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Oxygen transfer strategy modulates the productions of lipase and esterase enzymes by *Candida rugosa*

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ABSTRACT

Different oxygen transfer strategies were employed in a batch bioreactor system to compare the activities, productivities and yields of lipase and esterase enzymes by *Candida rugosa* as well as the biomass accumulation. The fermentation starting with full oxygen saturation and continuing with the level of 30% saturation provided the highest lipase activity whereas continuing with the level of 60% provided the highest esterase activity. Low biomass yield was obtained when the fermentation was conducted at the level of full oxygen saturation. Time courses of the extracellular and intracellular enzyme activities indicated that lipase activity was growth-associated and the cells secreted esterase into the medium after a considerable level of extracellular lipase activity was reached at all oxygen transfer strategies considered.

1. Introduction

Oxygen transfer condition is among the crucial factors that affect the product distribution in fermentation processes. The shortage of oxygen in the fermentation broth can restrict or enhance a metabolite formation by changing metabolic pathways. In some cases, controlled oxygen transfer rates can be required to regulate the oxygen uptake rate by the cells.

Candida rugosa is among the most extensively studied microorganisms by biotechnologists with respect to its powerful lipase (E.C. 3.1.1.3) production capacity. Wide substrate specificity provides *C. rugosa* lipase (CRL) to be successfully used in a variety of hydrolysis and esterification reactions, and its high stereoselectivity and regioselectivity make possible the synthesis of several pharmaceuticals [1]. *C. rugosa* synthesizes and secretes a mixture of lipase isoenzymes differing in biocatalytic properties. It has been established that at least seven genes are involved in the *C. rugosa* lipase-producing machinery and some isoenzymes exhibit esterase (E.C. 3.1.1.1) activity [2]. Additionally, carboxyl-thioesterases were also described and characterized from commercial samples [3,4]. Since *C. rugosa* has a non-universal genetic code [5], difficulties have been arisen in obtaining recombinant CRL to produce the desired isoenzyme. Consequently, many research activities have been diverted into modulating lipolytic enzyme activities towards desired enzyme through changing culture and fermentation conditions or operation mode of bioreactors. As Dominguez de Maria et al. [2] reviewed, carbon source (inducer), kind of operation (batch/fed-batch) and feed rate are important parameters in *C. rugosa* fermentation in terms of quantity and quality of the crude lipase. Despite comprehensive studies on CRL production, those investigate the effects of dissolved oxygen (DO) concentration on the fermentation progress are limited, and, moreover, none of them distinguishes the conditions that enhance lipase and esterase activities individually.

The overview of the literature on the effect of aeration in *C. rugosa* fermentation reveals that DO concentration greatly influences the production of lipase enzyme. It was reported that DO concentration above 20% of the medium saturation was enough to ensure the production of CRL [6]. Sokolovska et al. [7] proposed to use air enriched by pure oxygen in the fermentation of *C. cylindracea*, and reported the flow rates of the gases to maintain the oxygen concentration at the recommended value (20%) for optimum lipase production. Puthli et al. [8] changed the oxygen supply to the bioreactor by changing aeration rate, and observed that higher oxygen concentration beyond the optimum value was detrimental and decreased the biomass production, especially in the late logarithmic phase of growth cycle which in turn affected the overall lipase production. Although oxygen supply conditions are expected to affect not only lipase by also esterase enzyme production by *C. rugosa*, none of the authors have reported the DO concentration or oxygen transfer condition for increased esterase activity.

The aim of the present work is to propose different oxygen transfer strategies (OTSs) that increase lipase and esterase enzyme productions individually in *C. rugosa* fermentation. For all OTSs employed, time variations in extracellular and intracellular lipase

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and esterase activities as well as cell biomass were followed. The overall results indicated that oxygen transfer strategy (OTS) in a bioreactor system was among decisive parameters that affected the activity, productivity and yield of *C. rugosa* enzymes.

