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Nutrition and bioprocess development for efficient biosynthesis of an antitumor compound from marine-derived fungus

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Abstract An integrated nutrition and bioprocess strategy was developed for improving the biosynthesis of an antitumor compound, 1403C, by a marine-derived fungus, *Halorosellinia* sp. (no. 1403). First, statistical design strategies were synthetically applied to optimize the nutritional composition. The resulting 1403C production reached 2.07 g/l, which was 143.5 % higher than the original production. However, it only produced 0.44 g/l of 1403C in 5-l bioreactor fermentation. Thus, the operating parameters including culture pH, dissolved oxygen, agitation speed, impeller type and inoculum level were considered to improve the fermentation process, and an effective control strategy for 1403C production by *Halorosellinia* sp. submerged in a 5-l bioreactor was established. When inoculating 0.22 g/l dry biomass, controlling dissolved oxygen not lower than 30 % during the growth phase but ranging between 30 and 40 % during the stationary phase, using a double-layer six-flat-blade Rushton disc turbine agitated at 400 rpm, keeping short-term low pH and rapid-rising pH with glucose starvation, the highest 1403C production was finally obtained at 1.32 g/l, which was promoted by 200 % compared to before optimization. Fermentation scale-up was finally performed in a 500-l bioreactor, and 1403C production of 1.09 g/l was obtained.

Keywords *Halorosellinia* sp. (no. 1403) · Medium optimization · 1403C · Fermentation process · Antitumor

Introduction

Recently, marine fungi have proven to be a rich source of bioactive natural products, most of which grow in a unique and extreme environment [4, 8]. Over 1,000 new compounds have been isolated from marine fungi, and the number of compounds is increasing [24]. Breast cancer is the most common malignancy among women worldwide. Approximately 230,480 new cases of invasive breast cancer and 39,520 breast cancer deaths were expected to occur among US women in 2011 according to American Cancer Society statistics [9]. Therefore, currently an effective medicine is desparately needed.

The compound 1403C (also called SZ-685C) is synthesized by mangrove endophytic fungus *Halorosellinia* sp. (no. 1403) collected from the South China Sea [7]. The compound can induce apoptosis in breast cancer cells by suppression of the Akt/FOXO pathway and shows great potential as a new antitumor drug [29, 32]. So far, preclinical research has needed to use a large amount of 1403C. Thus, fermentation optimization and scale-up for production enhancement and compound accumulation are urgently needed.

Generally, medium optimization is regarded as the most effective measure to improve fermentation productivity directly. This operation relates to several methods of statistical experimental design. The one-factor-at-a-time

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strategy has the advantage that the individual effects of medium components can be seen on a graph, without the need to revert to statistical analysis. Orthogonal and Plackett-Burman designs are often applied to reduce the number of trials and determine the critical variables. Response surface methodology (RSM) is a mathematical tool based on polynomial regression fitting, significance analysis and stationary point location. Soaring production of aspergiolide A by the marine-derived fungus *Aspergillus glaucus* HB1-19 and in (+)-terrein by *Aspergillus terreus* PF26 has been reported via medium optimization using statistical methodology [6, 31].

Different parameters represent the corresponding phases of growth and metabolism. The broth pH reflects the metabolic balance of organic acid produced from the respiration of cells. Dissolved oxygen (DO) is determined by the oxygen transfer rate (OTR) and oxygen uptake rate (OUR). Oxygen concentrations in the saturated gas-liquid interface and the bulk phase together with the volumetric oxygen transfer coefficients (K_La) affect the OTR [20]. The viscous nature of the fermentation broths of some filamentous fungi during their submerged cultivation could weaken the oxygen transfer capacity directly [14]. The effect of DO on fungal fermentation can be either positive or negative, as energy generation or oxygen toxicity [26]. Meanwhile, the OUR is obtained by the specific oxygen consumption rate multiplied by the concentration of biomass [2]. As to the shear intensity and the mixing effect in the bioreactor, a modest combination of the agitation speed and the impeller type is necessary for the submerged culture of filamentous fungi [5]. Freely dispersed clumps and short hyphae always come with high shear intensity, whereas the inhomogeneous mass transfer capacity usually occurs at a low agitation speed.

The purpose of this study was to enhance the production of 1403C by *Halorosellinia* sp. (no. 1403) in bioreactor fermentation. Medium, culture pH, agitation speed, impeller type, inoculum level and DO were considered and systematically optimized, and an integrated nutrition and bioprocess strategy was established.

Materials and methods

Strain and culture conditions

Halorosellinia sp. (no. 1403) (CCTCCM 201018) was kindly provided by Sun Yat-sen University. The culture was maintained in petri dishes of agar medium composed of 10 g glucose, 1 g yeast extract, 2 g tryptone and 18 g agar per liter of artificial seawater I (ASW I). The dishes were incubated at 28 °C and relative humidity (RH) above 50 % for 5 days, and then used for liquid culture. To prepare ASW I, 24.5 g NaCl, 4.0 g Na_2SO_4, 0.56 g $CaCl_2$, 5.0 g $MgCl_2{\cdot}6H_2O$, 0.026 g H_3BO_3, 0.664 g KCl, 0.1 g KBr and 0.2 g $NaHCO_3$ were dissolved in 1 l distilled water.

The seed medium consisted of 10 g glucose, 1 g yeast extract and 2 g tryptone in 1 l ASW I; 15 μl antifoam 204 (Sigma-Aldrich) was added to every baffled Erlenmeyer flask (500 ml) with 100 ml seed medium before sterilization. The solution was autoclaved at 121 °C for 20 min. After cooling down to room temperature, a rounded agar lump (10 mm diameter) from the dishes, divided into four pieces, was inoculated into each baffled Erlenmeyer flask. The first stage seed was incubated at 170 rpm and 28 °C on a rotary platform shaker for 72 h. The second stage seed was inoculated with 5 ml of first stage seed and incubated identically for 36 h.

