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Significant Improvement of *Serratia marcescens* Lipase Fermentation, by Optimizing Medium, Induction, and Oxygen Supply

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Abstract Production of an extracellular lipase from *Serratia marcescens* ECU1010, which is an industrially important biocatalyst for the stereospecific synthesis of Diltiazem precusor, was carefully optimized in both shake flasks and a fermenter, using Tween-80 as the enzyme inducer. Dextrin and beef extract combined with ammonium sulfate were indicated to be the best carbon and nitrogen sources, respectively. With the increase of Tween-80 from 0 to 10 g l^{-1}, the lipase production was greatly enhanced from merely 250 U l^{-1} to a maximum of 3,340 U l^{-1}, giving the highest lipase yield of ca 640 U g^{-1} dry cell mass (DCW), although the maximum biomass (6.0 g DCW l^{-1}) was achieved at 15 g l^{-1} of Tween-80. When the medium loading in shake flasks was reduced from 20 to 10% (*v*/*v*), the lipase production was significantly enhanced. The increase in shaking speed also resulted in an improvement of the lipase production, although the cell growth was slightly repressed, suggesting that the increase of dissolved oxygen (DO) concentration contributed to the enhancements of lipase yield. When the lipase fermentation was carried out in a 5-l fermenter, the lipase production reached a new maximum of 11,060 U l^{-1} by simply raising the aeration rate from 0.5 to 1.0 vvm, while keeping the dissolved oxygen above 20% saturation via intermittent adjustment of the agitation speed (≥400 rpm), in the presence of a relatively low concentration (2 g l^{-1}) of Tween-80 to prevent a potential foaming problem, which is easy to occur in the intensively aerated fermenter.

Keywords Lipase fermentation · Medium optimization · *Serratia marcescens* · Tween-80 · Dissolved oxygen · Oxygen transfer

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Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), distributed widely in nature, can be found in animals, plants, and microorganisms. The lipases from microorganisms are more attractive for bioindustry because they are readily available in large quantities. Microbial lipase has been used extensively in foods, pharmaceuticals, fine chemicals, detergents, and wastewater treatment [1–3], and the interest in its production had greatly increased since the last decade. Moreover, lipases are the most frequently used enzymes in organic synthesis, catalyzing the chemo-, regio-, and/or stereoselective hydrolysis of carboxylic acid esters or catalyzing the reverse reactions (ester synthesis) in organic solvents [1–7].

The lipase from *Serratia marcescens* is an industrially relevant enzyme, which can catalyze the enantioselective hydrolysis of 3-(*p*-methoxyphenyl)glycidic acid methyl ester [(±)-MPGM], a key intermediate for the synthesis of Diltiazem hydrochloride [8–11]. In our laboratory, an extracellular lipase produced by *S. marcescens* ECU1010 was shown to be an efficient biocatalyst for the kinetic resolution of (±)-MPGM [12].

Tween-80 (polyoxyethylene sorbitan monooleate), a nonionic surfactant, can be used as carbon source in lipase fermentation [13, 14]. In our previous work [12], Tween-80 was found beneficial for the lipase production by *S. marcescens* ECU1010. As Tween-80 added in the initial medium was utilized slowly by the microorganism [14], and in our further work, we found that large amounts of Tween-80 used in fermentation might cause difficulties in the foaming control, and largely excessive Tween-80 left in the culture broth at the end of fermentation resulted in difficulties in the downstream processing of the lipase. In this work, with a low amount of Tween-80 as the enzyme inducer, we carefully optimized other carbon and nitrogen sources that support the cell growth and lipase production and successfully developed an optimal fermentation process in a 5-l fermenter for a much higher production of *S. marcescens* ECU1010 lipase.

Materials and Methods

Microorganism, Medium, and Culture Conditions

The strain of *S. marcescens* ECU1010 used in this work was a Gram-negative bacterium, which is now deposited in China General Microbiological Culture Collection Center with an accession number of CGMCC No. 1219 and maintained on agar slants in a medium composed of (per liter): peptone, 10 g; yeast extract, 5 g; and agar, 20 g in addition to a mineral salt mixture (MSM) containing KH_2PO_4, 2.0 g; NaCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; and $FeSO_4 \cdot 7H_2O$ 0.01 g. The pH of the medium was adjusted to 7.0 before autoclaving. The fermentation media were also composed of MSM and other components, which will be described in the corresponding figure legends or table footnotes.