2. Materials and methods

2.1. Chemicals

The chemicals were of reagent grade and purchased from commercial suppliers (Merck, Germany; Sigma–Aldrich, USA).

2.2. Microorganism, culture media and conditions

C. rugosa DSMZ 2031 obtained from Deutsche Sammlung von Mikrooganismen and Zellkulturen GmbH (DSM, Braunschweig, Germany) was used in the study. The stock cultures were maintained on universal yeast medium (UYM).

The microorganism incubated at 30 °C for 22 h was inoculated into 100 mL pre-culture medium of the following composition (per liter): 5 g soy peptone, 3 g yeast extract, 3 g malt extract, 10 g dextrose. After incubation at 30 °C and 150 rpm conditions for 22 h in an orbital shaker (Edmund Bühler SM-30, Germany), the cells were transferred to the bioreactor that contained the enzyme production medium (per liter): 2 g triolein, 4 g urea, 15 g KH_2PO_4, 5.5 g K_2HPO_4, 1 g $MgSO_4{\cdot}7H_2O$, 10 mg $FeCl_3{\cdot}6H_2O$ and 10 ml vitamin solution (pH 6.2). The vitamin solution contained per liter 400 mg of thiamine, 2 mg of biotin and 2 g of inositol.

Batch bioreactor experiments were conducted in 2 L bioreactor systems (Sartorius Biostat B Plus, Germany), consisting of temperature, pH, foam, agitation rate, gas flow rate and DO concentration measurements and controls with 1 L working volume. Enzyme production employing different OTSs was carried out at $T = 30$ °C temperature, $N = 500$ rpm agitation rate and Q/V = 0.6 vvm gas flow rate conditions. The pH of the medium was not controlled throughout the fermentation. The changes in extracellular and intracellular activities of lipase and esterase enzymes as well as cell biomass concentration with time were followed. Extracellular and intracellular protease activities were also measured at the end of fermentations.

2.3. Enzyme assays

Alkalimetric final titration was used to determine the lipase enzyme activity by adapting the method by Cernia et al. [9]. The mixture, containing 2.5 mL phosphate buffer solution (0.1 M pH 7.2), 0.5 mL olive oil and 0.1 mL sample, was incubated at 37 °C under magnetic stirring for 30 min. After terminating the reaction with 2.5 mL acetone:ethanol mixture 1:1 (v/v), the solution was titrated with 0.1 M NaOH in the presence of phenolphthalein as indicator. One unit lipase activity (U) was defined as the amount of enzyme that catalyzes the release of fatty acid per min under the conditions mentioned above.

The esterase enzyme activity was measured spectrophotometrically (Shimadzu 1601, Tokyo, Japan) by using *p*-nitrophenyl acetate as substrate [10]. One unit enzyme activity (U) was defined as the amount of enzyme produces 1 μmol *p*-nitrophenol per min at pH 7.5 and 25 °C.

For intracellular enzyme activity assay, the cells were disrupted by adapting the procedure reported by Dalmau et al. [11]. After harvesting by centrifugation (Hettich Rotina 35R) at 12,000 × *g* for 10 min at 4 °C, the cells were washed in Tris–HCl buffer (10 mM, pH 8.0) and resuspended to a 3 mL final volume with the same buffer. The cell suspension was disrupted with glass beads (Biospec Mini-Beadbeater, USA) for 8 periods of 30 s. The disrupted cells were centrifuged at 12,000 × *g* at 4 °C for 10 min and the supernatant was used as the cell extract for the determination of intracellular activity. The cell viability was inspected with a microscope (Olympus CX21FS1, USA) after staining the cells with methylene blue.

Protease activity was assayed spectrophotometrically (Shimadzu 1601) using casein as substrate [12]. One unit of enzyme activity is defined as 4 nmol tyrosine released/min per mL.

Biochemical assays were carried out in duplicate. The range of duplicate values was within 5%.

