Production of Microbial Lipids by \textit{Yarrowia Lipolytica}

Received for publication, May 14, 2015
Accepted, October 4, 2015

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Abstract

Experiments performed in this work have aimed to establish conditions that allow fermentation
directing with a Yarrowia lipolytica strain to the biosynthesis of lipids at a high and reproducible level.
Evaluation of lipid accumulation was achieved by Soxhlet method. The effects of several culture
conditions on growth and lipid production of Yarrowia lipolytica were studied in this research. Lipid
accumulation during fermentation was significantly influenced by the medium composition, the most
important factor proving to be the C/N ratio. Results showed that maximum productivity, of 7.5 g/L
lipid, corresponding to 50.3% of lipid in dry biomass (g/g), was accomplished in the presence of
-glucose 40 g/L, (NH₄)₂SO₄ as nitrogen source and a 50:1 C/N ratio. This study allowed the
identification of parameters affecting the production of lipids by Yarrowia lipolytica. Optimum
conditions for biosynthesis of lipids were obtained using the following parameters: inoculum 10% (v/v),
temperature 28°C, initial pH 6, agitation rate 240 rpm, intense aeration (1.0 l air/l medium/minute) and
duration 96 hours. Results showed that Yarrowia lipolytica exhibited impressive cell growth and lipid
production, accumulating up to 50% of lipid in dry biomass.

Keywords: fermentation, lipid, C/N ratio, Yarrowia lipolytica

1. Introduction

Biotechnology of lipids covers the microbial production and the biotechnological
transformation of lipid and lipid-soluble compounds. Storage lipid in the form of
triacylglycerols and their different fatty acid types are the main targets for biotechnological
product development. Biotechnological processes have recently started entering the
oleochemical business [1]. Positive examples for biotechnological developments are found
mainly in the field of specialty products for the cosmetic, health food and pharmaceutical
industry, with microbial steroid transformation representing a prominent example for the
production of active pharmaceutical ingredients [2].

The aim of all biotechnological processes is to obtain products that are either cheaper
than can be obtained from other sources. Within the field of lipids, the opportunities to
produce triacylglycerol lipids are limited to the highest valued materials [3].

Not all microorganisms can be considered as abundant sources of oils and fats, though,
like all living cells, microorganisms always contain lipids for the normal functioning of
membranes and membranous structures. Those microorganisms that do produce a high
content of lipids may be termed “oleaginous” in parallel with the designation given to oil-bearing plant seeds. Of about 600 different yeast species, only 25 or so are able to accumulate more than 20% lipids; of 60,000 fungal species, fewer than 50 accumulate more than 25% lipid [4]. The lipids which accumulate in oleaginous microorganisms are mainly triacylglycerols. Of current interest are oils containing the polyunsaturated fatty acids: g-linolenic acid (18:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6). With few exceptions, oleaginous microorganisms are eukaryotes and thus representative species include algae, yeasts, and molds. Bacteria do not usually accumulate high amounts of triacylglycerols [1].

The key to lipid accumulation lies in allowing the amount of nitrogen supplied to the culture to become exhausted within about 24-48 h. Exhaustion of nutrients other than nitrogen can also lead to the onset of lipid accumulation [1] but, in practice, cell proliferation is effected by using a limiting amount of N (usually NH₄ or urea) in the medium. The excess carbon which is available to the culture after N exhaustion continues to be assimilated by the cells and, by virtue of the oleaginous organism possessing the requisite enzymes, it is converted directly into lipids [2].

The essential mechanism which operates is that the organism is unable to synthesize essential cell materials because of nutrient deprivation and thus cannot continue to produce new cells. Because of the continued uptake of carbon and its conversion to lipids, the cells can then be seen to become engorged with lipid droplets [2].

The medium has to be formulated with a high carbon-to-nitrogen ratio, higher than 20:1. The culture must be grown at a rate which is about 25-30% of the maximum. Under this condition, the concentration of nitrogen in the medium is virtually nil and then the organism has sufficient residence time within the chemostat to assimilate the excess carbon and convert it into lipids [5].

At the end of the lipid accumulation phase, it is essential that the cells are promptly harvested and processed. If glucose, or other substrate, has become exhausted at the end of the fermentation, then the organism will begin to utilize the lipids, as the role of the accumulated material is to act as a reserve storage of carbon, energy, and possibly even water [2].

For this research, oleaginous yeast *Yarrowia lipolytica* was chosen because it is one of the most extensively studied “nonconventional” yeasts. This yeast is able to accumulate large amounts of lipids (in some cases, more than 50% of its dry weight [7, 8]. Several technologies including different fermentation configurations have been applied for single-cell oil production by strains of *Yarrowia lipolytica* grown on various agro-industrial by-products or wastes (i.e., industrial fats, crude glycerol, etc.) [7, 9].

### 2. Materials and methods

**Microorganism and maintenance**

The laboratory strain (wild type) *Yarrowia lipolytica* used in this study was provided by the Collection of Microorganisms of the Faculty of Biotechnology – University of Agronomical Sciences and Veterinary Medicine, Bucharest.

