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ORIGINAL PAPER

Influence of agitation and aeration on growth and antibiotic production by *Xenorhabdus nematophila*

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Abstract The effect of agitation and aeration on the growth and antibiotic production by *Xenorhabdus nematophila* YL001 grown in batch cultures were investigated. Efficiency of aeration and agitation was evaluated through the oxygen mass transfer coefficient (K_La). With increase in K_La, the biomass and antibiotic activity increased. Activity units of antibiotic and dry cell weight were increased to 232 U ml^{-1} and 19.58 g l^{-1}, respectively, productivity in cell and antibiotic was up more than 30% when K_La increased from 115.9 h^{-1} to 185.7 h^{-1}. During the exponential growth phase, DO concentration was zero, the oxygen supply was not sufficient. So, based on process analysis, a three-stage oxygen supply control strategy was used to improved the DO concentration above 30% by controlling the agitation speed and aeration rate. The dry cell weight and activity units of antibiotic were further increased to 24.22 g l^{-1} and 249 U ml^{-1}, and were improved by 24.0% and 7.0%, compared with fermentation at a constant agitation speed and a constant aeration rate (300 rev min^{-1}, 2.5 l min^{-1}).

Keywords *Xenorhabdus nematophila* · Aeration · Agitation · Oxygen mass transfer coefficient (K_La) · Antibiotic production · Growth

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Introduction

Xenorhabdus nematophila is a Gram-negative bacterium, belonging to Enterobacteriaceae. The bacterium is mutually associated with an insect-pathogenic nematode in the genus *Steinernema* (Steinernematidae) (Thomas and Poinar 1979), and is carried in the intestine of the infective juvenile (IJ) stage of the nematode. The IJ penetrates an insect host and releases the bacteria into the hemocoel. The bacteria multiply rapidly, resulting in a bacterial septicemia and death of the host within 24–48 h. The nematode feeds on the multiplying bacteria. This nematode–bacteria complex has been developed commercially as biological control agent of insect pests. The secondary metabolites produced by the *Xenorhadus* overcome the insect immune system (Forst and Nealson 1996), kill the insect, and inhibit the growth of various fungal and bacterial competitors (Akhurst 1982; Chen et al. 1994; Chen et al.1996). By doing so, the bacterial symbionts are believed to prevent putrefaction of the insect cadaver and establish conditions that favor the development of both the nematode and bacterial symbionts (Gaugler and Kaya 1990).

The foremost applied interest in studying these bacteria concerns nutritional requirements for improving mass production of the nematodes for biological control of insects. For biocontrol purposes, mass production of these nematode–bacteria complexes is perferably performed in liquid culture (Ehlers 1996). Liquid media are pre-incubated with the bacteria 24 h prior to inoculation of IJs from monoxenic cultures (Ehlers et al. 1998). Beside the function of serving as a food source for nematode, the bacterial symbiont also produces signals that influence

nematode development. A food signal is excreted by the bacteria into the liquid medium, triggering the recovery of the IJs and thus permits the production of biocontrol nematodes in artificial media. The second interest of these bacteria is to use their secondary metabolites, which have a commercial potential, in the pharmaceutical and agroforestry industries (Webster et al. 1998).

For mass production of the biocontrol nematode, bacterial cultures at high cell density are required for IJ recovery, offspring production, and high antibiotic productivity. There are many factors influencing bacterial growth and antibiotic production. Temperature, pH, nutrients, agitation, and aeration are the most typical factors. Especially, aeration and agitation of the culture influence bacterial growth and, consequently, affect antibiotic production (Akhurst 1982; Chen et al. 1996). *Xenorhabdus* spp. and *Photorhabdus* spp. are facultative anaerobes, but aeration is essential for antibiotic production. No antibiotic activity was detected in cultures in sealed vials that were not agitated (Akhurst 1982; Chen et al. 1996). In a previous work, we had chosen a strain with high antibiotic activities in aerobic batch cultures. Then we optimized the culture medium composition and culture condition in shake flasks. Among the parameters of culture condition, the medium volume in flask and rotary speed of shake flask had significant influence on growth and antibiotic production. These results indicate that aeration is a important parameter in fermentation of *Xenorhabdus* spp. and *Photorhabdus* spp. (Wang 2004). In this paper, we investigate the effect of various aeration and agitation conditions on growth and antibiotic production of the symbiotic bacteria associated with *Steinernema* sp. YL001 from Shanxi, China.