The experiments for medium optimization were performed in 250-ml Erlenmeyer flasks containing 50 ml fermentation medium, which was inoculated with 2.5 ml second stage seed. The composition of the original medium was 10 g glucose, 2 g tryptone and 1 g yeast extract dissolved in 1 l 20 % ASW I. The flasks were incubated at 28 °C and 170 rpm on a rotary platform shaker for 120 h. All cultures were performed in triplicate.

A 5-l stirred-tank bioreactor (Shanghai Guoqiang Bioengineering Equipment Co., Ltd.) was employed and equipped with pH, DO, temperature and foam sensors. DO was measured using a polarographic probe calibrated to 100 % saturation for aeration of 1 vvm at agitation of 600 rpm and tank inside pressure of 0.02 Mpa. The working volume was 3.5 l, and the impeller equipment was a double-layer six-blade Rushton disc turbine (RDT, 6.8 cm i.d.). The lower impeller was 2.5 cm above the reactor bottom, and the vertical distance between two impellers was 7.2 cm. The agitation speed was controlled at 300 rpm through electromagnetic impulse. The aeration system was an air inlet through a ring sparger with an air-flow meter. A jacketed water bath was designed to maintain the temperature at 28 °C. Initially, the lower limit of DO was set at 30 % saturation for the whole fermentation. A 175-ml second stage seed was sterilely inoculated into the bioreactor.

Analytical methods

The off-line pH of the broth was measured by an FE20 pH meter (Mettler-Toledo). The total biomass was determined by dry cell weight (DCW) [30]. Broth was centrifuged at 13,000*g* for 5 min, and the obtained supernatant was immediately stored at −20 °C for use in the analysis of residual glucose with the biosensor instrument SBA-40E (Biology Institute of Shandong Academy of Science, China). Ten milliliters of fermentation broth and 20 ml acetic acid were transferred to a 50-ml Eppendorf centrifugal tube and mixed well. Then the mixture was placed in the ultrasonic equipment (KQ-800, Kunshan Ultrasonic

Instruments Co., Ltd.) with a frequency of 40 kHz and a nominal power of 800 W for 30 min in order to obtain further dispersion and extraction. Subsequently, the tubes were transferred to a 65 °C water bath for 30 min. Then the ultrasonic process and water bath operation were repeated again. Finally, 1 ml mixture was centrifuged at 13,000*g* for 3 min. The supernatant was prepared for further HPLC analysis after proper dilution. The analytical method for 1403C was adapted from the former report [30].

Experiment design

Medium optimization by statistical designs

Statistical design strategies were implemented to optimize the fermentation medium. The one-factor-at-a-time strategy was first used to determine the basic medium composition. Then, a two-level Plackett-Burman design was selected to analyze the significance of each factor. Finally, a central composite design was used to locate the optimum values given by response prediction. The polynomial regression model was fitted as follows:

$$Y = a_0 + \sum a_i X_i + \sum a_{ii} X_i^2 + \sum a_{ij} X_i X_j$$

Here, Y is the response; a_0 is the value of the fitted response at the center point of the design. a_i, a_{ii} and a_{ij} are the linear, quadratic and cross term coefficients, respectively. The coefficients of the equation were obtained by SAS 9.2 software (SAS Institute Inc).

Bioprocess optimization in 5-l bioreactor fermentation

Cell growth and metabolite biosynthesis are often related to the culture conditions. Five operating parameters, including culture pH, impeller type, agitation speed, DO and inoculum level, were investigated in submerged culture of *Halorosellinia* sp. (no. 1403) in a 5-l bioreactor. Each factor was tested on at least two levels. After analyzing the corresponding influences of different factors on 1403C production, a comprehensive control strategy for the fermentation in a 5-l bioreactor was established to modify the bioprocess. Samples were taken every 12 h for determination of the off-line pH, residual glucose, DCW and 1403C production.

Results and discussion

Medium optimization

A one-factor-at-a-time design was first applied to determine the favorable medium ingredients (Suppl. Fig. 1). Glucose, maltose and sucrose were more productive than other selected carbon sources. However, their combinations made no advantageous contribution to 1403C production. Glucose was finally selected because it is easily consumed, inexpensive and widely used in industrial fermentation. An organic nitrogen nutrient rich in vitamins and biotin seemed to be favored by this fungus. Beef extract, yeast extract and tryptone were relatively productive but not as good as the control. However, the combination of the organic nitrogen sources played an important role in 1403C production. Especially the mixture of beef extract and tryptone with the ratio of 1:1 resulted in the highest 1403C production of 1.15 g/l, which was nearly two fold the production in the original medium.

The microbes acquire appropriate quantities of the trace element at the initiation of the shift from primary to secondary metabolism [27]. The unique high-salinity surroundings of natural seawater contain 22 kinds of trace elements, whose average concentrations range between 0.05 and 50 μmol/kg [15]. In this work, the effects of six trace elements (i.e., Mn^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Co^{2+}) on 1403C production were investigated. Each trace element was added to the medium at a final concentration of 100 mg/l, but only manganese sulfate monohydrate promoted 1403C production. Then, four levels of Mn^{2+} were further tested. Adding 100 mg/l manganese sulfate highly improved 1403C production, but manganese sulfate of more than 150 mg/l inhibited it.

The composition of artificial seawater was modified from seven inorganic salts [13] by orthogonal design $L_8(2^7)$ and named ASW II. ASW II contained 19.624 g NaCl, 4.908 g Na_2SO_4, 1.392 g $CaCl_2$, 6.24 g $MgCl_2{\cdot}6H_2O$, 0.032 g H_3BO_3, 0.081 g KBr and 0.161 g $NaHCO_3$ in 1 l distilled water. A desirable condition of osmotic pressure and trace elements was chosen by shifting the level of ASW II, and 40 % ASW II was very adaptive for *Halorosellinia* sp. (data not shown).

