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High cell density fed-batch fermentation for the production of a microbial lipase

M.N.I. Salehmin[a], M.S.M. Annuar[a,*], Y. Chisti[b]

[a] Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[b] School of Engineering, PN 456, Massey University, Private Bag 11 222, Palmerston North, New Zealand

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ABSTRACT

Extracellular lipase of the yeast *Candida rugosa* was produced via high cell density fed-batch fermentations using palm oil as the sole source of carbon and energy. Feeding strategies consisted of a pH-stat operation, foaming-dependent control and specific growth rate control in different experiments. Compared to foaming-dependent feeding and the pH-stat operation, the specific growth rate control of feeding proved to be the most successful. At the specific growth rate control set at 0.05 h^{-1}, the final lipase activity in the culture broth was the highest at ~700 U L^{-1}. This was 2.6-fold higher than the final enzyme activity obtained at a specific growth rate control set at 0.15 h^{-1}. The peak enzyme concentration achieved using the best foaming-dependent control of feeding was around 28% of the peak activity attained using the specific growth rate control of feeding at 0.05 h^{-1}. Similarly, the peak enzyme concentration attained using the pH-stat feeding operation was a mere 9% of the peak activity attained by specific growth rate control of feeding at a set-point of 0.05 h^{-1}. Fed-batch fermentations were performed in a 2 L stirred-tank bioreactor (30 °C, pH 7) with the dissolved oxygen level controlled at 30% of air saturation.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are versatile enzymes that are widely used in production of fine chemicals and other industrial processes [1–8]. All commercially relevant lipases are produced as extracellular enzymes via microbial fermentation processes. Microbial lipases have been discussed extensively in the literature [9–11].

For a given producer species, the lipase titer depends on the biomass concentration in the broth, the nutritional characteristics of the culture medium and the other production conditions. A high concentration of the microbial biomass is desired in a fermentation to maximize the volumetric productivity of the enzyme and attain a high titer. High cell density fermentations (HCDF) have the potential for enhancing lipase productivity and titer [12]. HCDFs do have drawbacks [13]. For example, the control of culture conditions (e.g. dissolved oxygen concentration) can be difficult in an HCDF operation and this may adversely impact the production [12]. Notwithstanding their shortcomings, high cell density operation is considered economically attractive overall [13].

A high cell density production process typically involves fed-batch fermentation [12]. The feeding is controlled to ensure that the substrate concentration does not build up to an inhibitory level [12,14,15]. Various feeding strategies have been expounded in the literature, including a constant rate feeding and specific growth rate control feeding [12]. In specific growth rate control, the feed rate increases exponentially with time so that the specific growth rate is maintained at some predetermined value. Other feeding strategies include pH-stat operation and foam-dependent feeding. In pH-stat operation, the culture is fed in response to a change in pH from a specified value. In foam-dependent feeding, the complete consumption of a foam-suppressor substrate such as a vegetable oil may lead to onset of foaming and this becomes the signal for further feeding. Prior work on high cell density fermentations for the production of lipases has been reviewed [12].

Several published works reported lipase production from *Candida rugosa* ATCC 14830 [16–18]. Various feeding strategies with oleic acid as carbon source were studied for highest lipase yield. Constant substrate feeding rate and constant specific growth rate control feeding were employed in these works [16–18]. Constant specific growth rate control feeding at low rate yielded high cell concentration and lipase production in this strain as shown by Kim and Gordillo [16,18]. A relatively high cell concentration of 90 g L^{-1} and extracellular lipase activity of 23.7 U mL^{-1} were obtained

* Corresponding author. Tel.: +60 379674003; fax: +60 379674178.
E-mail addresses: suffian_annuar@um.edu.my, suffian72@gmail.com (M.S.M. Annuar).

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from using this feeding strategy [16]. On the other hand, similar substrate feeding method resulted in a much lesser cell concentration at 6.9 g L^{-1} but high extracellular lipase activity at 117 U mL^{-1} [18]. Yet in another similar study, good lipase yield was obtained using constant substrate feeding strategy instead of constant specific growth rate control [17]. This clearly indicated that no single feeding strategy could be made universally applicable for producing high lipase yield even from similar microbial strain.

To date no other published work dealt with lipase production from *Candida rugosa* ATCC 10571. Likewise, even though the utilization of palm oil as carbon substrate for the production of lipase was reported in other microorganisms [20–23], no literature is available on the use of palm oil in HCDF to produce microbial lipase, and its application as a supplied carbon source in fed-batch fermentations. While the physical behavior of palm oil in aqueous fermentation medium may be approximated with the behavior of other immiscible substrate such as pure oleic acid, its chemical composition as triglycerides that are made up of different types of fatty acids is in contrast to oleic acid. Thus, it is worthwhile to examine the behavior of fed-batch HCDF when palm oil is used as sole carbon and energy source for growth and lipase by *C. rugosa* ATCC 10571. This would certainly add to the comparison data for extracellular lipase yield for the particular yeast strain and the implemented feeding strategies.