Materials and methods

Organism

Xenorhabdus nematophila YL001, obtained from Biorational Pesticides Research and Service Center, Yangling, China, was isolated from its nematode symbiont, *Steinernema* sp. YL001, which was obtained from the soil from Yangling, China. The bacteria used in this study were phase I variant.

Culture condition and media

Xenorhabdus nematophila YL001 was maintained on nutrient agar (NA) medium and subcultured monthly. Due to the instability of the phase I under normal culture conditions, the phase I, but less so the phase II exhibits a significant level of antibiotic activity, glycerinated stocks of the bacteria frozen at –70°C were frequently used as starting material for culture. To insure that it was phase I of this bacteria species, plate culture was incubated in darkness at 25°C on nutrient agar supplemented with 0.004% triphenyltetrazolium chloride (W/V) and 0.0025% bromothymol blue (W/V) (NBTA). Phase I is distinguished from phase II by its adsorption of bromothymol blue to produce a red core colony overlaid by dark blue and surrounded by a clear zone after 3–5 days of incubation.

Seed cultures for growth and antibiotic production were prepared by inoculating a loopful of phase I *X. nematophila* YL001 from a 72 h culture growing on an NBTA plate into a 250-ml flask containing 50 ml fresh fermentation medium. The medium contains (g l^{-1}): glucose 6.13, peptone 21.29, $MgSO_4 \cdot 7H_2O$ 1.50, $(NH_4)_2SO_4$ 2.46, KH_2PO_4 0.86 , K_2HPO_4 1.11 and Na_2SO_4 1.72. Media was adjusted to a final pH of 7.0 to provide optimal conditions for growth for *X. nematophila* YL001. The flasks were incubated in darkness at 25°C on an Eberbach rotary shaker at 150 rev min^{-1} for 18–24 h, at which time the optical density (600 nm) and pH readings were approximately 2.0 and 7.0, respectively.

Batch cultures was carried out in a 7-l Setric fermentor (4.5 l working volume). The fermentors were equipped for measurements of agitation, temperature and dissolved oxygen concentration (DO). The cultivation temperature was 28°C; aeration rate and agitation speed were controlled as indicated in Table 1. According to 9% inoculation volume, 400 ml of the seed culture was transferred into 4.5 l of sterile medium in fermentors. The fermentors were incubated according to the cultivation condition for 72 h .

Samples of ca. 20 ml were withdrawn each 3 h approximately. An amount of 10 ml aliquots of the fermentation broth were centrifuged (12,000 rev min^{-1}, 20 min, 4°C, Himac CR 22G) to separate the bacteria cells from the supernatant. The supernatants were store at 4°C until required for use.

Table 1 Determination of K_La values for various aeration rates and agitation speeds

Run no.	Aeration (l min^{-1})	Agitation (rev min^{-1})	K_La (h^{-1})
XB1	2.0	200	115.9
XB2	2.5	200	136.2
XB3	2.5	300	185.7

Analytical methods

Antibiotic activity was measured by agar diffusion plate assay with *B. subtilis* (Maxwell et al. 1994). Briefly, 1 ml containing 10^7–10^8 colonies of *B. subtilis* were applied to NA plate. After 2 h incubation at room temperature, the supernatants of culture following microfilteration, using a 0.22 μm syringe microfilter, were placed on 6-mm disk filters (Whatman 3 MM paper) and air dried. The dried disks were put on the NA plates and incubated at 28°C for 24 h to determine the relationship between the size of the zones of inhibited bacterial growth and the concentration of the antibiotic. Zones of inhibition were measured from the edge of antibiotic disk to the margin of the zone of inhibition. Antibiotic activity was expressed as units of activity per milliliter the supernatants of culture, where 1 U was defined as a 1.0-mm annular clearing around the antibiotic disk. The size of the zone of inhibition served as a measure of antibiotic titer.

