J Ind Microbiol Biotechnol (2004) 31: 133–136
DOI 10.1007/s10295-004-0126-9

ORIGINAL PAPER

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Studies on a novel carbon source and cosolvent for lipase production by *Candida rugosa*

Received: 6 March 2003 / Accepted: 18 February 2004 / Published online: 7 April 2004

Abstract Oleic acid esters were shown to be the best carbon source for both cell growth and lipase production by *Candida rugosa*. Use of a cosolvent, dodecane, in fermentations improved the solubility of solid substrates and increased oxygen solubility. This resulted in the highest lipase activity in batch fermentation with glycerol trioleate and dodecane. Lipase activity reached 77.1 units ml^{-1}.

Keywords Lipase production · Carbon source · Cosolvent · Fermentation · *Candida rugosa* · Dodecane

Introduction

Lipase (triacylglycerol lipase, EC 3.1.1.3), catalyzing the hydrolysis of triacylglycerols at water/oil interfaces, is attracting more and more attention for use in chemical reactions such as transesterifications [19], ester syntheses [15, 18] and optical resolutions [20]. Lipase can be produced by a number of microorganisms including bacteria [5], yeast and fungi [12]. The extracellular lipase from *Candida rugosa* is reported to be non-specific in the glycerol position [9] and suitable for the resolution of racemic acids and alcohols, due to its high stereospecificity [2].

Although *C. rugosa* lipase has been studied extensively, little information is available about factors and conditions that influence its biosynthesis. Genetic studies have identified up to seven genes encoding different lipases in *C. rugosa* [7], but successful expression of the lipase genes in heterologous host microorganisms is very difficult, because *C. rugosa* obeys a non-canonical codon usage [6]. Improvement of lipase production still depends on the optimization of culture conditions, including the composition of the culture medium, such as carbon and nitrogen sources, and on fermentation parameters, such as dissolved oxygen, temperature and aeration rate. It has been reported that the properties of lipases could be modulated by using different operational conditions [1]. There are also several reports on optimizing carbon and nitrogen sources [4, 10, 17]. It is generally accepted that carbohydrates, especially glucose, have an inhibitory effect on lipase production, but that lipid substrates and fatty acids promote it [11, 17]. Oleic acid is regarded as one of the best inducers.

Through orthogonal tests, we concluded that the carbon source was one of the most important factors influencing lipase production. Although Dalmau et al. [3] observed that palmitic acid was better than oleic acid, there existed operational problems because palmitic acid was insoluble [3]. In this paper, we report more beneficial carbon sources than oleic acid. Furthermore, the problem of insolubility of carbon source is also solved.

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Materials and methods

Microorganism, medium and culture conditions

Peptone and yeast extract were obtained from Oxoid Co. (UK). *C. rugosa* (ATCC 14830) was maintained at 4 °C on GM medium, which included 3.5 g peptone, 30 g yeast extract, 20 g glucose, 2 g KH_2PO_4, 1 g $MgSO_4{\cdot}7H_2O$ and 20 g agar in 1 l distilled water. Basal liquid medium contained (per liter): 15 g KH_2PO_4, 6 g Na_2HPO_4, 6 g $(NH_4)_2SO4$, 1 g $MgSO_4{\cdot}7H_2O$ and micronutrients (0.01 g $FeCl_3{\cdot}6H_2O$, $4{\times}10^{-6}$ g inositol, $8{\times}10^{-6}$ g biotin, $2{\times}10^{-4}$ g thiamine hydrochloride). Carbon sources were added at a final concentration of 10 g l^{-1}, if not mentioned specially. The pH value of the medium was adjusted to pH 6.3 with NaOH. The basal liquid medium was steam-sterilized and the micronutrients were sterilized by microfiltration (0.22 μm).