2.4. Cell biomass concentration

Biomass was determined by dry weight as follows [11]: samples were filtered (0.45 μm), washed with a mixture of dioxane–propionic acid (1:1), and washed with 20 mL of distilled water. The filters were then dried at 85 °C to constant weight.

3. Results and discussion

C. rugosa fermentation was carried out employing four different oxygen transfer strategies to investigate the effect of DO concentration (% saturation) adjustment and control throughout the fermentation on lipase and esterase enzyme activities.

Oxygen transfer strategies employed in the study are depicted in Fig. 1. *OTS I*. The fermentation was conducted at the level of full oxygen saturation; no decrease in DO concentration was allowed by continuous feeding of pure oxygen into the medium. *OTS II*. The fermentation was started with full oxygen saturation, a decrease was allowed in DO concentration until 60% saturation, and then this concentration was kept constant by feeding a mixture of oxygen and air gases. *OTS III*. The fermentation was started with full oxygen saturation, the level was gradually decreased to 80, 60, 40 and 30% saturations at 2-h intervals, and thereafter, 30% saturation was maintained by feeding a mixture of oxygen and air gases. *OTS IV*. The fermentation was started with full oxygen saturation, a decrease was allowed in DO concentration until 30% saturation, and then this concentration was kept constant.

The variations in pH with fermentation time for different OTSs are also depicted in Fig. 1. There were no considerable changes in pH values throughout the fermentations except for slight decreases in early periods.

3.1. Biomass

Fig. 2 shows the variations in cell biomass concentration with fermentation time for different OTSs. The accumulation rate of

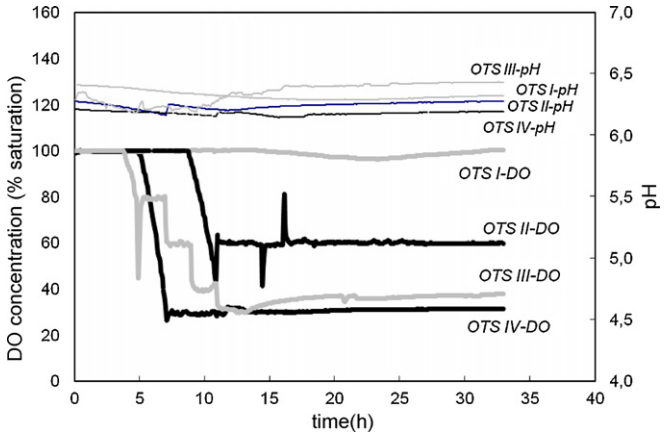

Fig. 1. The variations in DO concentration (% saturation) and pH with time in the fermentation of *C. rugosa* for different OTSs ($T = 30$ °C, $N = 500$ rpm, Q/V = 0.6 vvm, carbon source: 2 g/L triolein, nitrogen source: 4 g/L urea).

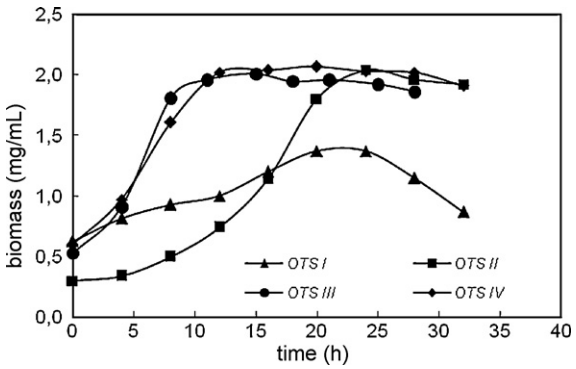

Fig. 2. Effect of OTS on biomass (T = 30 °C, N = 500 rpm, Q/V = 0.6 vvm, carbon source: 2 g/L triolein, nitrogen source: 4 g/L urea).