Cultivation in Shake Flasks

Seed cultures were prepared by inoculating cells grown on an agar slant into a 250-ml flask containing 30 ml of the above medium without agar, and subsequently incubated with shaking at 30°C and 150 rpm for 12 h.

The flask culture experiments were usually performed in 250-ml flasks containing 30 ml of the fermentation medium. After inoculated with 2.5% (*v*/*v*) of the seed culture, the flasks were subsequently incubated with shaking at 30°C and 150 rpm. Sampling was performed

every 4 h, and cultivation time was described in the corresponding figure legends or table footnotes. The culture broth was centrifuged at 10,000×*g* and 4°C for 15 min to obtain a clear supernatant, which was used for all assays.

Batch Fermentation in 5-l Fermenter

Seed cultures were grown in 500-ml Erlenmeyer flasks with a working volume of 100 ml, incubated at 30°C for 12 h in a shaker working at 150 rpm. Batch fermentations were carried out in a 5-l fermenter with a working volume of 3.0 l. Unless specified otherwise, the fermentation medium contained (per liter): dextrin, 15.0 g; beef extract, 10.0 g; $(NH_4)_2SO_4$, 5.0 g in addition to MSM, and a stock solution of Tween-80 (30 g l^{-1}, 200 ml) was fed at a flow rate of 50 ml h^{-1} at the end of the exponent growth phase (8 h) to a final concentration of 2.0 g l^{-1}. The pH was adjusted to 7.0 using 2 M NaOH or 2 M H_2SO_4. The fermentation conditions were as follows: inoculum size 2.5%, temperature 30°C. The dissolved oxygen (DO) concentration was controlled by regulating the agitation speed.

Analytical Methods and Enzyme Assay

Cell concentrations were determined by measuring the optical density at 600 nm (OD_{600}) and correlating it with dry cell weight (DCW). The activity of the extracellular lipase was routinely measured by an assay to measure the amount of *p*-nitrophenol formed from *p*-nitrophenyl acetate (pNPA) [15]. To 2.87 ml of 100 mM potassium phosphate buffer (KPB, pH 7.0) was added 100 μl of the culture supernatant or distilled H_2O (a blank). After preincubation at 30°C for 3 min, the reaction was initiated by a quick mixing with 30 μl of 100 mM pNPA solution in DMSO, and the change in absorbance at 405 nm was recorded with a spectrophotometer. One unit of the lipase activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the above conditions.

Results and Discussion

Many factors including nutritional compositions and physico-chemical factors, such as nitrogen and carbon sources, lipids, inorganic salts, culture temperature, pH, agitation, and dissolved oxygen concentration, may greatly influence the production of lipase. Among culture medium components, carbon and nitrogen sources are two major factors influencing the expression of lipase activity. Lipases are generally produced in the presence of inducers, such as oil and fats, fatty acids, sugar esters (e.g., Tween-80), and bile salts, and its production is also greatly influenced by other carbon sources, such as sugars, sugar alcohols, polysaccharides, whey, and other complex sources [16]. In this work, we use Tween-80 in a relatively low amount as an inducer to promote the lipase production and tried to find other carbon sources that could be easily utilized by *S. marcescens* ECU1010 to support its growth.

Effect of Tween-80 and Oxygen Supply in Shake-Flask Culture

Different concentrations of Tween-80 were used to evaluate its effect in detail, and the results are shown in Fig. 1 and Table 1. Lipase production was greatly enhanced from 240 to 3,340 U l^{-1} when Tween-80 was increased from 0 to 10 g l^{-1}. It was also shown that with the increase of Tween-80 concentration from 0 to 15 g l^{-1}, the cell mass increased from 3.7