The cell concentration was measured by the optical density (OD) at 600 nm with a spectral photometer. The dry cell weight (DCW) was determined gravimetrically after centrifugation of 2-ml sample at 10,000 rev min^{-1} for 20 min (Himac CR 22G, Japan) and drying of the cell pellet at 110°C for 24 h. The optical density (OD_{600}) value was converted to dry cell weight (DCW) using the equation that $OD_{600} = 1.0$ is equal to 0.37 g l^{-1} DCW.

The glucose concentration were measured by the 3,5-dinitrosalicylic acid spectrometric method (Miller 1960).

Oxygen mass transfer coefficient K_La was measured before inoculation following the dynamic method (Pouliot et al. 2000; Rainer 1990) .

Analyses were carried out in duplicate. The data is reported as the average of the measurement and the standard error was less than 5%.

Results and discussion

Determination of K_La

The supply of oxygen is a critical factor in all aerobic fermentation. An insufficient oxygen transfer leads to a decrease of microbial growth and product formation. The effect of oxygen on fermentation is generally viewed from two aspects: oxygen transfer rate (OTR) and DO concentration. These two factors have different dimensions and thus different physical meanings. The oxygen transfer rate, however, is not a constant during fermentation. Instead, it changes with time due to the change of DO in the fermentation medium. Thus, the oxygen mass transfer coefficient (K_La) is a useful tool in evaluating the effect of oxygen on fermentation process (Elibol and Ozer 2000; Sahoo and Agarwal 2002). In submerged fermentation, the oxygen mass transfer coefficient serves to compare the efficiency of bioreactors and their mixing devices and is one of the most important scale-up factors (Makni et al. 1995). The data of K_La under various aeration rates and agitation speeds are given in Table 1. It seems unlikely that the aeration rate affected the K_La as significantly as the agitation speed did. K_La increased sharply as agitation speed increased from 200 to 300 rev min^{-1}. However, K_La was not significantly affected by the aeration rate. Agitation speed is found to be a precise means to control the K_La.

Profiles of DO concentration in batch fermentation

Figure 1 shows the changes in DO concentration in the batch fermentation system of *Xenorhabdus nematophila* YL001 under various aeration rates and agitation speeds. The changes in DO concentration under different combinations of two agitation speeds and aeration rates control show a similar tendency: a rapid decrease in the first 8 h and stabilization at around zero level in 8–41 h of fermentation, then increased gradually. This phenomenon indicates that under various agitation and aeration, the oxygen uptake rate of cells is higher than the oxygen transfer rate in the first 8 h, and oxygen limitation took place during the exponential growth phase.

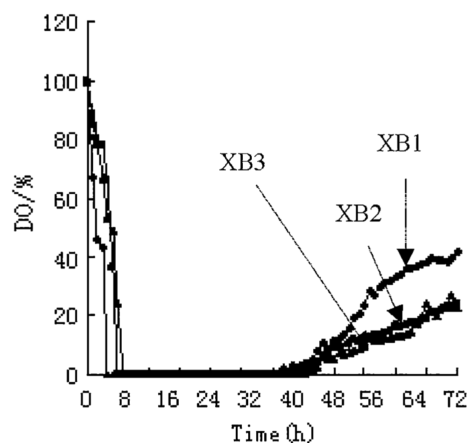

Fig. 1 Profiles of dissolved oxygen concentrations in batch fermentation by *X. nematophila* YL001 under various conditions: K_La = 115.9 h^{-1} (curveXB1); K_La = 136.2 h^{-1} (curve XB2), and K_La = 185.7 h^{-1} (curve XB3)