Subsequently, a two-level Plackett-Burman design was selected to analyze the significances of five factors according the 1403C production. Considering the enormous contribution of the interaction of X_2 and X_3, three terms, i.e., X_1, X_3 and X_2X_3, made the most remarkable contribution to 1403C production; their values of "Model Prob > F" were even less than 0.01. The analysis of variance (ANOVA) is shown in Suppl. Table 1, and three critical factors were eventually captured. Their coefficients offered the direction and step used in the steepest ascent method [12]. The steepest ascent method is an economically efficient procedure developed to move the experimental region of a response in the direction of the fastest change toward the optimum, and the corresponding gradient vector was a three-dimensional one, which included X_1, X_2 and X_3. The 1403C production reached its peak at

the first step with 9.0 g/l X_1, 1.05 g/l X_2 and 4.4 g/l X_3, set as the center point of the CCD design (Suppl. Table 2).

A three-factor, five-level central composite design was then used. The results of the model were analyzed by ANOVA (Table 1). Obviously, the model was proven to be quite significant, as the Fisher F test with a very low probability value of "Model Prob > F" was only 0.0007. The value of the determination coefficient (R^2) reached 0.898, which implied that 89.8 % of the experimental data would be explained by the quadratic model. The adjusted coefficient of determination (Adj R^2) was more suited for comparing models with different numbers of independent variables. The high value of Adj R^2 was 0.807, which indicated that 80.7 % of the data variability belonged to the range of the regression equation. At the same time, the coefficient of variation (CV) was as low as 6.15, which also indicated the precision and reliability of the fitted model.

The fitted polynomial regression equations were expressed as a three-dimensional curve to visualize the relationship between the response and experimental levels of two factors and to deduce the optimum conditions. Three-dimensional response surface plots for the effect of interaction between two variables on 1403C production when the rest variable was maintained at the zero level are presented in Suppl. Fig. 1. In general, exploration of the response surfaces indicated a complex interaction between the variables. The predicted maximum 1403C production (2.26 g/l) occurred at the coded point (+1.68, 0, +1.68), i.e., 12.36 g/l glucose, 1.05 g/l tryptone and 6.08 g/l beef extract, in real value. Thus, the predicted optimal medium was composed of 12.36 g/l glucose, 1.05 g/l tryptone, 6.08 g/l beef extract and 0.246 g/l $MnSO_4 \cdot H_2O$ dissolved in 40 % ASW II.

Additionally, verification of the 1403C production by predicted medium composition was performed in duplicate. The time profiles of *Halorosellinia* sp. (no. 1403) in submerged culture in 250-ml Erlenmeyer shake flask culture with the original and optimized medium were then analyzed (Suppl. Fig. 1). It seemed that plenty of organic acids accumulated in the optimized medium and caused lower pH during the fermentation process. The fungus grew better and produced more biomass (5.8 g/l) in the optimized medium. Moreover, the optimized medium greatly improved 1403C biosynthesis. The maximum production in the optimized medium was obtained as 2.07 g/l, which was 143.5 % higher than that in the original medium. The good correlation between the predicted and experimental results corroborated the effectiveness of the model.

pH control strategy

Batch fermentation of *Halorosellinia* sp. in a 5-l stirred-tanked bioreactor with optimized medium under the initial control strategy was performed first. Compared with the growth profiles in shake flask culture (Fig. 1a), an apparent divergence was discovered as the broth pH showed a lower value for a long period until the glucose was exhausted at 80 h (Fig. 1b). Under this condition, the corresponding cell growth reached the stationary phase at 48 h, and the DCW was maintained at 3.8 g/l for 42 h, which was 31 % lower than that in shake flask culture. It seemed that weaker metabolic enzyme activity took place when long-term low pH appeared, as the rate of glucose consumption declined and the lowest DO value was still as much as 55 %. For the production, 1403C accumulated quickly before the declining phase, whereas the highest production was only 0.44 g/l and 78.7 % lower than that harvested from shake flask culture. The death and autolysis of mycelia led to a sharp decline in 1403C production. Therefore, broth pH probably played a critical role in submerged culture of *Halorosellinia* sp. in a 5-l bioreactor.

A control strategy based on the pH signal has been widely applied to microbial batch and/or fed-batch

Table 1 The ANOVA for the response surface quadratic model

Source	DF	Sum of squares	Mean square	F value	P value probability > F
Model	9	1.063×10^6	1.182×10^5	9.80	0.0007*
X_1	1	7.919×10^5	7.919×10^5	65.66	<0.0001*
X_2	1	2,529.41	2,529.41	0.21	0.6568
X_3	1	59,561.84	59,561.84	4.94	0.0505
X_1X_2	1	421.98	421.98	0.035	0.8554
X_1X_3	1	52,132.11	52,132.11	4.32	0.0643
X_2X_3	1	13,024.7	13,024.7	1.08	0.3232
X_1^2	1	46,950.88	46,950.88	3.89	0.0768
X_2^2	1	1.034×10^5	1.034×10^5	8.57	0.0151*
X_3^2	1	822.30	822.30	0.068	0.7993
Residual	10	1.206×10^5	12,060.52		
Cor total	19	1.184×10^6			

* Significant at the 5 % level ($P < 0.05$); $R^2 = 0.8981$, CV % = 6.15, Adj $R^2 = 0.8065$, Adeq precision = 10.430