The strain was maintained by periodic transfer on YPD agar slants containing (g/L): glucose 20.0, bactopeptone 20.0, yeast extract 10.0, agar 15.0, pH 6. *Yarrowia lipolytica* was incubated for 48 hours at 30°C and maintained at 4°C for maximum three months [10, 11].
Inoculum, growth medium
From a YPD agar slant, a first preinoculum was inoculated into YPD medium (20 mL in 50 mL Erlenmeyer flasks) and incubated in a rotary shaker for 12 h at 30°C and 220 rpm. Cells from the preculture were used to inoculate (10%) the fermentation medium (50-200 mL in 500 mL Erlenmeyer flasks). Fermentation was carried out in different conditions, in order to determine the optimum conditions for lipid biosynthesis [10, 11].

Fermentation medium has the following basic composition (g/L): glucose, lactose, sucrose, fructose, maltose (as carbon sources) 10-50; KH₂PO₄, 5.0; Na₂HPO₄, 2.5; MgSO₄, 1.5; organic nitrogen sources (peptone, yeast extract) and inorganic sources (NH₄NO₃, NaNO₃ and (NH₄)₂SO₄) 1.0; CaCl₂, 0.1 [10, 11].

Fermentation conditions
Fermentations were carried out batch wise in 500 mL Erlenmeyer flasks, containing 50, 100, 150, 200 mL of fermentation medium [12]. The sterilized medium was inoculated with 10% (v/v) preculture and incubated at 25, 30, 35°C, initial pH 5.0, 5.5, 6.0, 6.5 and 7.0, for 96 hours, in a rotary shaker, at 150, 180, 210, 240 rpm [13, 14].

Determination of yeast growth and biomass
Biomass concentration was determined measuring the optical density (OD) of the fermentation medium by visible spectrophotometry (Helios γ-Thermo Electron Corporation-USA) at 570 nm and 8 hour intervals [15, 16].

For absorbance readings, 0.5 mL of culture medium were diluted in water (1:25) and measured with a spectrophotometer, at 570 nm. Calibration curves were performed using absorbance readings [16].

The quantity of yeast cells was assessed by dry weight determination [16].

Biomass separation
At the end of fermentation process, the cultivation medium was centrifuged for 20 minutes at 4000 rpm. The sediment was washed with ethanol (95%) and hexane, in order to remove extracellular fat from the cell surface. The wet biomass was dried at a temperature of 60°C to constant weight [7].

Lipid extraction and determination
Lipids were extracted by treating the dried biomass with chloroform: methanol 2:1 in a Soxhlet extraction apparatus and estimated gravimetrically [7, 17]. Quantitative determination of lipids was done by solvent evaporation and weighing the remaining product [3, 7].

Other analytical methods
Residual sugar content was determined by 3,5-dinitro-salicylic acid method, in order to monitor the glucose content at the end of the fermentation process [16].

3. Results and discussions
The effects of several culture conditions on growth and lipid production of *Yarrowia lipolytica* is given below.
Influence of carbon sources on biomass and lipid production

To study the effects of carbon sources on lipid production and biomass, various available substrates were used: glucose, lactose, sucrose, fructose and maltose. Cells were cultivated in a rotary shaker incubator at 30°C, 30 g/L carbon source, pH 6 and 220 rpm, for 96 hours. The results show that all tested substances were suitable carbon sources for the growth and lipid accumulation of the strain, among which glucose was the most suitable. The lipid production was 4.9 g/L, followed by sucrose with a yield of 4.1 g/L. Therefore, glucose was chosen as carbon source in the following experiments. Figure 1 shows the effect of carbon sources on lipid production and biomass.

Influence of nitrogen sources on biomass and lipid production

The effect of nitrogen sources on lipid production and biomass is shown in Table 1. Compared with organic nitrogen sources (peptone, yeast extract), when inorganic ones (NH₄NO₃, NaNO₃ and (NH₄)₂SO₄) were used, the cells grew more quickly and reached a higher lipid productivity. Among the inorganic nitrogen sources, (NH₄)₂SO₄ was the most suitable and the lipid productivity and biomass was 5.6 g/L and 13.5 g/L, respectively. It is reasonable that...
(NH₄)₂SO₄ was chosen as nitrogen source in following experiments. Experiments were carried out at 30°C, glucose 40 g/L, nitrogen source 1 g/L, pH 6 and 220 rpm, for 96 hours.

**Table 1. Effect of nitrogen source on cell growth and lipid production**

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Biomass (g/L)</th>
<th>Lipid production (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>11.0</td>
<td>3.8</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>11.3</td>
<td>3.6</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>12.5</td>
<td>4.9</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>12.3</td>
<td>4.8</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>13.5</td>
<td>5.6</td>
</tr>
</tbody>
</table>

**Influence of C/N ratio on lipid production**

Experiments were conducted covering a large range of C/N ratios from 10:1 to 50:1. The effect of the C/N ratio on biomass at different sugar concentrations is shown in Figure 3. As the C/N ratio increases, the biomass increases rapidly, to reach a maximum value at a C/N ratio of 50:1. At C/N ratios below this, the biomass declines.