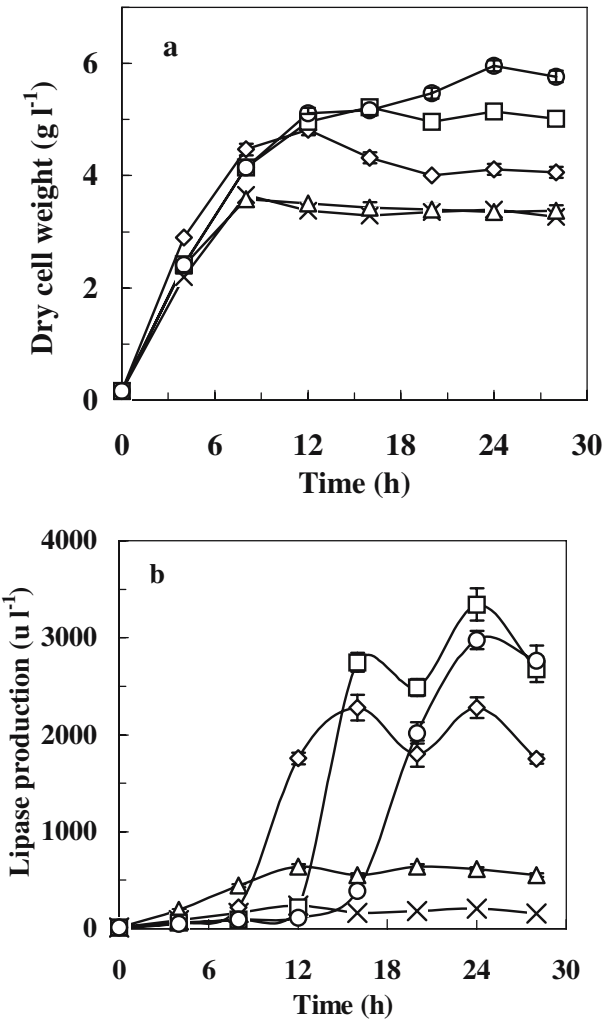

Fig. 1 Effect of initial Tween-80 concentration on cell growth (**a**) and production of extracellular lipase (**b**) by *S marcescens* ECU1010. Initial Tween-80 concentrations (g l^{-1}): 0 (×); 1 (*empty triangles*); 5 (*empty diamonds*); 10 (*empty squares*); and 15 (*empty circles*). The *error bars* in the figures indicate the standard deviations from two independent samples

to 6.0 g l^{-1}, and the time for the maximum lipase production was also delayed. As a result, both cell growth and lipase production were greatly affected by Tween-80, and the best concentration of Tween-80 was 10 g l^{-1} in shake-flask cultures.

Experiments involving medium load ratio of the shake flask and shaking speed suggested that oxygen supply affect both the cell growth and lipase production (Fig. 2). Less medium load may increase dissolved oxygen (DO) concentration, while increasing shaking speed can improve oxygen transfer rate (OTR) in shake flasks. As shown in Fig. 2, little difference was found in dry cell weight when the medium load was increased from 10 to 20% (*v*/*v*), whereas a slight repression of the cell growth was observed when the shaking speed was varied from 150 to 200 rpm. On the other hand, an increase in the shaking speed (from 150 to 200 rpm) appeared beneficial for the lipase production, while a reduction of the medium load ratio (from 20 to 10%) also resulted in an obvious increase of the lipase production (Fig. 2b). When the shake-flask culture was performed under conditions of 10% medium load and 200 rpm shaking speed, the specific production of the lipase reached the

Table 1 Effects of initial Tween-80 concentration on cell growth and extracellular lipase production by *S. marcescens* ECU1010.[a]

Initial Tween-80 concentration (g l^{-1})	Maximum DCW (g l^{-1})	Maximum lipase production (U l^{-1})	Specific lipase production (u g^{-1} DCW)
0	3.7±0.0[b] (8 h)[c]	237±18 (12 h)[d]	65±5
1	3.6±0.1 (8 h)	642±23 (12 h)	178±2
5	4.8±0.1 (12 h)	2,279±134 (16 h)	476±38
10	5.2±0.1 (16 h)	3,342±166 (24 h)	643±20
15	6.0±0.0 (24 h)	2,298±96 (24 h)	383±16

[a] The culture medium was composed of (per liter): dextrin, 10 g; yeast extract, 20 g; and a certain concentration of Tween-80, in addition to MSM (see Materials and Methods section), pH 7.0. The cultivation was carried out in 250-ml flask containing 30 ml medium and shaken at 30°C and 150 rpm for 28 h.

[b] The maximum errors were calculated from two independent samples.

[c,d] Cultivation time when maximum DCW or lipase production was obtained.