Effect of agitation speed and aeration rate on cellular growth and antibiotic production

Results of each batch cultivation are summarized in Table 2. To analyse the kinetic characteristics of these process, three kinetic parameters within the whole process: specific growth rates (μ), specific antibiotic production rates (q_P), and specific glucose consumption rates (q_s), were calculated by an interpolation method based on the data of DCW, antibiotic activity units, and glucose concentration. Using the same method, we also calculated the cell yield on glucose ($Y_{X/S}$) and antibiotic yield on glucose ($Y_{P/S}$).

As K_La increases, more oxygen was transferred into the fermentation broth, both the DCW and antibiotic activity units increased, and glucose concentration decreased in the culture medium. In the batch experiment XB1, the agitation speed of 200 rev min^{-1}, and the aeration rate of 2.0 l min^{-1}, antibiotic activity was first observed at 12 h, after which it increased rapidly to 24 h and developed slowly thereafter to reach a maximum level (181 U ml^{-1}) at 48 h. However, in XB3, antibiotic activity was detectable from the beginning of the experiment and reached a maximum level at 48 h. The maximal antibiotic activity was 232 U ml^{-1} and improved by 28.0%, compared with that in XB1. After reaching maximum antibiotic activity units, the antibiotic activities remained relatively constant throughout the remainder of the experiment (72 h). Such trends have been reported earlier for antibiotic activity by *Xenorhabdus* sp. RIO (Isaacson and Webster 2002). Ji et al. (2004) also report that as the bacterial population increased, the antibacterial activity of the culture broth during the growth of *X. nematophila* K1 in TSB medium increased and reached the maximal level at the stationary growth phase of the bacteria. These results indicate that antibiotic production was influenced by agitation speed and aeration rate, and lower agitation speed and lower aeration rate obviously inhibited antibiotic production by *X. nematophila* YL001. Thus, higher agitation rate (300 rev min^{-1}) and higher aeration rate (2.5 l min^{-1}) were chosen as the optimal condition for the antibiotic production of *X. nematophila* YL001.

The growth rate of cells was higher at a higher K_La (Table 2). Under agitation speed of 300 rev min^{-1}, and the aeration rate of 2.5 l min^{-1} (XB3), the maximum DCW (about 19.58 g l^{-1})was obtained in 57 h where the glucose (0.69 g l^{-1}) in the culture broth was almost exhausted. The maximum DCW attained during XB1, the agitation speed of 200 rev min^{-1}, and the aeration rate of 2.0 l min^{-1}, was about half that found in XB3, but the glucose consumption rate was not obviously different compared with that in XB3 where the glucose concentration was 0.89 g l^{-1}. The specific glucose consumption rate increased with increasing K_La while the antibiotic yield increased when K_La increased (Table 2). Furthermore, the specific growth rate and the cell yield on glucose in XB3 were both higher than those in XB1 and XB2. The cell productivity and antibiotic productivity were up 2.0 and 1.26 times, respectively, when the K_La was increased from 115.9 to 185.7 h^{-1}. So, the rise was not linear. Since oxygen limitation was detected for these fermentation process (Fig. 1), the cell productivity and antibiotic productivity can be improved. This indicates that the agitation and aeration could further be optimized. It can therefore be concluded that high concentration, high yield,

Table 2 Main characteristics in batch fermentation by *X. nematophila* YL001 under various conditions