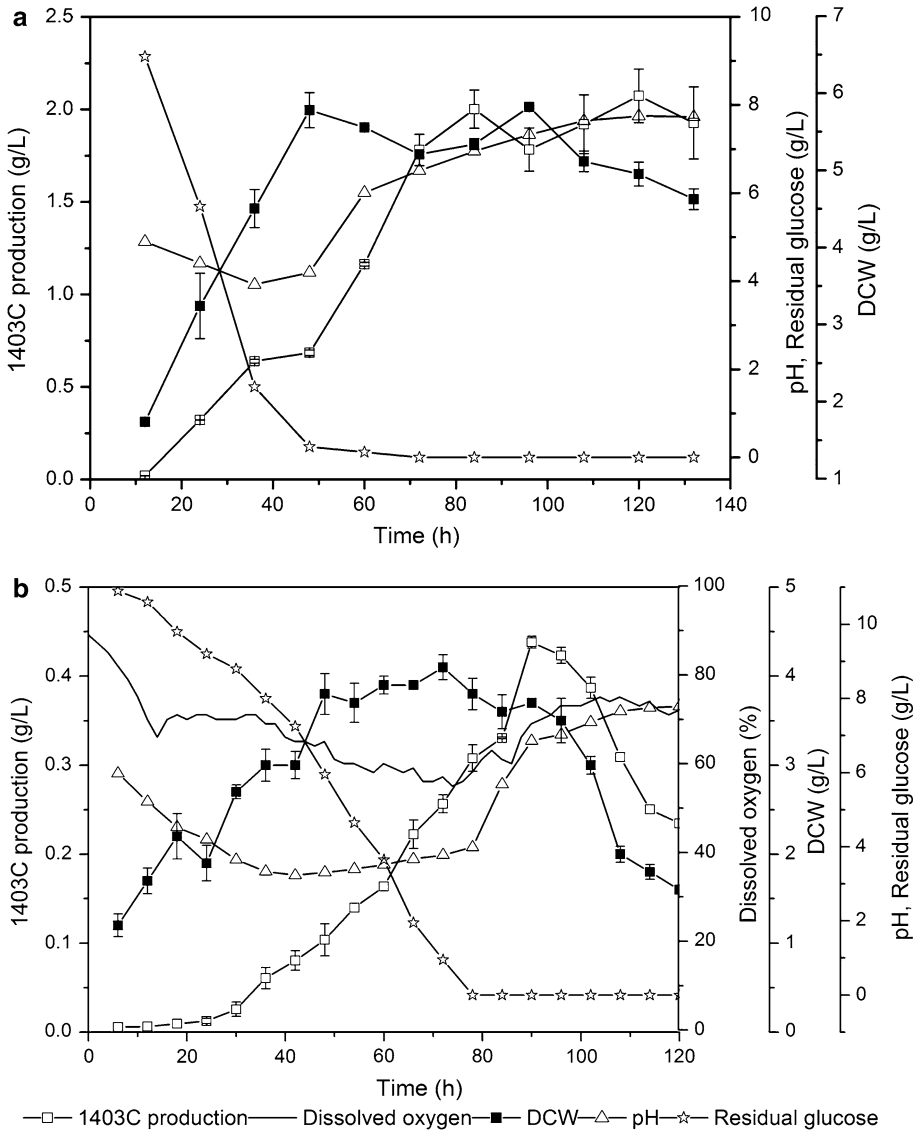

Fig. 1 Comparison of time profiles of pH, DCW, DO, residual glucose and 1403C production by submerged cultures of *Halorosellinia* sp. (no. 1403) in a 250-ml shake flask (**a**) and 5-l bioreactor (**b**)

fermentation recently, including pH-shift mode [19, 25] and pH–stat mode [22, 33]. Initially, a pH-simulating strategy, regulating the broth pH by simulating the pH tendency in the shake flask culture, was applied in a 5-l bioreactor. Then a batch process at various pH values ranging from 4.0 to 7.0 for production of 1403C by *Halorosellinia* sp. controlled using a pH–stat strategy was investigated. An intermittent pumping base (1 M NaOH) or acid (1 N HCl) according to the feedback of the pH signal was used to control pH.

Figure 2a depicts the time profiles of batch fermentation in a 5-l bioreactor with a pH-simulating strategy. By pumping base or acid under manual control, the pH was adjusted to simulate the pH tendency in the shake flask culture. The 1403C production rose dramatically to 0.92 g/l at 96 h, which was 109 % higher than the initial production. The turning point of this batch fermentation appeared at 48 h, as the residual glucose was less than 0.2 g/l and the DO level soared to 80 % within 24 h. The great change in the physical environment did not influence the biomass significantly, as the DCW fluctuated around 3.9 g/l for 96 h. Consequently, short-term low pH and rapid-rising pH at the point of carbon starvation were beneficial for the biosynthesis of 1403C.

As illustrated in Fig. 2b–e, a batch culture with pH–stat 7.0 had maximal production of biomass (5.1 g/l) and 1403C (0.86 g/l). The pH–stat strategy with low pH values had a negative influence on carbon consumption. For pH–stat 4.0, even 0.8 g/l glucose was unused. Moreover, the stationary phase shortened, the decline phase was prolonged, and the final biomass was lower than 2.0 g/l. The cell growth was inhibited as the pH–stat level decreased, which might be ascribed to the small and weak mycelium being obtained in low pH culture. Moreover, by-product

