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Enhancement of Hyaluronic Acid Production by Batch Culture of *Streptococcus zooepidemicus via* the addition of *n*-Dodecane as an Oxygen Vector

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This study aimed to examine the influence of adding an oxygen vector, n-dodecane, on hyaluronic acid (HA) production by batch culture of *Streptococcus zooepidemicus*. Owing to the high viscosity of culture broth, microbial HA production during 8–16 h was limited by the oxygen transfer coefficient K_La, which could be enhanced by adding n-dodecane. With the addition of n-dodecane to the culture medium to a final concentration of 5% (v/v), the average value of K_La during 8–16 h was increased to 36±2 h^{-1}, which was 3.6 times that of the control without n-dodecane addition. With the increased K_La and dissolved oxygen (DO) by adding 5% (v/v) of n-dodecane, a 30% increase of HA production was observed compared with the control. Furthermore, the comparison of the oxygen mass transfer in the absence and presence of n-dodecane was conducted with two proposed mathematical models. The use of n-dodecane as an oxygen vector, as described in this paper, provides an efficient alternative for the optimization of other aerobic biopolymer productions, where K_La is usually a limiting factor.

Keywords: Hyaluronic acid, *Streptococcus zooepidemicus*, oxygen vector, n-dodecane, batch culture

Hyaluronic acid (HA) is a polysaccharide that is consisted of alternated β-(1→4)-glucuronic acid (GlcUA) and β-(1→3)-*N*-acetylglucosamine (GlcNAc) moieties [17]. With its unique physicochemical and biological properties, such as high water-holding capacity, viscoelasticity, and biocompatibility [25], HA is widely applied in tissue engineering, drug delivery, ophthalmology, rheumatology, healthcare, and cosmetic fields [8, 16, 19, 23, 24]. The estimated world market value of HA is about USD 500 million, far exceeding that of the other exopolysaccharides [9].

The commercial HA can be extracted from rooster combs or produced by microbial production of group A and C *Streptococci*. Owing to the fact that the use of animal-derived biochemicals for human therapeutics is being met with opposition because of the risk of cross-species viral infection, the microbial production is gradually replacing extraction as the preferred source of HA. However, a major problem concerning the microbial HA production is the high broth viscosity, which results in a low oxygen mass transfer and limited HA yield [9].

The conventional approaches to increase oxygen mass transfer rate usually include increasing agitation or aeration rates, raising the partial pressure of oxygen in the gas phase, or improving the bioreactor designs [14]. However, turbulence and shear stress associated with the intensified mixing are harmful to the fragile cells and thus the final product yield decreases [3, 6].

A novel approach to achieve better oxygen supply is to increase the solubility of oxygen in the culture medium by the addition of oxygen vectors. This approach includes the application of oxygen vectors such as hemoglobin, hydrocarbons, and perfluorocarbons [1, 11, 13, 15, 18, 27, 29]. The oxygen solubility in these compounds is about 15 to 20 times higher than that in water [30]. Rols *et al.* [26] have elucidated the mechanism of enhanced oxygen transfer with oxygen vectors. Oxygen vectors have no toxicity against the cultured microorganisms, and in some cases, they could be used as the supplementary sources of energy and carbon source [10].

Although oxygen mass transfer is a major bottleneck for microbial biopolymer production, surprisingly little work has been done to improve the oxygen transfer rate through the addition of oxygen vectors. The aim of this work was

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to study the application of n-dodecane as an oxygen vector to improve HA production by batch culture of *Streptococcus zooepidemicus*. Here, n-dodecane is selected as the oxygen vector for it is biologically inert, has no toxicity, and is very cheap. To the best of our knowledge, this is the first report in the literature regarding the use of n-dodecane as an oxygen vector to improve HA production.

MATERIALS AND METHODS

Microorganism and Media

S. zooepidemicus WSH-24 was utilized in this study. Fresh slants were cultured at 37°C for 12 h and were used for inoculation.

Seed culture medium consisted of (in g/l) sucrose 20, yeast extract 20, $MgSO_4 \cdot 7H_2O$ 2.0, $MnSO_4 \cdot 4H_2O$ 0.1, KH_2PO_4 2.0, $CaCO_3$ 20, and 1 ml of a trace elements solution. The trace element solution consisted of (g/l) $CaCl_2$ 2.0, $ZnCl_2$ 0.046, and $CuSO_4 \cdot 5H_2O$ 0.019. The initial pH of the seed medium was adjusted to 7.2 by adding 5 mol/l of NaOH solution.