The microorganism was grown in 500-ml flasks. Inoculum for the flasks was cultured on plates of GM medium for 48 h at 30 °C. Cells on the plates were suspended in 0.9% (w/v) NaCl solution; and 15 ml NaCl solution was needed to suspend all the cells on each plate. The suspensions were collected in a sterilized flask and 5 ml was inoculated into each flask, containing 45 ml basal liquid medium. The flasks were incubated at 30 °C for 24 h in a rotary shaking bath at 200 rpm (10-cm throw, model HYG-II; Shanghai Xin-xin Automated Equipment, China). Fermentation experiments were carried out in a 5 l Biostat B fermentor (3 l working volume; Braun, Germany). The inoculum was prepared in flasks which contained 5 g l^{-1} olive oil and basal liquid medium and was grown at 30 °C for 20 h, at 200 rpm. Standard operational conditions were: 10% inoculum, pH 6.3, agitation rate 500 rpm (in a fermentor equipped with a flat impeller with six blades), temperature 30 °C and airflow 0.5–5 l min^{-1}, to ensure a dissolved oxygen level not lower than 20% of air saturation.

Biomass

Samples (10 ml per sample) were filtered (0.45 μm), washed with a mixture of dioxane:propionic acid (1:1) and then washed with distilled water. The filters were dried at 105 °C to a constant weight.

Lipase assay

Lipase activity in the culture broth was measured by titrating the fatty acid liberated from olive oil with 0.05 mol l^{-1} NaOH, as described by Sugihara [16]. One unit of lipase activity was defined as the lipase quantity that liberated 1 μmol fatty acid in 1 min.

Cell-bound lipase analysis

Cells from 10 ml broth were harvested by centrifugation at 5,000 *g* for 10 min at 4 °C, washed with 10 mM Tris-HCl buffer (pH 8.0) and suspended in the same buffer, at a final volume of 5 ml. The cell suspension was disrupted at 0 °C with a cell disintegrator (JY92-II; Ningbo Xin-zhi Scientific Instrument Institute, China) for ten periods of 30 s each. The disrupted cells were centrifuged at 4,000 *g* at 4 °C for 10 min. The supernatant was used as the cell extract for the determination of cell-bound lipase.

Protein analysis

Protein was determined by the method of Lowry et al. [8], with bovine serum albumin as standard.

Results and discussion

Effect of fatty acids on cell growth and lipase production

Obradors et al. [11] reported that fatty acids with an odd number of carbons did not induce lipase production. Fatty acids with an even number of carbons were, therefore, selected as the sole carbon source to investigate their effect on *C. rugosa* (Table 1).

Except for acetic acid, which was soluble, and caprylic acid, which was slightly soluble, the remaining fatty acids were insoluble and floated on the medium surface as oil drops (oleic acid) or lumps (dodecanoaic, myristic, palmitic, stearic acids). In spite of their solubility, acetic acid and caprylic acid did not support cell growth; and no lipase or extracellular protein was detected. The insoluble fatty acids supported cell growth and produced lipase to different extents. The longer the carbon chain of the fatty acids, the higher the lipase activity and the more extracellular protein. Although oleic acid possesses the same length of carbon chain as stearic acid, growth and activity were less on stearic acid, suggesting that the unsaturated fatty acid was a more appropriate carbon source.

Other proteins besides lipase were increased in the medium supplemented with oleic acid, which supported more growth than palmitic acid or stearic acid (Table 1). Cell-bound lipase was not detected, except the with substrates giving high extracellular activity. In three replicate experiments, palmitic acid as carbon source led to a higher cell-bound lipase activity than stearic acid.

Table 1 Effect of different fatty acids on cell growth and lipase production by *Candida rugosa* (based on three replicates). *ND* Not determined

Carbon source	Biomass (g l^{-1})	Extracellular lipase activity (units ml^{-1})	Cell-bound lipase activity (units g_{cells}^{-1})	Extracellular protein (μg ml^{-1})	Specific lipase activity (units $\mu g_{protein}^{-1}$)
Acetic acid	0	0	0	0	0
Caprylic acid	0	0	0	0	0
Dodecanoaic acid	183	0	0	ND	–
Myristic acid	0.325	0.223	0	3.001	0.074
Palmitic acid	1.447	3.329	24.3	14.119	0.236
Oleic acid	3.741	8.538	31.2	31.457	0.271
Stearic acid	2.063	4.250	22.9	16.284	0.261