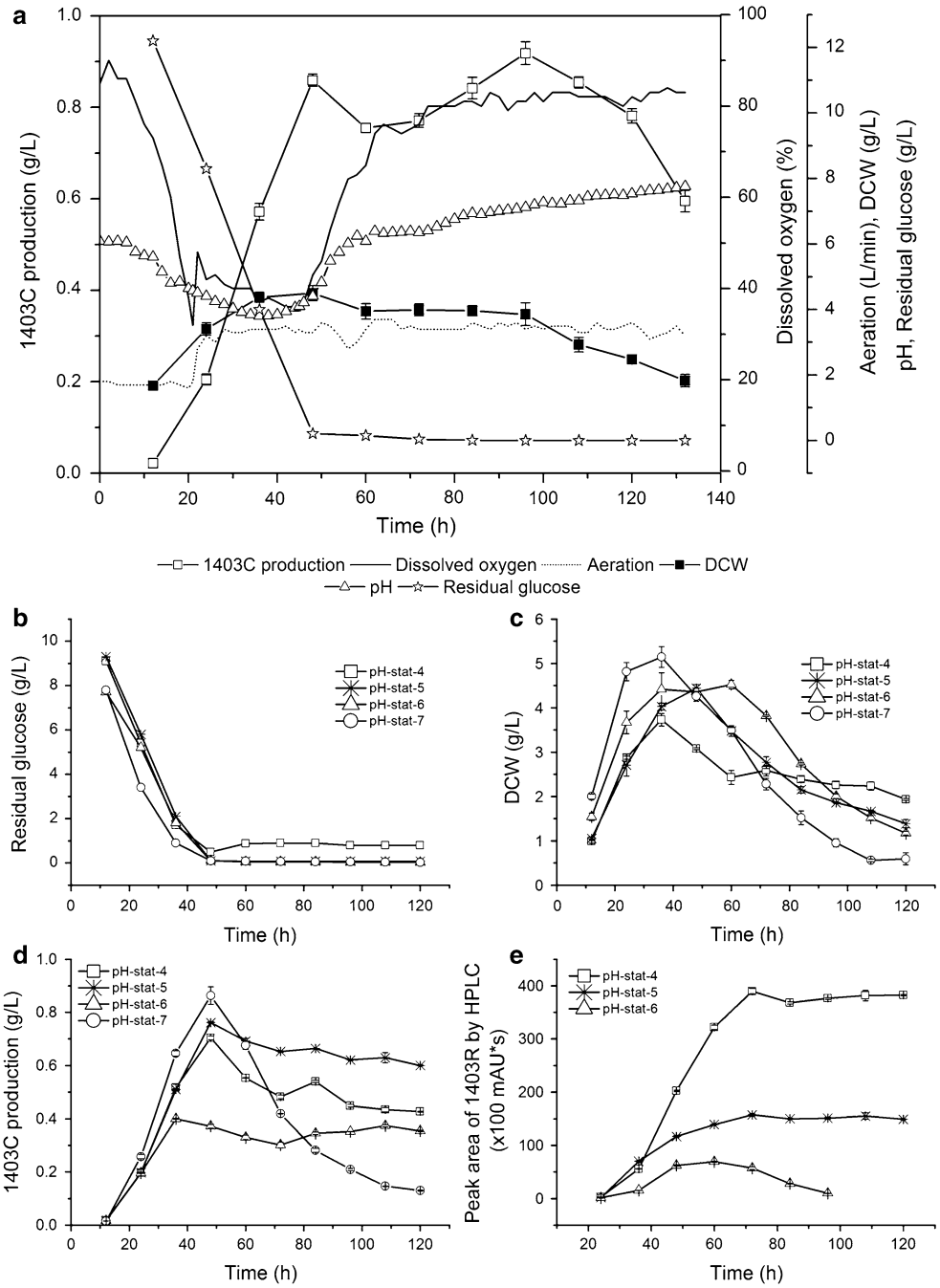

Fig. 2 Effect of the pH-control strategy on growth and metabolism of *Halorosellinia* sp. (no. 1403) submerged culture in a 5-l bioreactor. **a** pH-simulating strategy, comparison of (**b**) residual glucose, (**c**) DCW, (**d**) 1403C production and (**e**) 1403R under the pH–stat strategy

1403R seemed to prefer accumulating in an acidic environment, and its production increased as pH declined. According to the above results, the pH–stat strategy was not beneficial for 1403C biosynthesis in the batch culture.

Effect of shear stress on 1403C production

It was reported that modest shear intensity was beneficial for 1403C production. Adding 5–10 glass beads (3 mm diameter) to the 250-ml Erlenmeyer flask could promote the metabolic synthesis of 1403C [17]. To investigate the influence of shear stress and mixing on 1403C production, different agitation speeds and impeller types were employed. Agitation speed, as is well known, affects both the air bubble distribution and mixing effect [3]. Fermentation in a 5-l bioreactor was performed to verify the appropriate agitation speed and impeller type. First, three bioreactors were controlled at 300, 400 and 500 rpm. They were each equipped with double-layer six-flat-blade Rushton disc turbines (RDT). Second, double RDT