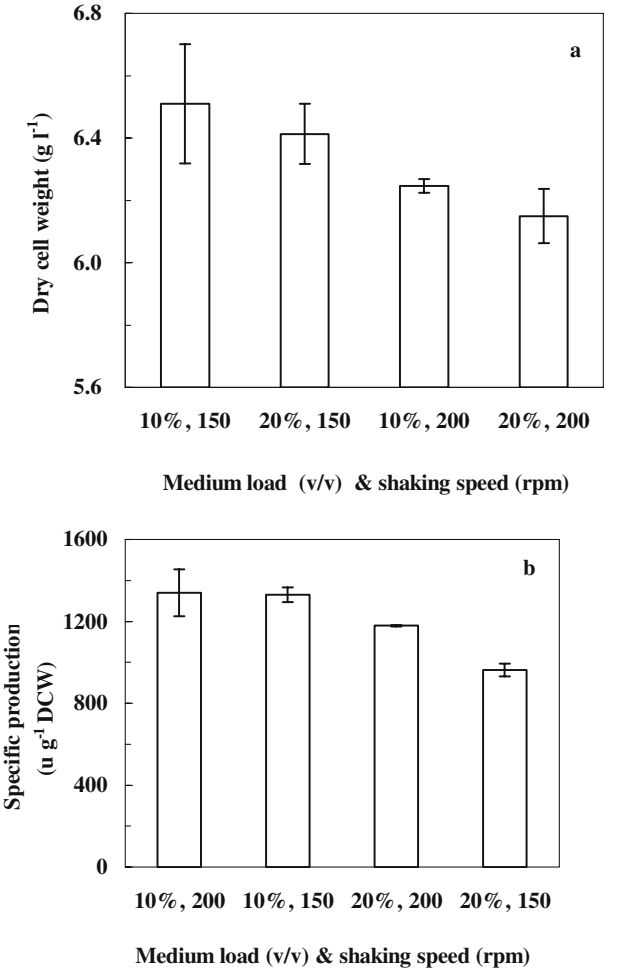

Fig. 2 Effects of medium load ratio (%, *v*/*v*) and shaking speed (rpm) on cell growth (**a**) and extracellular lipase production (**b**). The culture medium was composed of (per liter): Tween-80, 10 g; dextrin, 10 g; peptone, 20 g; in addition to MSM, pH 7.0. The cultivation was carried out in 250-ml flask and shaken at 30°C for 24 h. The *error bars* in the figures indicate the standard deviations from three independent samples

highest level (1317±35 U g^{-1} DCW), which was much higher than that obtained (1,012± 32 U g^{-1} DCW) under 20% medium load and 150 rpm shaking speed. These results suggested that oxygen supply might be the key factor limiting lipase production by *S. marcescens* ECU1010 in shake flasks. Giuseppin [17] also reported that the effect of oxygen limitation on lipase production by *Rhizopus delemar* was significant when the dissolved oxygen concentration was below 47 μmol l^{-1}, suggesting that oxygen was a limiting substrate in shake-flask cultures.

Combined Optimization of Carbon and Nitrogen Sources

Several carbon sources (mainly sugars) and organic nitrogen sources were tested to perform combined optimization in our work (Table 2). Besides carbon source, the type of nitrogen source also influences the lipase titers in the fermentation broth. Organic nitrogen sources are generally preferred, although inorganic nitrogen sources are also used in some cases [16]. Poor cell growth of *S. marcescens* ECU1010 was observed when inorganic nitrogen source was used as sole nitrogen source, so organic source was first selected, and the effect of inorganic nitrogen source was investigated separately in the next step. As indicated in Table 2, the cell growth was closely related to the type of carbon source. The lowest biomass and lipase production were obtained when glucose was utilized, while the sucrose gave the maximum cell concentration in all cases. Dextrin supported cell growth with a moderate biomass but gave the maximum lipase production in each case. Among nitrogen

Table 2 Effect of carbon and organic nitrogen sources on the growth and production of extracellular lipase by *S marcescens* ECU1010.[a]