Consequently, the present study attempted to compare three feeding strategies, i.e., the pH-stat operation, the foaming-dependent control of feeding and the specific growth rate control feeding, in HCDF of the yeast *Candida rugosa* ATCC 10571 for producing lipases using palm oil as the sole source of carbon and energy. *C. rugosa* lipases have been reviewed by Domínguez de María et al. [24]. Production of native and recombinant lipases by *C. rugosa* has been reviewed by Ferrer et al. [25].

2. Materials and methods

2.1. Yeast strain and growth medium

The yeast *Candida rugosa* ATCC 10571 was obtained from the American Type Culture Collection (ATCC) and maintained on YM agar medium. The medium contained (per liter): yeast extract 3.0 g; malt extract 3.0 g; peptone 5.0 g; glucose 10.0 g; and agar 15.0 g. The same medium without the agar was used in shake flask cultures. The production medium used in the bioreactor contained (per liter): 5.74 g K_2HPO_4; 3.7 g KH_2PO_4; 1.0 mL of a trace element solution [18], 10 mL of 0.1 M $MgSO_4{\cdot}7H_2O$; 2 g $(NH_4)_2SO_4$; 4×10^{-4} g inositol; 2×10^{-4} g thiamine, 8×10^{-4} g biotin; and 10 mL of commercial palm oil as a sole source of carbon and energy. The solution of magnesium salt was sterilized separately by autoclaving (121 °C, 15 min). Solutions of trace elements and vitamins were filter sterilized. Palm oil was added nonsterile.

2.2. Shake flask culture

Inocula were produced in a 250 mL shake flask containing 100 mL of the YM liquid medium. The pre-culture was incubated (180 rpm, 25–27 °C) for 28 h. Palm oil (1 mL) was then added and incubation was continued for a further 12 h at 300 rpm. The cells were then harvested by centrifugation (7,650 *g*, 4 °C, 10 min) and rinsed twice, each time by re-suspending in 100 mL of a saline solution (0.9% w/v (g/100 mL) NaCl). The harvested cells were aseptically transferred to the production medium in the bioreactor. The initial biomass concentration (X_0) in the freshly inoculated bioreactor was 0.2–0.3 g L^{-1}.

2.3. Bioreactor cultures

A Biostat® B+ 2 L stirred tank bioreactor (Sartorius Stedim Biotech GmbH, Goettingen, Germany) was used. The initial culture volume (V_0) was 1 L. Batch and fed-batch modes of operation were used in different experiments. In all cases, the culture pH was controlled at 7 using 2 M H_2SO_4 and a 25% (w/v) solution of ammonia. The fermentation temperature was controlled at 30 °C at a fixed aeration gas flow rate of 1 L min^{-1}. The oxygen content in the aeration gas was controlled by mixing oxygen with air such that the dissolved oxygen level in the fermentation broth remained at 30% of air saturation.

2.4. Batch operation

In batch operations, for the above specified culture conditions, the agitation speed of the impeller was set to 400, 600 and 800 rpm in different experiments. The concentration of the biomass and the lipase activity in the culture supernatant were measured via periodic sampling.

2.5. Fed-batch operation

Each fed-batch operation began as a batch operation as above and was switched to the fed-batch mode at 24 h when foaming was observed. Three feeding strategies were tested: a pH-stat operation, a foaming-dependent feeding and specific growth rate control feeding. Foaming was suppressed by adding palm oil with each feeding as an alternative to the commercial antifoam agents. All culture conditions were as previously specified for the batch operation. The agitation speed remained fixed at 600 rpm in all experiments.

In pH-stat feeding, the automatic pH control system of the batch operation was switched off. The limiting substrate (see below) was fed when the pH rose to above 7.0 indicating a depletion of the substrate. For each feeding, 60 mL of the culture broth was pumped out of the bioreactor and replaced with a mixture of 50 mL of double-strength fresh production medium and 10 mL of palm oil so that the final volume of the broth in the bioreactor was 1 L.

In foaming-dependent feeding, the feeding was initiated in response to a massive accumulation of foam on the surface of the culture broth. This apparently occurred because the substrate (palm oil) was consumed and no longer able to provide its antifoaming action. A microscopic examination of the culture samples confirmed an absence of oil droplets whenever massive foaming occurred. Each time foaming occurred, 60 mL of the culture broth was withdrawn and replaced with an equal volume of the fresh medium as explained above for the pH-stat feeding operation.

For the specific growth rate controlled feeding [16,18,26–30], the feed rate F varied exponentially with time according to the following equation

$$F = \frac{\mu X_0 V_0 e^{\mu t}}{X - X_0} \quad (1)$$

In the above equation, μ is the desired constant specific growth rate. The following set point values of the constant specific growth rate (μ) were used in different experiments: 0.05, 0.10 and 0.15 h^{-1}. The initial working volume (V_0) was 1 L and the final volume was 1.5 L. X_0 was the initial concentration of the biomass and X was the biomass concentration at time t. A peristaltic pump (Masterflex model no. 7551-10; www.masterflex.com) was controlled to provide the necessary feeding rate during the exponential feeding operation. The feed was palm oil, the carbon source. Nitrogen was supplied via the ammonia solution that was used for pH control.