Parameters	XB1	XB2	XB3
Initial glucose concentration (g l^{-1})	6.0	6.0	6.0
Residual glucose concentration (g l^{-1})	0.65	0.61	0.55
Maximal dry cell weight (g l^{-1})	10.08	12.08	19.58
Maximal antibiotic activity units (U ml^{-1})	181	197	232
Final dry cell weight (g l^{-1})	9.02	11.1	17.45
Final antibiotic activity units (U ml^{-1})	171	152	229
Culture time (h)	72	72	72
Time of maximal dry cell weight (h)	57	60	57
Time of maximal antibiotic activity units (h)	48	54	48
Cell productivity (g l^{-1} h^{-1})	0.13	0.15	0.24
Antibiotic productivity (U ml^{-1} h^{-1})	2.38	2.11	3.18
Maximal specific growth rates (h^{-1})	0.17	0.19	0.23
Maximal specific antibiotic production rates (h^{-1})	1.04	2.07	2.89
Maximal specific glucose consumption rates (h^{-1})	0.1	0.11	0.16
Average specific growth rates(h^{-1})	0.038	0.044	0.044
Average specific antibiotic production rates (h^{-1})	0.396	0.551	0.707
Average specific glucose consumption rates (h^{-1})	0.026	0.026	0.027
Cell yield on glucose (g g^{-1})	1.69	2.06	3.20
Antibiotic yield on glucose (U mg^{-1})	32.0	28.2	42.0

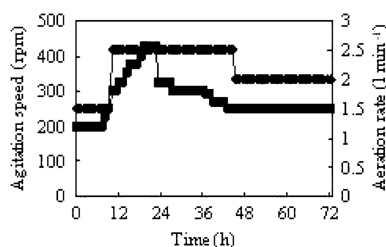

Fig. 2 The agitation speed and aeration rate under control DO concentration

and high productivity of cell and antibiotics could not be achieved under low DO concentration or without DO control within the whole culture process (Fig. 2).

As show in Table 2, not only biomass but also antibiotic activity increased as the value of $K_{L}a$ increased, indicating that higher agitation speed and higher aeration rate is beneficial to both cell growth and antibiotics production. The question therefore arises: which parameter, a high agitation speed or a high aeration rate, is responsible for the high cell productivity and antibiotic productivity? As can be seen in Table 1, the agitation speeds in both XB1 and XB2 were the same, where the aeration rate were 2.0 l min^{-1} and 2.5 l min^{-1}. In addition, in XB2 and XB3 the effect of identical aeration rate but different agitation speeds was compared to determine the effect of agitation speed on the productivity of cell and antibiotics. When the data in XB1 and XB2 are compared, the aeration rate can be evaluated because the agitation speeds were the same. Moreover, when you compare the data in XB2 and XB3, the effect of agitation speed can be evaluated because the aeration rate were the same in both cases (Table 2). It can be observed that both agitation speed and aeration rate have significant positive effects on the cell growth and antibiotics activity. However it seems unlikely that the aeration rate affected the cell growth and antibiotics activity as significantly as the agitation speed did, and demonstrated the profound effect of the agitation speed rather than the aeration rate, which is in good agreement with the results showing little influence of aeration rate on the $K_{L}a$ (Table 1).

Effect of DO control on cellular growth and antibiotic production

In general, during the culture process of an aerobic microorganism, oxygen demand is different at different growth stages. Using a constant agitation speed and a constant aeration rate is not beneficial to the cell growth and metabolism. This also results in prolonged culture time and wasted energy sources. So, the oxygen supply should be controlled according to the characteristics of cell growth and metabolism. Under optimal conditions for cell growth and antibiotic production of *X. nematophila* YL001 (300 rev min^{-1}, 2.5 l min^{-1}, 185.7 h^{-1}), oxygen limitation took place during cultivation. Dissolved oxygen concentration fell to zero during the exponential growth phase (Fig. 1). Oxygen limitation concomitant with an increase in the cell concentration in culture is well known to have a detrimental effect on cell activity and to decrease the productivity of antibiotics (Vardar and Lilly 1982). Enhancement of antibiotic productivity by increasing the DO concentration has been shown in penicillin and cephalosporin C fermentations, among others. During the growth phase, control of DO at high level increased the rate of specific cephamycin C production, 2 to 3-fold, compared to the experiments without DO control (Rollins et al. 1988; Yegneswaran et al. 1991). In order to investigate the effect of dissolved oxygen concentration on the fermentation *X. nematophila* YL001, a three-stage oxygen supply control strategy was designed, and dissolved oxygen concentration was controlled above 30% by manipulating agitation speed and aeration rate (Fig. 3). When dissolved oxygen concentration was controlled above 30%, Both the DCW and antibiotic activity reached the maximal values (24.22 g l^{-1} and 249 U ml^{-1}), and improved by 23.2% and 7.0%, compared with that under without DO