biomass was higher when *OTS III* and *OTS IV* were employed than other OTSs. Almost the same biomass concentration was achieved for the stationary phase of growth (2.1 mg/mL) for all strategies except for *OTS I*. Dissolved oxygen concentration rapidly decreased in the middle of the exponential phase of growth (Figs. 1 and 2) possibly due to the low oxygen transfer rate to the system compared with the oxygen uptake rate of the cells. The growth rate as well as the concentration of the biomass achieved was quite low when *OTS I* was employed indicating that high DO concentration throughout the fermentation repressed the cell growth. A possible limitation of the cell growth and an extremely long lag phase by an excess of dissolved oxygen were also reported by Sokolovska et al. [7]. Similarly, Puthli et al. [8] demonstrated that there was an optimum oxygen concentration for growth of *C. rugosa* and any further increase in dissolved oxygen concentration resulted in a detrimental effect. Considering these remarks, the cells were exposed to higher concentration of DO in the exponential phase by employing *OTS III* compared with other OTSs. However, no difference was observed between *OTS III* and *OTS IV* in terms of biomass concentrations achieved. The delays in the cell growth and consumption of oxygen in *OTS II* were due to the low inoculum size (Fig. 2).

3.2. Extracellular lipase and esterase activities

The effects of OTS on extracellular lipase and esterase enzyme activities by *C. rugosa* are shown in Fig. 3. Lipase activity was growth-associated and the level of maximum activity decreased in the order (U/mL): *OTS IV* (23.3) > *OTS III* (16.7) > *OTS II* (10.0) = *OTS I* (10.0). These results showed that high concentration of DO throughout the fermentation restricted the secretion of lipase enzyme

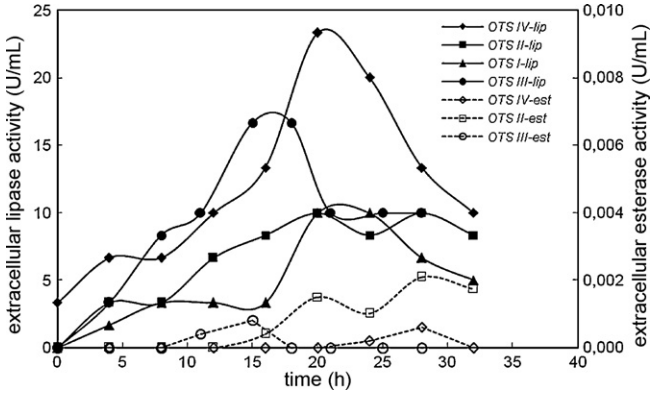

Fig. 3. Effect of OTS on extracellular lipase and esterase activities by *C. rugosa* (T = 30 °C, N = 500 rpm, Q/V = 0.6 vvm, carbon source: 2 g/L triolein, nitrogen source: 4 g/L urea).

whereas the concentration of 30% saturation in the late exponential and stationary phases of growth stimulated lipase secretion by *C. rugosa*. Low biomass accumulation in *OTS I* was another reason for low lipase activity. No esterase activity was detected in the fermentation medium in *OTS I*. The highest extracellular esterase activity was achieved in *OTS II* as 0.002 U/mL, which was followed by *OTS III* (0.0008 U/mL) indicating that high concentration of DO in the stationary phase of growth stimulated esterase secretion by *C. rugosa*.

The studies that investigate the influence of oxygenation on the lipase production by *C. rugosa* have a consensus that lipase production could be restricted by oxygen limitation and 20% of the full oxygen saturation was sufficient to ensure lipase production [6–8]. The data of the present study also demonstrated that lipase activity by *C. rugosa* was higher when DO concentration was kept constant at 30% saturation beyond the late exponential phase of growth (*OTS IV* and *OTS III*) than that was obtained at higher DO concentrations (*OTS I* and *OTS II*). In addition, the most original inference of the present work was that the DO concentration of 60% saturation (*OTS II*) in the stationary phase of growth promoted higher esterase activity than that was obtained at lower saturations (*OTS III* and *OTS IV*). In supplementary experiments, where DO concentration was kept constant at 30% and 60% saturations throughout the fermentations, we could not detect any considerable lipase activity as the accumulation of the cell biomass was quite low. Hence, we concluded that although full oxygen saturation was necessary in the beginning of the fermentation, it should be decreased in the middle of the exponential phase so as to the cell growth repression to be prevented and enzyme production to be stimulated.