Table 2 Effect of different esters on cell growth and lipase production by *C. rugosa* (based on three replicates)

Carbon source	Biomass ($g\ l^{-1}$)	Extracellular lipase activity (units ml^{-1})	Cell-bound lipase (units g_{cells}^{-1})	Extracellular protein ($\mu g\ ml^{-1}$)	Specific lipase activity (units $\mu g_{protein}^{-1}$)
Oleic acid	3.833	8.667	30.5	30.851	0.281
Glycerol tributyrate	0.592	1.121	11.4	5.007	0.224
Methyl stearate	2.546	4.274	23.9	17.096	0.260
Glycerol monostearate	3.238	5.163	26.4	19.337	0.267
Ethyl oleate	5.945	10.413	38.4	30.447	0.342
Glycerol trioleate	7.236	14.326	44.1	38.824	0.369

Effect of fatty acid esters

The effects of several fatty esters on *C. rugosa* growth and lipase production were investigated with oleic acid as the control (Table 2).

The short-chain glycerol tributyrate was not a good carbon source for growth. It gave only a low level of lipase activity and biomass. Glycerol trioleate, which had the target number of carbon atoms, significantly benefited lipase production, giving an extracellular lipase activity 65.3% higher than that with oleic acid. The effects of the saturated carbon source were obviously different from those of oleic acid or its esters. Consequently, the chain length of the carbon source should be considered first in lipase production. The unsaturation of the carbon source is also important.

Effect of cosolvents

Carbon sources suitable for *C. rugosa* lipase production are lipid-related, as indicated above. However, they cannot be absorbed easily due to their insolubility. Therefore, we investigated the use of a cosolvent to ensure the substrates are distributed well in the culture medium.

Ethanol

Carbon sources dissolved in ethanol were added to media at a final concentration of $2\ g\ l^{-1}$, as described by Shimada et al. [14]. Although ethanol improved substrate dispersion and increased growth and lipase production with respect to methyl stearate and glycerol monostearate, in general we found that ethanol had an inhibitory effect on growth (data not shown). For this reason, we concluded that it was not a good cosolvent.

Dodecane

Dodecane has been used in fermentations as an oxygen vector. It is non-toxic to microorganisms. Others using dodecane in fermentations have shown a rapid transfer of oxygen, low energy consumption, fewer air bubbles and less shear stress [13]. Therefore, dodecane was used as a cosolvent.

Methyl stearate or glycerol trioleate dissolved in dodecane was presented as the sole carbon source at a final concentration of $10\ g\ l^{-1}$ in shake-flask experiments. Esters performed better than acids for lipase production (Fig. 1). Compared with ethanol, dodecane resulted in a very good dispersion of both methyl stearate and glycerol trioleate.

Dodecane was also evaluated as sole carbon source. No cell growth or lipase activity occurred after incubation for 3 days.

The fermentation time-courses in a 5-l fermentor with glycerol trioleate as carbon source were investigated; and the results are shown in Figs. 2, 3, with and without dodecane. The level of dissolved oxygen was maintained above 68% during the fermentation with dodecane (Fig. 3), but it declined rapidly over 5–26 h without dodecane, dropping to 23% (Fig. 2). Dodecane improved both cell growth and lipase production. The highest lipase activity of *C. rugosa* reached 77.1 units ml^{-1}. Furthermore, the addition of dodecane shortened the fermentation by approximately 2 h.