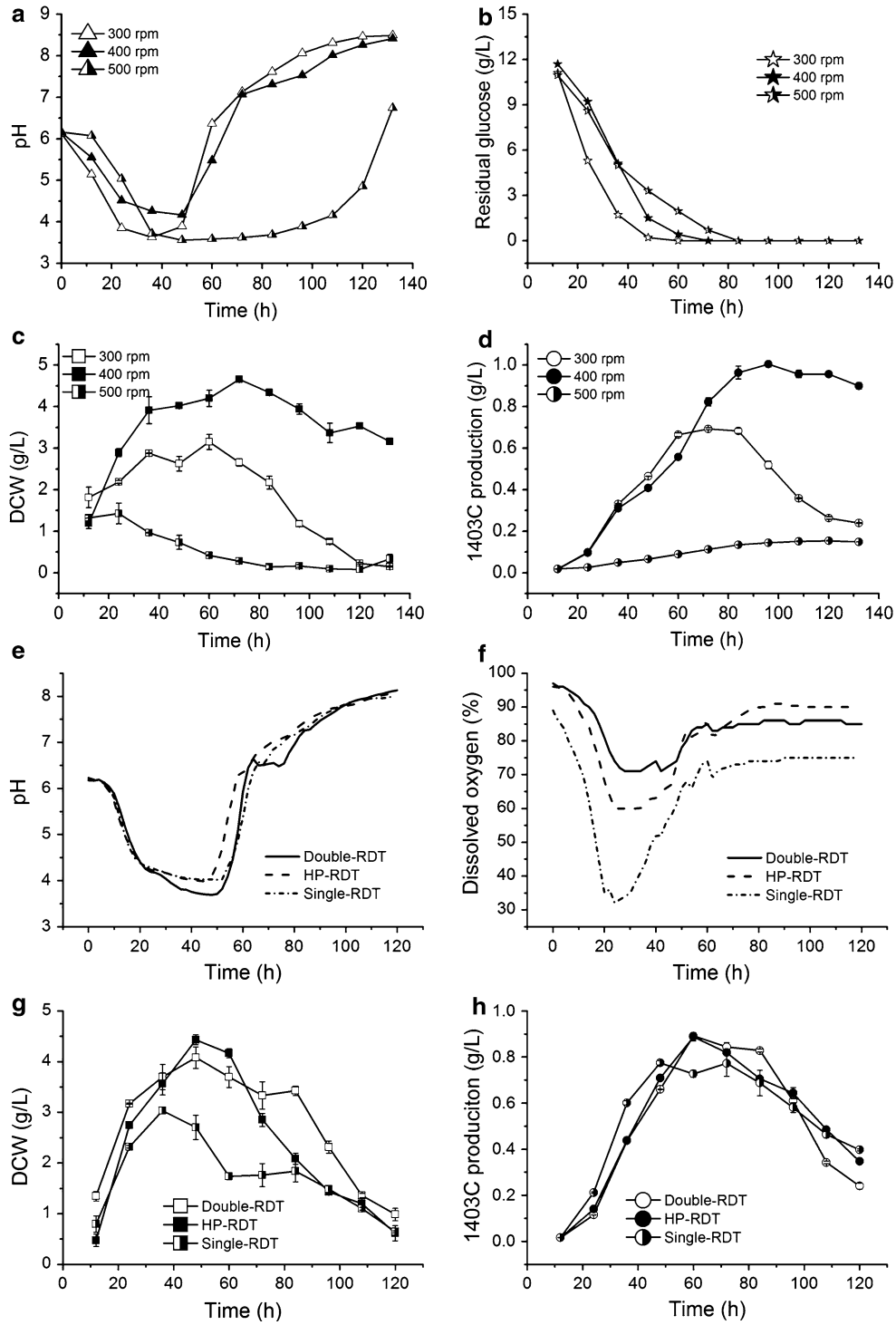

Fig. 3 Effects of shear stress on *Halorosellinia* sp. (no. 1403) submerged culture in a 5-l bioreactor. **a–d** Different agitation speeds; **e–h** different combinations of impeller types. RDT, six-blade Rushton disc turbine; HP, 3-curved-blade hydrofoil propeller

(control), an impeller combination (3-curved-blade hydrofoil propeller and RDT, HP-RDT) and single RDT were applied to three bioreactors agitated at 400 rpm, respectively. The hydrofoil propeller offers attractive benefits in improved axial flow mixing and reduced shear stress for mycelial cultivations [16]. Time profiles of pH,

residual glucose, DCW and product concentration were compared among three fermentations with different speeds and are shown in Fig. 3a–d.

Obviously, high agitation speed (500 rpm) caused serious metabolic inhibition of *Halorosellinia* sp. During the whole fermentation period, the mycelium suffered from a low culture pH of 3.0–4.0 for about 60 h. The excessively acidic environment was the direct consequence of the unbalanced metabolism of organic acid. The consumption of glucose was disturbed by the harsh shear stress of the speed at 500 rpm, and the corresponding residual glucose was not exhausted until 84 h. On the contrary, the pH values of the other two fermentations almost witnessed a similar variation trend, i.e., they decreased gently to the valley, then increased sharply to 7.0 within 24 h. During the whole period, the pH value of fermentation with 400 rpm was maintained at the intermediate state, which probably indicated a balanced metabolic flux of organic acids. The corresponding consumption rate of glucose also verified this supposition. Additionally, a low agitation speed (300 rpm) was a disadvantage as an inadequate mixing capacity weakens the efficiency of mass transfer. As to the residual glucose, the average consumption rate reduced as the agitation speed increased. Additionally, both the DCW and 1403C production experienced diverse processes when cultured under different agitation speeds. The highest biomass, as much as 4.66 g/l, appeared at 72 h and 400 rpm, which was 47.9 % higher than the control (300 rpm). Meanwhile, the highest 1403C production reached 1.0 g/l at 96 h, 44.9 % higher than the control. Besides, the stationary phase of the 400 rpm group was maintained up to 60 h. However, cell growth seemed to be severely damaged by intense agitation of 500 rpm, and the resulting 1403C production only reached 0.15 g/l. Accordingly, agitation at 400 rpm was favorable in this case.

As for different impeller combinations (Fig. 3e–h), the DO was correlated to a mixing effect and aeration. Computational fluid dynamics (CFD) models illustrated that an impeller combination could enhance the mixing efficiency and bring down the shear stress [28]. Therefore, the single-RDT type must provide the poorest mixing and weakest shear intensity. The minimum levels of DO for double-RDT, HP-RDT and single-RDT were 71, 60 and 32 %, respectively, under identical agitation speed and aeration. Generally, impeller combinations in HP-RDT could balance both the radial and axial mixing capacity. However, in terms of oxygen transfer in low-viscosity broth, oxygen dispersion was much more homogeneous in double RDT than in HP-RDT.

For fermentations performed with double RDT and HP-RDT, the DCW and 1403C production showed no obvious difference. Either double RDT or HP-RDT fermentation could obtain 1403C production of 0.89 g/l. However, the single-RDT fermentation produced the lowest biomass and 1403C production. The impeller choice seemed to have no impact on the highest 1403C production. A similar phenomenon was also reported in submerged fed-batch fermentation of *Aspergillus oryzae* in 550-l pilot plant stirred tank reactors for enzyme production [1]. Considering the fact that the double-RDT impeller is widely used in industrial fermentation, it was then selected for further research.

Inoculum optimization

The inoculum strongly influences the cell growth and metabolism, also concerning the inoculum age [23] and size [21], and more precisely the seed morphology [18, 30] for filamentous fungi. In order to optimize the inoculum concentration, different inoculum levels were first used in 250-ml shake flask culture. The results indicated that inoculum of 5 % (equal to 0.22 g/l dry biomass) produced strong mycelia and led to the highest 1403C production (data not shown). Then, bioreactor fermentation with the optimal inoculum size was carried out to verify the influences. Figure 4 depicts the time curves of *Halorosellinia* sp. (no. 1403) submerged culture in a 5-l bioreactor inoculated with different seed volumes equal to a dry biomass of 0.22 and 0.11 g/l. The 1403C biosynthesis was greatly improved with the high inoculum level, and the production vertex was obtained as 1.21 g/l, which was 98.4 % higher than that with the low inoculum level. However, the highest DCWs between both batches differed only slightly even though the high inoculum ratio caused faster glucose utilization. Interestingly, broth pH in the fermentation with a low inoculum level showed a quite long period of low value, indicating a metabolism abnormality, which might be related to its low 1403C production. Overall, it was obvious that critical facilitation worked by inoculum optimization.