The most important difference between lipase and esterase enzymes is the physicochemical interaction with their substrates. In contrast to esterases, lipases exhibit their activities only when they contact the interface of a biphasic water–oil system [13]. In our experiments, high level of DO concentration gave rise to the medium homogenization leading to high level of esterase activity in *OTS II* (DO concentration of 60% saturation). In the literature, no study reports that the DO concentration makes any difference between esterase and lipase activities by *C. rugosa* both extracellularly and intracellularly.

3.3. Time course of the fermentation

The data of the present study provide us also to evaluate the fermentation progress in addition to comparison of the enzyme activities for different OTSs. We observed that lipase activity was growth-associated and decreased after giving a maximum in the middle of the stationary phase of growth. On the other hand, esterase enzyme was released after a considerable level of lipase activity was reached in the medium which corresponded to the early period of the stationary phase of growth (Figs. 2 and 3).

We also followed intracellular enzyme activities with fermentation time and found that the intracellular lipase activity decreased with time starting from very beginning of the fermentation due to the secretion of the enzyme into the medium (Fig. 4). The pattern of the intracellular lipase activity showed a plateau at the end of the exponential phase of growth. Higher lipase activities were detected within the cell in *OTS II* and *OTS III* in the stationary phase of growth than other OTSs in consistent with the extracellular lipase activity courses. The formation of esterase enzyme started later than that of lipase. The highest intracellular esterase activity was obtained in *OTS III*, and no intracellular esterase activity was detected in *OTS I*.

When the time course of lipase activity is compared with that available in the literature, a similarity is found between the report of Gordillo et al. [14] and this paper. Gordillo et al. [14] reported that extracellular lipase production was growth-associated, and the maximum of intracellular lipase activity was observed in the

Table 1
Effect of OTS on the extracellular lipase and esterase productivity, specific productivity and yield of *C. rugosa* enzymes (T = 30 °C, N = 500 rpm, Q/V = 0.6 vvm, carbon source: 2 g/L triolein, nitrogen source: 4 g/L urea; *Y*: yield; Lip: lipase activity (U/mL); Est: esterase activity (U/mL); So: initial substrate concentration (mg/mL); X: biomass concentration (mg/mL)).

Oxygen transfer strategy	Maximum extracellular productivity (U/mL/h)		Maximum extracellular specific productivity (U/mg dw/h)		Maximum yield of lipase activity, $Y_{Lip/So}$ (U/mg)	Maximum yield of esterase activity, $Y_{Est/So}$ (U/mg)	Maximum yield of biomass, $Y_{X/So}$ (mg/mg)
	Lipase	*Esterase*	*Lipase*	*Esterase*			
OTS I	0.50	–	0.36	–	5.00	–	0.68
OTS II	0.50	6.56×10^{-5}	0.27	3.42×10^{-5}	5.00	1.05×10^{-3}	1.02
OTS III	1.11	5.46×10^{-5}	0.55	2.72×10^{-5}	8.33	4.10×10^{-4}	1.00
OTS IV	1.16	2.18×10^{-5}	0.56	1.10×10^{-5}	11.66	3.05×10^{-4}	1.03
Shake flasks	0.53	3.00×10^{-5}	0.37	2.80×10^{-5}	8.33	8.70×10^{-4}	0.74