Conclusions

Long-chain unsaturated lipid substrates were appropriate as the carbon source for lipase production by

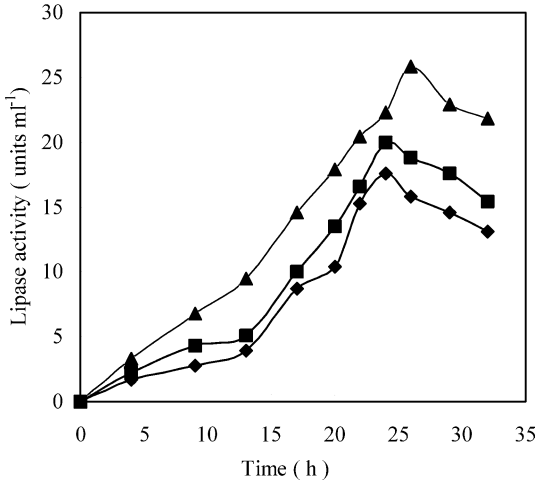

Fig. 1 Time-course of lipase production with different carbon sources and dodecane in flasks. *Diamonds* Oleic acid, *squares* methyl stearate, *triangles* glycerol trioleate

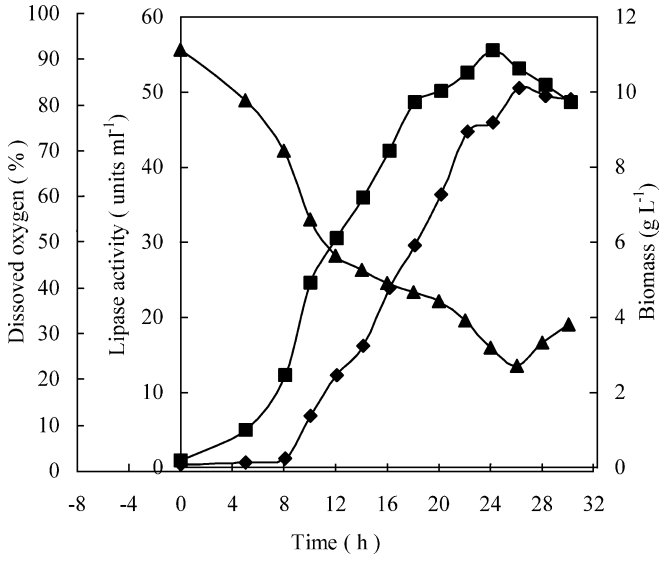

Fig. 2 Time-course of fermentation with glycerol trioleate as carbon source in a 5-l fermentor. *Diamonds* Biomass ($g\ l^{-1}$), *squares* lipase production (units ml^{-1}), *triangles* dissolved oxygen (%)

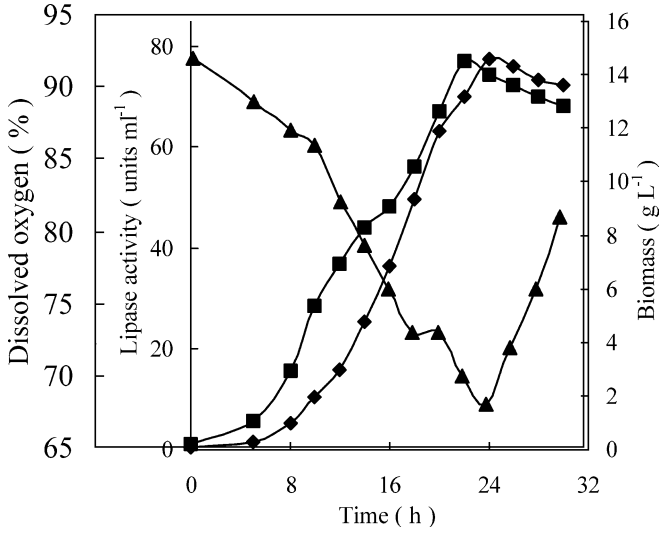

Fig. 3 Time-course of fermentation with glycerol trioleate as carbon source and dodecane as oxygen vector in a 5-l fermentor. *Diamonds* Biomass, *squares* lipase production, *triangles* dissolved oxygen

C. rugosa. Esters, such as oleic esters, were better than their corresponding fatty acids, which is inconsistent with a previous report [3]. It is necessary to keep the carbon source in a soluble state by dissolving it in a cosolvent such as dodecane. Dodecane also increased the dissolved oxygen level and consequently improved cell growth and lipase production.

Acknowledgement This work was supported by the Key Discipline Foundation of Shanghai.

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