The effect of the C/N ratio on lipid productivity is shown in Figure 3. As the C/N ratio increases, the accumulation of lipids increases to reach a maximum, giving an optimum C/N ratio for lipid production of 50:1. The highest value of lipid reached 6.1 g/L. From Figure 4, the effect of the C/N ratio on lipid productivity appears to be more pronounced at high values. At low C/N ratio, nitrogen limiting conditions necessary for lipid overproduction are not attained, resulting in low quantity of lipids, even though the biomass may be high.

**Influence of temperature on biomass and lipid production**

In order to investigate the effects of incubation temperature on biomass and lipid production, experiments were carried out at varying temperature, 25, 28, 31 and 35°C. Experiments were carried out at 220 rpm, pH 6, for 96 hours. The result (Figure 4) shows that both the biomass and lipid production enhanced with an increase in culture temperature at 28-31°C. The highest lipid production was obtained at 28°C (biomass yield=13.6 g/L, lipid production=6.2 g/L, corresponding to 45.6% of its dry weight), whereas at 25°C and 35°C, lower amounts of lipids were accumulated. Therefore, for further experiments, a temperature of 28°C was selected as optimum parameter for lipid production.
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Influence of initial pH on biomass and lipid production

To study the effects of initial pH on biomass and lipid production, *Yarrowia lipolytica* was incubated at 28°C, 220 rpm and varying initial values of pH (5.0, 5.5, 6.0, 6.5 and 7.0). Substantial growth was observed (Figure 5) at pH 6 and 6.5 (13.5 and 12.6 g/L, respectively), whereas lipid ($L$) accumulation was favoured at pH 6 ($Y_L$=6.3 g/L). At pH 5 and 7, restricted microbial growth was observed. The pH decreased slightly during growth, as low amounts of organic acids were produced. The result suggests that initial pH had a significant effect on the lipid accumulation and the optimum initial pH value was 6.0.

Influence of culture volume on biomass and lipid production

The study of this parameter was performed in fermentation experiments carried out in 500-mL Erlenmeyer flasks, using 50, 100, 150, 200 mL medium, in the following conditions: temperature 28°C, pH 6, for 96 hours, 240 rpm.

The effects of medium volume on biomass and lipid production are shown in Figure 6. Both the biomass and lipid yield decreased with an increase in culture volume. This suggests that the strain is an aerobic strain because the less culture volume the better aeration provided in the flask. When culture volume was 50 and 100 mL/500 mL, the biomass yield was 14.5 and 14.3 g/L, respectively. Considering economic benefits, small volumes of medium is not advantageous. Therefore, 100 mL (in a 500 mL flask) was selected as the suitable culture volume for lipid biosynthesis.
In order to investigate the effects of agitation speed on biomass and lipid biosynthesis, the yeast *Yarrowia lipolytica* was incubated at 28°C, pH 6 and varying agitation speed (150, 180, 210, 240, 280 rpm). The results (Figure 7) show a gradual and significant increase in biomass and lipid content with the increase of agitation speed during the bioprocess, up to 240 rpm. At 240 rpm, the biomass and lipid production were 14.7 and 7.3 g/L, respectively, corresponding to 49.7% (g/g) lipids of dry biomass. Higher agitation speed was advantageous to oxygen supply, resulting only a higher biomass yield. Therefore, further experiments will be conducted at 240 rpm.

Verification of the optimum conditions for lipid production in a stirred-tank bioreactor

Based on the results obtained from the previous experiments, further investigations aimed at the verification of the reproducibility of optimum conditions for biosynthesis of lipids in a stirred-tank bioreactor. Figure 8 shows the time courses of cell growth and lipid production under the optimized culture conditions. The concentration of residual sugars in the fermentation medium decreased to 15.2 g/L at 48 h, and 0.5 g/L at 96 h. The amount of lipids increased from 24 h to 96 h, when it reached the maximum productivity (7.5 g/L). The quantity of biomass kept rising from 12 h to 96 h, and reached the maximum weight (at 96 h, 14.9 g/L). It was obvious that the accumulation of biomass and lipids were achieved during the stationary growth phase.
4. Conclusions

The experiments performed in order to establish the influence of the medium composition on the biosynthesis of lipids by *Yarrowia lipolytica*, pointed out that maximum production (7.5 g/L) was accomplished in the presence of glucose 40 g/L, (NH$_4$)$_2$SO$_4$ as nitrogen source and a 50:1 C/N ratio.

This study also allowed the identification of parameters affecting the production of lipids by *Yarrowia lipolytica*. Optimum conditions for biosynthesis of lipid were obtained using the following parameters: inoculum 10% (v/v), temperature 28°C, initial pH 6, agitation rate 240 rpm, aeration corresponding to a medium/flasks ratio of 1:5 (v/v) at laboratory level and 1.0 l air/l medium/minute in bioreactor level, fermentation time 96 hours.

*Yarrowia lipolytica* exhibited impressive cell growth and lipid production, accumulating up to 50% of lipids in dry biomass.

5. Acknowledgements

This research was carried out with the support of the program “Invest in people!”, project co-financed by the European Social Fund POSDRU 2007-2013, POSDRU/159/1.5/S/132765

6. References