Fermentation medium contained (in g/l) yeast extract 25, sucrose 70, K_2SO_4 1.3, $MgSO_4 \cdot 7H_2O$ 2.0, $Na_2HPO_4 \cdot 12H_2O$ 6.2, $FeSO_4 \cdot 7H_2O$ 0.005, and 2.5 ml of the trace element solution (pH 7.2). Culture medium was sterilized at 121°C for 15 min.

Batch Culture of *S. zooepidemicus* in a 7-l Fermentor

HA production by batch culture of *S. zooepidemicus* was conducted with an initial sucrose concentration of 70 g/l. One loopful of cells from a fresh slant was transferred to 50 ml of seed culture medium and cultured on a rotary shaker at 200 rpm and 37°C for 12 h. The seed culture was inoculated into a 7-l fermentor (Model KL-7L, K3T Ko Bio Tech, Korea) with a working volume of 4.0 l. The pH was automatically controlled at 7.0 by adding 5 mol/l NaOH solution, and temperature was maintained at 37°C. Unless otherwise mentioned, the aeration rate and agitation speed were 0.5 vvm and 200 rpm, respectively.

Analytical Methods

Lactic acid, residual sucrose, and cell concentration were analyzed with the method previously described [20]. HA concentration was measured by the carbazole method based on uronic acid determination [2]. Dissolved oxygen (DO) concentration was measured by the DO electrode ASY3871 online, and the oxygen mass transfer coefficient K_La was determined by the dynamic gas-out/gas-in method [4]. HA molecular weight and polydispersion index were determined by high performance gel filtration chromatography (HPGFC) with a multi-angle laser light scattering detector (MALLS), as previously described [21].

RESULTS AND DISCUSSION

Influence of N-Dodecane Addition on Broth Rheology and Oxygen Mass Transfer During Batch Culture of *S. zooepidemicus*

The oxygen mass transfer coefficient, K_La, is one of the most important parameters in aerobic bioprocesses and depends on many factors, such as the geometrical and operational characteristics of vessels, media composition, and morphology of microorganisms [11]. There are many reports in the literature about the application of oxygen vectors to improve K_La [1, 11, 13, 15, 18], whereas the study concerning the use of n-dodecane as an oxygen vector to improve K_La during microbial HA production of *S. zooepidemicus* has not been found in the literature.

The n-dodecane at different concentrations (0, 1, 3, 5, 7, 9%, v/v) was added to the culture broth at 8 h, at which DO became a limiting factor for microbial HA production by *S. zooepidemicus* [22]. The batch culture without n-dodecane addition was referred to as the control, and the values of K_La and DO were the average values during 8–16 h. Fig. 1 shows K_La and DO as a function of n-dodecane concentration. Within a low n-dodecane concentration range (not more than 5%), K_La almost increased linearly with the increase of n-dodecane concentration, and increased from 10±1 h^{-1} of the control to 37±2 h^{-1} at an n-dodecane concentration

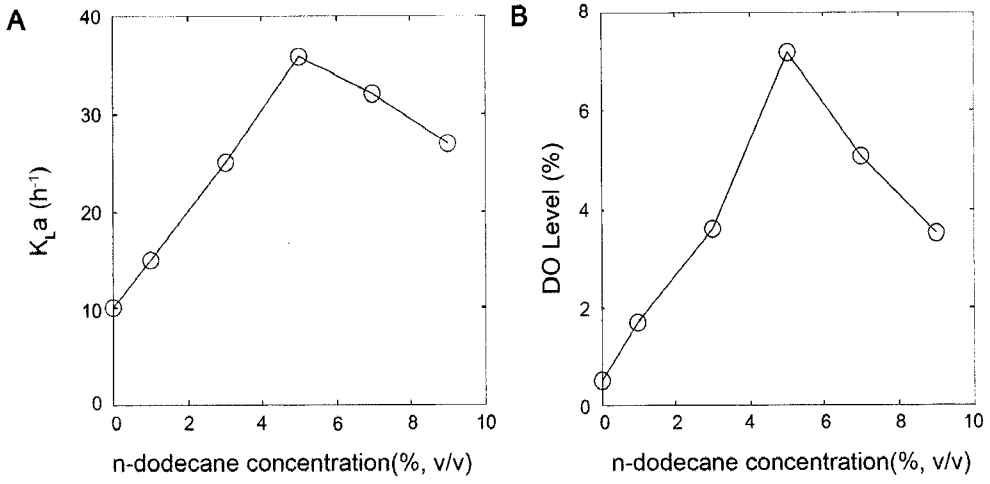

Fig. 1. Influence of n-dodecane addition on oxygen mass transfer during batch culture of *S. zooepidemicus*. **A.** Oxygen transfer coefficient K_La. **B.** Dissolved oxygen (DO).

