L glycerol and more (Table 2). Both the lipid production and the lipid content of biomass were positively affected by the increase of glycerol concentration from 4 to 40 g/L. Therefore, 40 g/L glycerol gave the highest lipid production (4.6 g/L), lipid content of biomass (33 %), and lipid volumetric productivity (0.15 g/L/h) in 30-h batch processes.

Bioreactor batch cultures were carried out to study the kinetics of lipid accumulation by *C. freyschussii* DBVPG 6208. Growth and lipid production in nitrogen limited GMY medium (40 g/L glycerol) proceeded through two consecutive phases (Table 3). During the first 10 h of cultivation, exponential balanced growth occurred and approx. 12 g/L glycerol were consumed. In this phase, a substantial increase of cell counts and biomass concentration occurred (from 1.0e7 to 1.1e9 cells/ml and from 0.2 to 6.1 g/L, respectively) and scarce production of intracellular lipids took place. In fact, 1.1 g/L lipids were produced at the rate of 0.11 g/L/h, accounting for the 18 % of biomass weight. At the end of the

balanced growth, the glycerol excess continued to be consumed and get exhausted after 30 h of cultivation. During this phase, cell counts did not increase and the carbon source was directed toward the synthesis of cellular storage lipids. Lipid production occurred with an increased rate (0.18 g/L/h) and yielded 4.7 g/L lipids, accounting for the 32 % of biomass, after 30 h.

Table 2: Effect of glycerol concentration on biomass and lipid production by C. freyschussii DBVPG 6208. Values are significantly different (P < 0.05, n = 3), unless they have a common superscript.

Glycerol	Cells	Total biomass	Biomass lipids	Lipids / biomass content	Lipids / glycerol conversion
g/L	Cells/mL	g/L	g/L	%	%
4	$3.9 \cdot 10^{8}$	3.0	0.4	15	11.0 [a]
8	$8.0 \cdot 10^{8}$	4.7	0.9	22	12.0 [a]
16	$9.4 \cdot 10^{8}$ [a]	7.4	1.7	24	10.7 [a]
24	$1.1 \cdot 10^{9}$ [a]	9.1	2.7	29	11.1 [a]
40	$1.1 \cdot 10^{9}$ [a]	13.9	4.6	33	10.2 [a]

Table 3: Batch cultures of C. freyschussii in GMY medium (40 g/L glycerol): biomass and lipid production at the end of the balanced growth and the lipogenic phase. Values are significantly different (P < 0.05, n = 3), unless they have a common superscript.

Phase	Culture time	Cells	Total biomass	Biomass lipids	Lipids / biomass content	Lipids volumetric productivity
		Cells/mL	g/L	g/L	%	g/L/h
Balanced growth	0 - 10 h	$1.1 \cdot 10^{9}$ [a]	6.1	1.1	18	0.11
Lipogenic phase	10 - 30 h	$1.2 \cdot 10^{9}$ [a]	14.5	4.7	32	0.18

3.3 Fed-batch cultures of *C. freyschussii*

In order to prevent growth inhibition over 40 g/L glycerol and extend the lipogenic phase, different fed-batch strategies were tried. Fed-batch processes were initiated batchwise in 2 L GMY medium containing 40 g/L glycerol, then different feeding strategies were applied as glycerol run out after 30 h (Table 4). When two glycerol pulses were repeatedly given to reinstate 40 g/L glycerol at the depletion of the carbon source, lipids increased up to 9.1 g/L, accounting for the 33 % of biomass, in 290 h. Nonetheless, glycerol consumption rate progressively decreased, causing the production rate to decline and negatively affecting the volumetric productivity of the process as well (0.03 g/L/h).

Table 4: Overview of performances obtained with batch and fed-batch processes. Values are significantly different (P < 0.05, n = 3), unless they have a common superscript.