Nitrogen source	Carbon source	Dry cell weight (g l^{-1})[b]	Lipase activity (U l^{-1})[b]
Yeast extract	Glucose	2.9±0.1	100±3
Yeast extract	Corn starch	2.9±0.1	839±79
Yeast extract	Dextrin	3.8±0.0	1,595±5
Yeast extract	Maltose	4.3±0.0	789±14
Yeast extract	Sucrose	5.8±0.0	770±14
Peptone	Glucose	1.6±0.0	125±3
Peptone	Corn starch	2.8±0.1	1,188±12
Peptone	Dextrin	3.2±0.0	2,061±483
Peptone	Maltose	4.3±0.1	1,012±100
Peptone	Sucrose	6.1±0.1	374±27
Corn steep liquor	Glucose	1.9±0.0	100±3
Corn steep liquor	Corn starch	3.3±0.1	1,665±46
Corn steep liquor	Dextrin	3.9±0.1	2,788±260
Corn steep liquor	Maltose	4.0±0.1	2,268±340
Corn steep liquor	Sucrose	5.5±0.0	269±5
Beef extract	Glucose	2.3±0.1	196±5
Beef extract	Corn starch	3.0±0.1	1,968±56
Beef extract	Dextrin	3.5±0.0	5,852±201
Beef extract	Maltose	4.5±0.2	5,538±360
Beef extract	Sucrose	6.3±0.3	1,942±109

[a] The culture medium was composed of MSM, Tween-80 (5.0 g l^{-1}), plus a specified carbon and nitrogen source (each 15.0 g l^{-1}), pH 7.0. The cultivations were carried out in 250-ml flasks containing 30 ml medium and shaken at 30°C and 150 rpm for 16 h.

[b] Average of two independent cultures±SD.

Table 3 Effect of initial NH_4^+ concentration on cell growth and production of extracellular lipase by *S. marcescens* ECU1010.[a]

Initial $(NH_4)_2SO_4$ concentrations (g l^{-1})	Maximum DCW (g l^{-1})	Maximum lipase production (U l^{-1})	Specific lipase production (U g^{-1} DCW)
0	4.0±0.1[b] (12 h)[c]	4,394±310 (16 h)[d]	1,090±148
2	4.0±0.0 (12 h)	5,360±73 (16 h)	1,334±134
5	4.1±0.1 (12 h)	5,670±711 (16 h)	1,370±186
10	4.2±0.1 (12 h)	5,287±0 (16 h)	1,274±16

[a] The culture medium was composed of (per liter): MSM, Tween-80, 2.0 g; dextrin, 15.0 g; beef extract, 15.0 g; and a certain concentration of $(NH_4)_2SO_4$, pH 7.0. The cultivations were performed in 250-ml flasks containing 30 ml medium and shaken at 30°C and 150 rpm for 24 h.

[b] Average of two independent cultures±SD.

[c,d] Cultivation time when maximum DCW or lipase production was obtained.

sources, no significant difference was observed in cell growth. However, the four nitrogen sources, yeast extract, peptone, corn steep liquor, and beef extract, showed large differences in supporting the lipase production by *S. marcescens* ECU1010 with activities of 1,600, 2,060, 2,790, and 5,850 U l^{-1}, respectively.

To evaluate the effect of inorganic nitrogen sources, different concentrations of $(NH_4)_2SO_4$ (0, 2, 5, and 10 g l^{-1}) were initially added to the culture medium. As illustrated in Table 3, although no significant effect was found on cell growth, the lipase production was positively supported by the presence of NH_4^+, and the maximum lipase production (5,670 U l^{-1}) achieved at 5 g l^{-1} of $(NH_4)_2SO_4$ was 29% higher than that of the control [no $(NH_4)_2SO_4$]. In addition, the feeding time of $(NH_4)_2SO_4$ was also examined, indicating that the $(NH_4)_2SO_4$ should better be added directly to the initial medium (data not shown).

Then, to investigate the best lipase fermentation conditions in shake flasks, three levels of dextrin and beef extract (i.e., 10, 15, and 20 g l^{-1}) were tested to determine their exact effect on the lipase production and cell growth, and the best concentrations of Tween-80 (10 g l^{-1}) and $(NH_4)_2SO_4$ (5 g l^{-1}) were used. As shown in Table 4, at each concentration of dextrin, the final cell concentration increased with the increase in beef extract concentration as the organic

Table 4 Combined optimization of carbon and nitrogen sources for lipase production by *S. marcescens* ECU1010.[a]

Dextrin (g l^{-1})	Beef extract (g l^{-1})	DCW (g l^{-1})[b]	Lipase activity (U l^{-1})[b]
10	10	4.2±0.0	11,683±380
10	15	5.0±0.1	12,291±410
10	20	5.5±0.2	12,367±213
15	10	4.6±0.0	12,838±349
15	15	5.2±0.1	11,941±122
15	20	6.0±0.0	11,379±471
20	10	5.2±0.0	11,167±380
20	15	5.7±0.1	12,017±46
20	20	6.4±0.0	12,120±46

[a] The culture medium was composed of (per liter): Tween-80, 10 g; $(NH4)_2SO_4$, 5 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g and different amounts of dextrin and beef extract (pH 7.0). The cultivations were carried out in 250-ml flasks containing 30 ml medium and shaken for 24 h at 30°C and 150 rpm.