DO-shift strategy

In aerobic processes, oxygen must be continuously supplied in order to achieve acceptable productivities, since the role of oxygen in microorganism growth and its metabolism is of vital importance; both the OUR by the cell and the OTR into the system have to be emphasized [10, 11]. DO in the broth reflected the balance of OTR and OUR.

In this work, the oxygen demand for 1403C production by *Halorosellinia* sp. was first analyzed by a broth volume shift strategy in a shake flask culture. Broth volume was changed in the growth and production phase to create

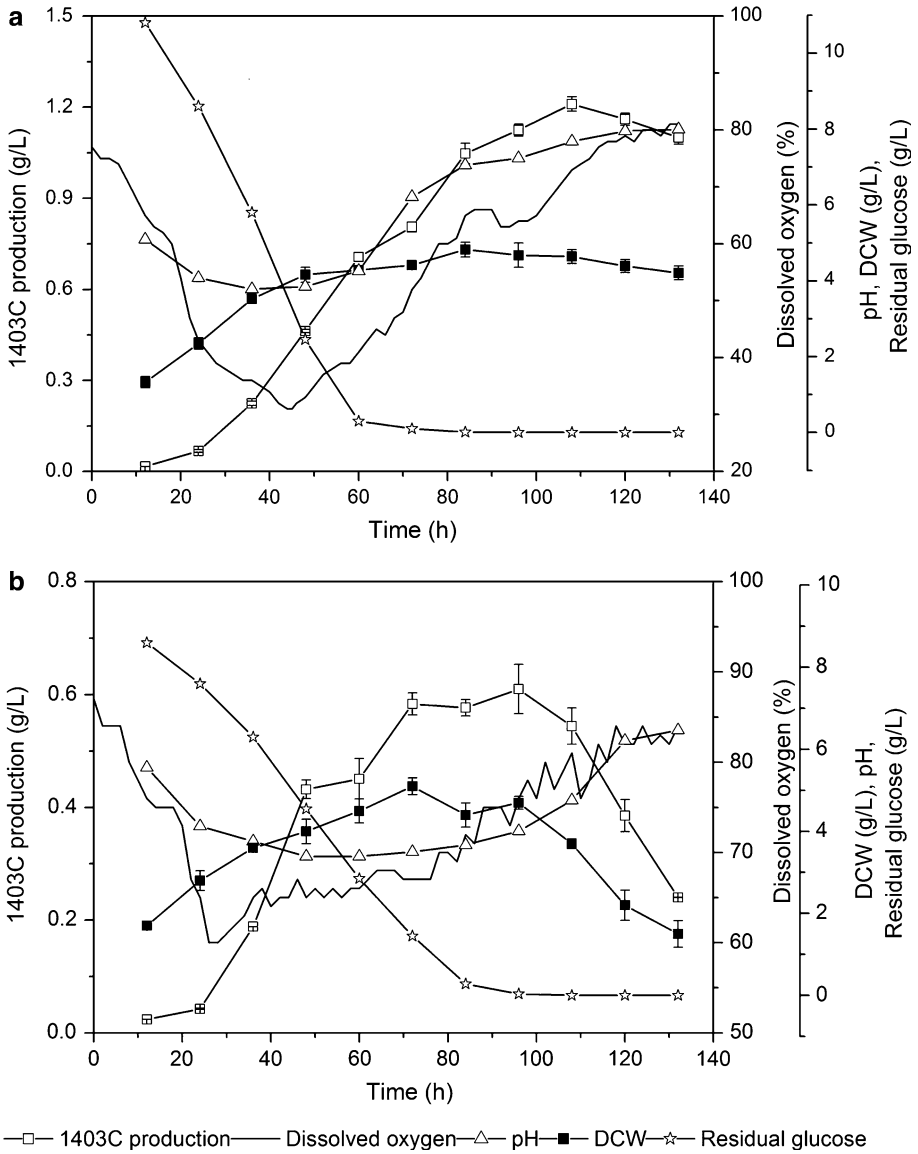

Fig. 4 Time profiles of pH, DCW, DO, residual glucose and 1403C production by submerged culture of *Halorosellinia* sp. (no. 1403) in a 5-l bioreactor with different inoculum levels, i.e., seed amounts equal to DCW of 0.22 g/l (**a**) and 0.11 g/l (**b**)

various DO conditions. The starting point of the stationary phase (60 h) was regarded as the shift point. It was demonstrated that a varying DO demand occurred in different phases in submerged culture. Broth volume shifts between the growth phase and production phase improved 1403C production by 11.7 % as compared to the control (2.07 g/l) (data not shown). On the basis of the above results, an optimized oxygen control strategy was proposed that involves supplying sufficient oxygen in the growth phase and limiting it during the production phase. Thus, different step-wise DO-shift strategies in the production phase (60–120 h) were then investigated to improve the DO control in a 5-l bioreactor.

As to the step-wise DO-shift strategy operated in a 5-l bioreactor, batch fermentation was controlled based on the DO signal, controlling DO to be not lower than 30 % during the growth phase but ranging between different DO levels (20–30, 30–40 and 40–50 %) during the stationary phase by simultaneous regulation of aeration and agitation speed. The impeller type of double RDT agitated at 400 rpm was controlled at the beginning of batch culture. In general, aeration adjustment was made prior to that of agitation speed as change in agitation speed could cause an intense change in both the DO level and shear stress.