Feeding mode		Time	Biomass lipids	Lipids / biomass content	Lipids volumetric productivity
		h	g	%	g/L/h
Batch		30	4.7	32[a]	0.15
Fed-Batch	Glycerol, pulsed	300	9.1	33[a]	0.03
Fed-Batch	10X-CMM, pulsed	115	20	32[a]	0.22
Fed-Batch	20X-CMM, continuous	100	29	32[a]	0.30

Since glycerol pulses did not improve the productivity, 30-h batch cultures were fed with concentrated media (10X-CMM and 20X-CMM) exhibiting the same C:N ratio (48:1) of GMY medium. When 4

repeated pulses of 10X-CMM were given, the culture grew up to $4.0 \cdot 10^9$ cell/mL and 65 g/L biomass. The high cell density culture consumed glycerol and produced lipids with improved rates, and generated 20 g/L lipids, accounting for the 32 % of biomass, with a mean volumetric productivity of 0.22 g/L/h. Conversely, the continuous feeding of 20X-CMM (5.5 g/L/h) for 70 h caused the culture to grow up to 5.6e9 cells/ml and to produce 29 g/L lipids, accounting for the 32 % of biomass, with a mean volumetric productivity of 0.30 g/L/h.

4. Discussion

Oleaginous yeasts have been demonstrated to be very efficient in the accumulation of intracellular TAGs which may be exploited as feedstock for biodiesel manufacturing. Nowadays microbial TAGs are still too costly to compete with plant oils, but it is expected that they can be used by the biofuel industry in the future if they will be produced from cheap substrates (e.g. crude glycerol, the main waste product of biodiesel industry) with high yield and productivity (Liu and Zongbao, 2007; Zhu et al., 2008; Azocar et al., 2010). This study succeeded in identifying an environmental strain of *Candida freyschussii* as an oleaginous yeast that may find application in TAGs production from glycerol. The strain was capable to grow abundantly and rapidly on glycerol and to accumulate lipids within intracellular lipid bodies. Due to the composition of its storage lipids, the oleaginous biomass of *C. freyschussii* may represent a valuable feedstock for biodiesel production (Satyarthi et al., 2009). In fact, the storage lipids of this yeast did not contain detectable amounts of free FAs and were mostly composed by TAGs with a FAs profile similar to plant oils and particularly rich in the monounsaturated FAs.

Nitrogen limitation is the most efficient condition for inducing lipogenesis in oleaginous yeasts (Granger et al., 1993; Ratledge and Wynn, 2002). During the growth phase, nitrogen is necessary for the synthesis of proteins and nucleic acids, while the carbon flux is distributed among energetic and anabolic processes yielding carbohydrates, lipids, nucleic acids and proteins. When nitrogen gets limited, the growth rate slows down and the synthesis of proteins and nucleic acids tends to cease. In non-oleaginous species, the carbon excess remains unutilized or is converted into storage polysaccharides. On the contrary, oleaginous species preferentially channel the carbon excess toward lipogenesis and accumulate TAGs within the lipid bodies (Ratledge and Wynn, 2002; Granger at al., 1993), the maximum theoretical yield of lipid production from hexoses or glycerol being approx. 30 % w/w (Rossi et al. 2011).

Consistently, growth and lipid production by *C. freyschussii* in nitrogen limited media occurred through two consecutive phases and the extent of lipid accumulation was positively affected by the increase of glycerol concentration. On the other hand, high glycerol concentrations inhibited growth and, thus the best performing batch process was obtained with 40 g/L glycerol. In these conditions, 4.7 g/L lipids (i.e. the 33 % of biomass) were produced in 30 h, with a volumetric productivity of 0.15 g/L/h and with a 10 % conversion of glycerol.

In order to prevent growth inhibition over 40 g/L glycerol and extend the lipogenic phase, different fed-batch strategies were tried. The best performing fed-batch processes took advantage from the feeding of concentrated media exhibiting the same C:N ratio of GMY medium, leading to highly productive high cell density cultures. With the continuous feeding of 20X-CMM (5.5 g/L/h glycerol) 29 g/L lipids (i.e. the 32 % of biomass) were produced in 100 h of cultivation, with a mean volumetric productivity of 0.30 g/L/h. The values herein reported are among the highest yields, productivities, and conversions ever obtained for fermentative processes exploiting oleaginous fungi to produce lipids from glycerol (Meesters et al., 1996; Easterling et al. 2009; Makri et al. 2010; Liang et al., 2010). Therefore, *C. freyschussii* could be considered as a promising microorganism for the production of single cell oils. This strain is intriguing, since it exploits the waste-product of biodiesel industry to produces TAGs that exhibit the optimal features for biodiesel manufacturing itself. Furthermore, the present study is the first proposed biotechnological application for *C. freyschussii*, an understudied species of *Candida* yeast genus.

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