[b] Average of two independent cultures±SD.

nitrogen source. The optimal lipase production achieved among various combinations of dextrin/beef extract was around 11,000~13,000 U l^{-1} and the maximum lipase production 12,838±349 U l^{-1} was obtained at 15 g l^{-1} of dextrin and 10 g l^{-1} of beef extract. The optimal medium was used subsequently for an up-scaled cultivation in a 5-l jar fermenter, whereas the amount of Tween-80 was intentionally reduced to 2 g l^{-1}, to prevent potential foaming problem, which is easy to happen in a rigorously aerated bioreactor.

Lipase Fermentation in a 5-l Bioreactor

Microbial lipase fermentation is affected by many factors including medium composition, pH, temperature, aeration, and agitation rates. In many cases, production of lipase was significantly influenced by dissolved oxygen in culture broth. Frost and Moss [18] summarized that improvements of oxygen supply by agitation or air sparging are beneficial to lipase production for single-cell organisms in all cases and for filamentous molds in most

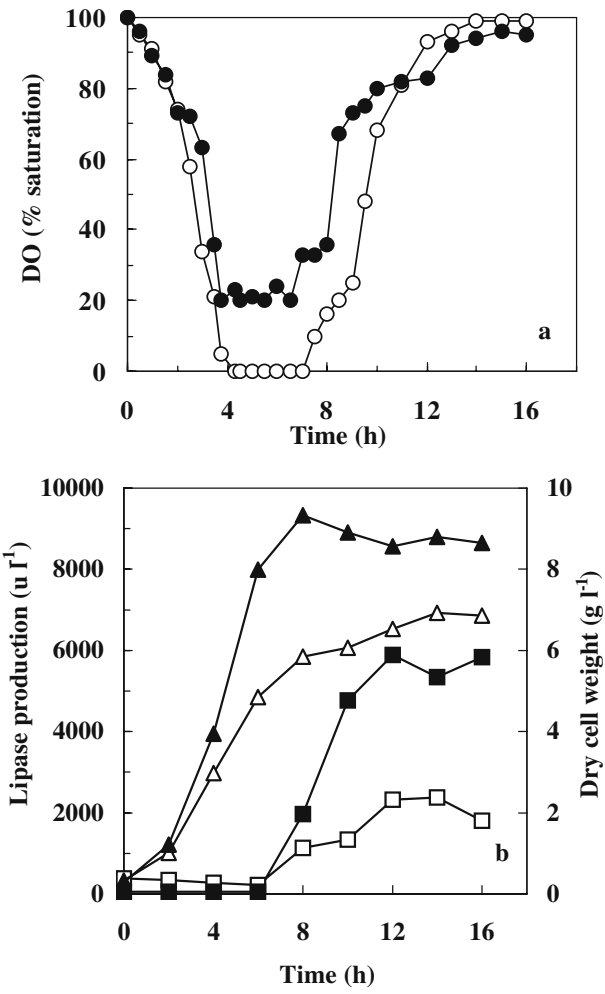

Fig. 3 Effect of the lowest dissolved oxygen level (% saturation) on cell growth (**a**) and extracellular lipase production (**b**). Dissolved oxygen conditions: (*empty circles*) uncontrolled (≥0%); (*filled circles*) controlled (≥20%). Symbols: (*empty triangles*, *filled triangles*) dry cell weights (DCW) and (*open squares*, *filled squares*) lipase production, at DO≥0% (*empty triangles*, *open squares*) or DO≥20% (*filled triangles*, *filled squares*)

cases. The intrinsic factor affecting cell growth and lipase production by *Acinetobacter radioresistens* was OTR rather than DO concentrations [19].