As shown in Fig. 5a–d, the highest production of 1403C and DCW reached 1.32 g/l (108 h) and 4.4 g/l (84 h) with DO fluctuating at 30–40 %, and it reached 1.2 g/l (96 h) and 3.3 g/l (72 h) with DO fluctuating at 40–50 %, respectively. Maintaining the DO level at 20–30 % seemed unreasonable because a harsh decline of biomass and small increase of 1403C production had occurred since the startup of the DO shift. The corresponding DCW reached up to 5.4 g/l (66 h), higher than in the other two groups, whereas the peak production was only 0.81 g/l. Great divergence in 1403C

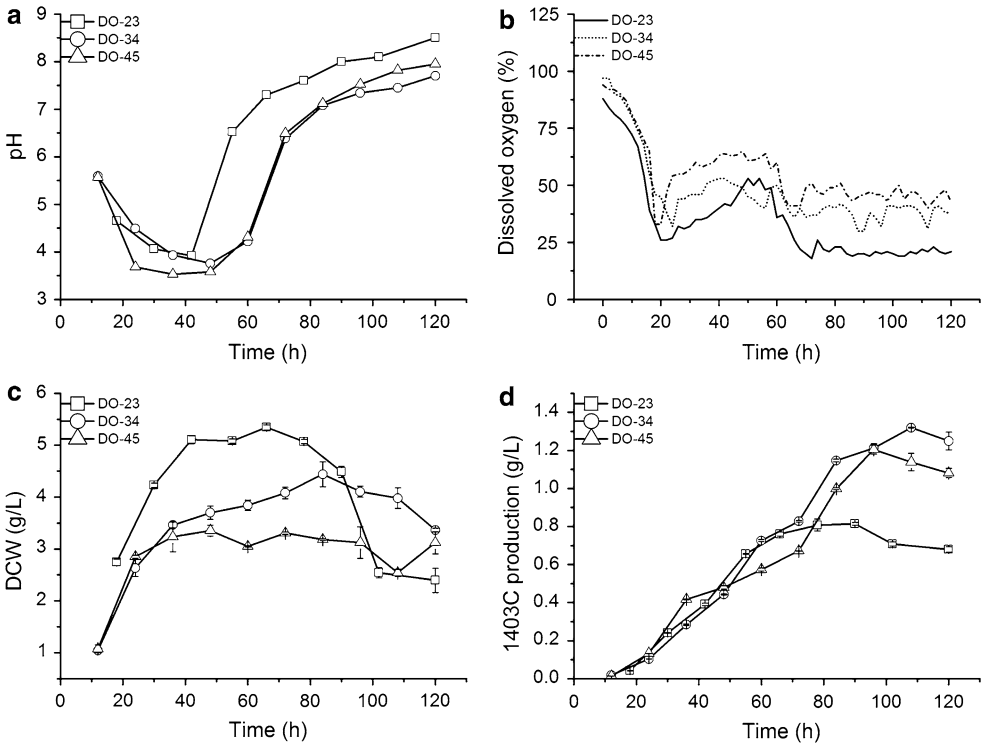

Fig. 5 Effect of the DO level (after 60 h) on *Halorosellinia* sp. (no. 1403) submerged culture in a 5-l bioreactor. **a** pH, **b** DO, **c** DCW, **d** 1403C production

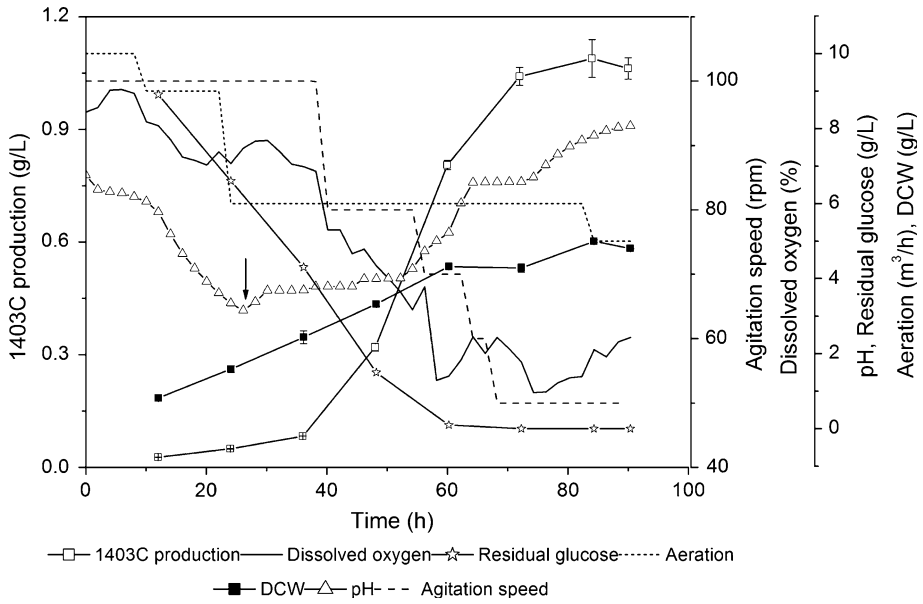

Fig. 6 Time profiles of submerged culture of *Halorosellinia* sp. (no. 1403) in a 500-l bioreactor

accumulation did occur at the production phase when supplied with different oxygen concentrations. Overall, there was no doubt that a relatively low DO (30–40 %) in the late period was beneficial for 1403C production.

Scale-up fermentation in a 500-l bioreactor

Finally, a pilot scale in 500-l bioreactor fermentation based on the criterion of the constant aeration rate (vvm) and peak impeller tip speed at early phase was proposed and performed according to the muti-parameter control strategy established via the successive cases in a 5-l bioreactor. The initial agitation speed and aeration were 100 rpm and 10 m^3/h (0.5 vvm), respectively. As a result of the good mixing efficiency in the 500-l bioreactor, the DO level could only be kept lower than 60 % when both the agitation speed and aeration were halved. The pumping base was used to adjust the broth pH gradually for 26–36 h in

order to avoid a long-term low pH level. As illustrated in Fig. 6, 1403C production was successfully reproduced in a 500-l bioreactor, and the highest production was 1.09 g/l.

Conclusion

Nutrition and bioprocess were optimized to enhance the production of an antitumor compound, 1403C, by marine-derived fungus *Halorosellinia* sp. (no. 1403). Statistical design strategies were first applied to improve the nutritional composition, which led to a 143.5 % production increase. Then, the bioprocess was improved by synthetically modifying the important process parameters including pH, agitation speed, impeller type, inoculum level and DO. With these efforts, 1403C production in a 5-l bioreactor was greatly improved and reached 1.32 g/l, which was promoted by 200 % as compared to that before optimization. Fermentation scale-up to 500 l was finally performed, and the maximal 1403C production reached 1.09 g/l.

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