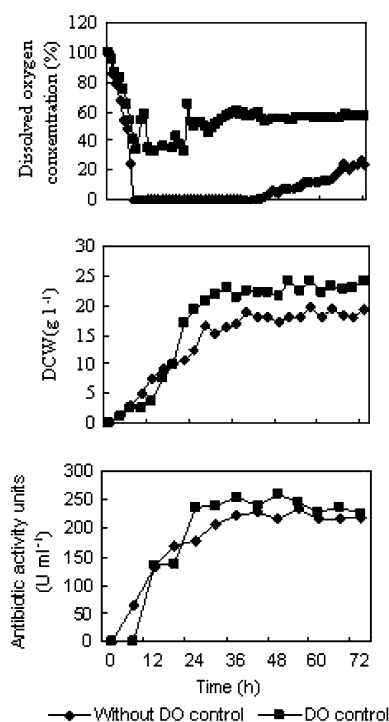

Fig. 3 Effects of dissolved oxygen concentration on growth and antibiotic activity by *X. nematophila* YL001

control which were 19.58 g l^{-1} and 232 U ml^{-1} (Fig. 3). The results indicate that DO control can be used to increase the cell density and thus provide a better nutritional basis for subsequent propagation of the symbiotic nematodes, as the number of offspring per nematode is positively correlated with the provision of the bacteria cells (Johnigk and Ehlers 1999). Therefore, it was essential to keep the DO level high to achieve a high cell growth and antibiotics activity. But in the beginning of DO control, the DCW, antibiotic activity, were lowered (Figure 3). The reason is clear, it is due to the difference of oxygen transfer rate. In the beginning of batch culture, the agitation speed and aeration rate without DO control (300 rev min^{-1}, 2.5 l min^{-1}) are higher than that under DO control (200 rev min^{-1}, 1.5 l min^{-1}), more oxygen was transferred into the fermentation broth, and sufficient oxygen is beneficial to both the cell growth and metabolism. So, the three-stage oxygen supply control strategy should be further optimized. Furthermore, in the experiment of DO control, the antibiotic activity increased only 7.0% compared to that without DO control, and only one dissolved oxygen concentration was designed, the cell productivity and antibiotic productivity can be improved, so the optimum dissolved oxygen concentration for cell growth and antibiotic production by *X. nematophila* YL001 will be further investigated in a future study.

Conclusion

In batch fermentation experiments using *X. nematophila* YL001, we controlled the agitation speed and aeration rate to create different values of K_La in the fermentor. It is interesting to find that agitation speed is a precise means to control the K_La rather than aeration rate. The cell growth and antibiotic production are strongly affected by K_La, activity units of antibiotic and dry cell weight were increased to 232 U ml^{-1} and 19.58 g l^{-1}, productivity in cell and antibiotic was up more than 30% when K_La increased from 115.9 h^{-1} (200 rev min^{-1}, 2.0 l/min) to 185.7 h^{-1} (300 rev min^{-1}, 2.5 l min^{-1}). DO concentration also had strong effects on the cell growth and antibiotic production. Increasing the DO concentration above 30% air saturation by using a three-stage oxygen supply control strategy, The dry cell weight and activity units of antibiotic were further increased to 24.22 g l^{-1} and 249 U ml^{-1}. Therefore, it was essential to keep the DO level high to achieve a high cell growth and antibiotics activity. In addition, the idea of controlling different environmental conditions at different culture stages, according to the characteristics of cell growth and metabolism, could be applied to the fermentation process of other aerobic microorganism.

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