In a preliminary experiment of shake-flask culture, the problem we found was that growth of *S. marcescens* ECU1010 and production of lipase were probably limited by oxygen supply, which may be solved by improving oxygen transfer rate in bioreactors. The experiments in shake flasks showed that 10 g l^{-1} of Tween-80 gave the maximum lipase production, but to avoid the potential foaming problem during the fermentation and to prevent excessive amount of Tween-80 being left in the culture broth at the end of fermentation, its amount was decreased to 2 g l^{-1}. Two strategies, control of the lowest DO by tuning the agitation speed and enhancement of the aeration rate, were used to improve the oxygen supply. The first batch of fermentation was performed at temperature 30°C, pH 7.0, 400 rpm of agitation, and 0.5 vvm of aeration, with Tween-80 fed at the end of the exponential growth phase in a rate of 50 ml h^{-1} to a final concentration of 2.0 g l^{-1}, without any control or regulation of DO during the whole fermentation process. In the second batch of fermentation, the dissolved oxygen was stably maintained above 20% saturation by increasing the agitation speed when it fell to 20%, and the agitation speed was reduced to 400 rpm when the DO increased. In the third batch of fermentation, the initial aeration rate was raised up to 1.0 vvm, while other fermentation conditions were kept the same as the second fermentation. As shown in Fig. 3, when the lowest DO was uncontrolled during the fermentation, the maximum cell concentration and lipase production in fermenter were merely 6.9 and 2,382 U l^{-1}, respectively. They were markedly increased to 9.3 g l^{-1} and 5,883 U l^{-1} when the aeration rate was kept at 0.5 vvm, while the lowest DO concentration was maintained above 20% saturation. Moreover, when the initial aeration rate was further increased from 0.5 to 1.0 vvm, no significant effect on cell growth was observed, whereas the enzyme activity was greatly enhanced up to 11,060 U l^{-1} (Fig. 4). These results suggested that both the cell growth and lipase production of *S. marcescens* ECU1010 were closely related to dissolved oxygen concentration and/or oxygen transfer rate during the lipase fermentation. By optimization of the batch fermentation, the enzyme activity reached the highest level (11,060 U l^{-1}), which was almost the same as that obtained in shake-flask culture in the presence of 10 g l^{-1} Tween-80.

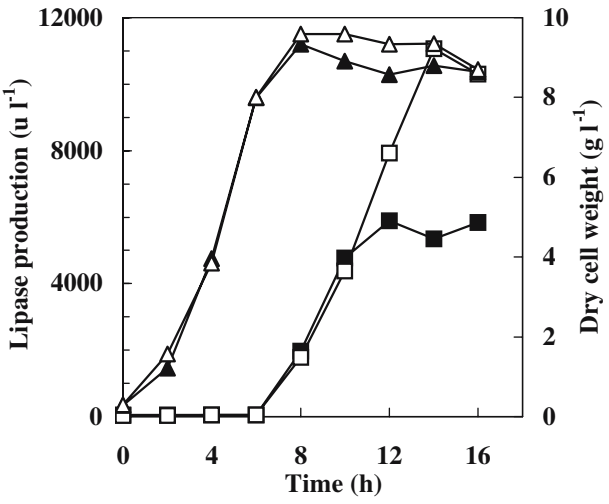

Fig. 4 Improvement of lipase production by increasing aeration rate and keeping DO above 20% saturation. Symbols: (*empty triangles*, *filled triangles*) cell growth and (*empty squares*, *filled squares*) lipase production, at an aeration rate of 0.5 vvm (*filled triangles*, *filled squares*) or 1.0 vvm (*empty triangles*, *empty squares*)

Conclusions

Using Tween-80 as an inducer, a combined optimization of carbon and nitrogen sources was performed in this work, and the results showed that dextrin and beef extract plus $(NH_4)_2SO_4$ were the best carbon and nitrogen sources, respectively. A further investigation on the effect of medium load ratio of the shake flask and shaking speed in the lipase fermentation indicates that oxygen supply is probably the limiting factor in shake-flask cultures. The amount of Tween-80 has been significantly decreased down to 2 g l^{-1}, while the lipase production has been successfully improved up to 11,060 U l^{-1} by increasing aeration rate and maintaining the lowest DO concentration above 20% saturation. Such a high production of lipase in 5-l fermenter was almost the same as that obtained in shake-flask cultures with as much as 10 g l^{-1} of Tween-80.

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