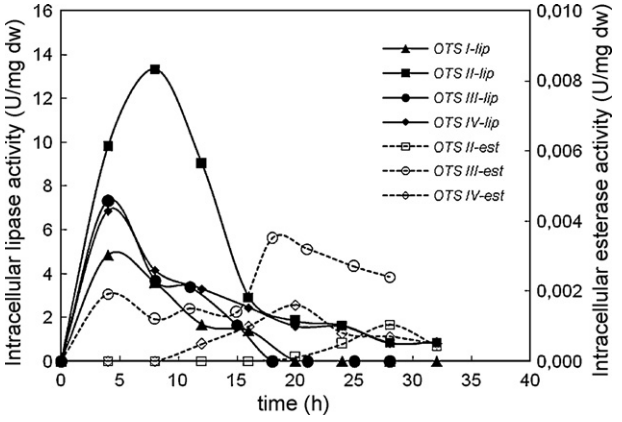

Fig. 4. Effect of OTS on intracellular lipase and esterase activities by *C. rugosa* (T = 30 °C, N = 500 rpm, Q/V = 0.6 vvm, carbon source: 2 g/L triolein, nitrogen source: 4 g/L urea).

middle-exponential phase. The authors showed that intracellular lipase activity remained constant at the end of the batch. In the present study, we additionally represented the time course of esterase enzyme and demonstrated that *C. rugosa* cells produced lipase and esterase enzymes in a sequential pattern.

We measured extracellular and intracellular proteolytic activities at the end of fermentations to detect if protease was responsible for the decrease in enzyme activities. The extracellular protease activity could only be detected in *OTS II* (3.5 U/mL) and *OTS I* (1.8 U/mL), and no intracellular protease activity was detected in all OTSs employed. This result revealed that *C. rugosa* did not produce any significant amount of protease under the conditions employed for lipolytic activity, and the decrease in extracellular enzyme activity was not mainly due to the proteolytic activity. However, when the total (extra- and intracellular) activity of lipase was calculated, a decrease towards the end of fermentation was also observed for all OTSs. Therefore, we can conclude that the decrease in cell growth or adsorption of the produced enzyme onto the aqueous-organic (disperse triolein) interface might have been responsible for the decrease in extracellular lipase activity. Gordillo et al. [14] explained this decrease with an adsorption phenomenon relating to lipase concentration, produced between the aqueous and organic phase. Besides, Sokolovska et al. [7] described that lipase concentrated in the foam that developed due to high gas flow rate caused an abrupt decrease in lipase activity.

3.4. Yield and productivity comparison

The overall results showed that OTS modulated the levels of lipase and esterase activities. The data of this study are also compared in terms of maximum extracellular productivity and specific productivity of *C. rugosa* enzymes and in terms of yields with respect to the initial carbon source concentration for all OTSs in Table 1. The highest productivity, specific productivity and yield of lipase were obtained in *OTS IV* whereas those for esterase were obtained in *OTS II*. Maximum yields of biomass were similar in all OTSs except for *OTS I*. We tabulated also the results of shake flask study of *C. rugosa* under the same conditions except for aeration in Table 1. *OTS IV* and *OTS II* had considerable advantages over shake flask cultivation in terms of lipase and esterase productions, respectively. The cell biomass yield was also found to be higher in bioreactor experiments than shake flask results except for *OTS I*. These results indicated that oxygen transfer with diffusion was sufficient for cell growth, however, cannot ensure high enzyme production.

4. Conclusion

In the present work, we demonstrated that OTS was a crucial parameter that regulated lipase and esterase formations by *C. rugosa*. The control of DO concentration throughout the fermentation resulted in different levels of enzyme activities and also changed the enzyme distribution. The results showed that the excess of DO in the stationary phase of growth enhanced the esterase activity. Additionally, we presented herein for the first time the relationship among cell growth, intracellular and extracellular enzyme activities for different OTSs. Further studies on the aeration and agitation rates in *C. rugosa* fermentation may improve the activity and productivity of CRL isoenzymes to higher values. These novel research findings also provide an important insight on the enzyme or isoenzyme differentiation of a microorganism beyond and/or in addition of genetic studies.

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