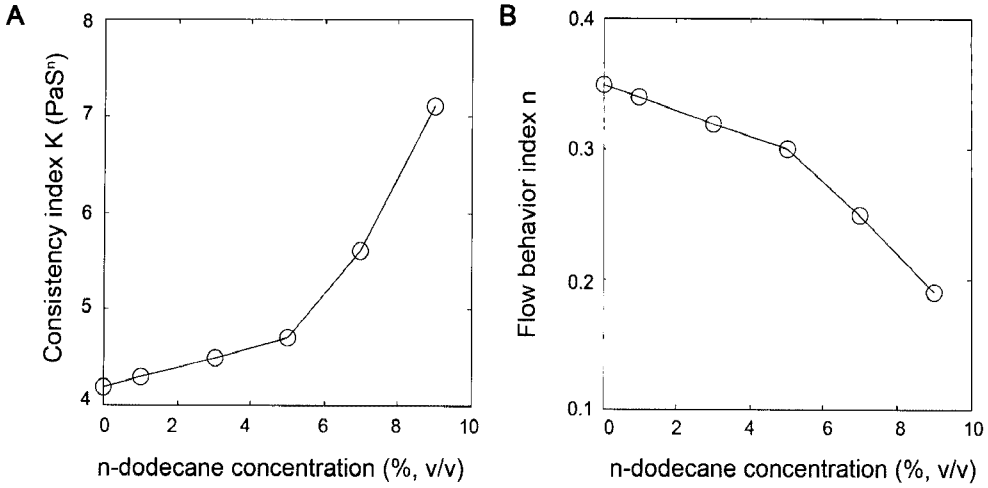

Fig. 2. Influence of n-dodecane addition on broth rheology during batch culture of *S. zooepidemicus*. **A.** Consistency index K. **B.** Flow behavior index n.

of 5%. Corresponding to the kinetics of K_La, the DO level showed the same trends as K_La towards the addition of n-dodecane, and increased from 0.5±0.1% of the control to 7.3±0.5% at an n-dodecane concentration of 5%. However, both K_La and DO decreased with the further increase of n-dodecane concentration to higher than 5%.

The decrease of K_La and DO at higher n-dodecane concentrations was possibly attributed to the increased broth viscosity, which resulted from the addition of n-dodecane. To verify this point, the influence of n-dodecane addition on broth rheology was investigated. Broth rheology is usually described in terms of the Ostwald-de Waele (Power-law) model, as shown in Equ. (1):

$$\tau=K\cdot\gamma^n \quad (1)$$

where τ is the shear stress (Pa), γ is the shear rate (s^{-1}), K is the consistency index ($Pa\cdot s^n$), and n is the flow behavior index. For a Newtonian fluid, n=1, and for a pseudoplastic fluid, n<1. The increase of K means the increase of broth viscosity, whereas the decrease of n indicates the increased pseudoplasticity of the culture broth.

Fig. 2 shows the influence of n-dodecane addition on the rheological parameters K and n. It was indicated that K and n were slightly affected by the addition of n-dodecane with a concentration lower than 5%. However, K increased while n decreased significantly with the increase of n-dodecane concentration higher than 5%, indicating the increased apparent viscosity and pseudoplasticity of the culture broth. The influence of n-dodecane addition on broth rheology was in accordance with the kinetics of K_La and DO in the presence of n-dodecane. The similar influence of oxygen vector on broth rheology was also observed by Rols *et al.* [26]. Since the significant enhancement of K_La and DO was achieved by the addition of n-dodecane, the impact of n-dodecane addition on microbial HA production of *S. zooepidemicus* was studied further.

Influence of N-Dodecane Addition on the Microbial HA Production by Batch Culture of *S. zooepidemicus*

Fig. 3 shows the influences of n-dodecane addition on the cell growth, sucrose consumption, HA production, and lactic acid synthesis. The n-dodecane could stimulate cell growth and sucrose consumption at concentrations lower than 5%, but had little impact on cell growth and sucrose consumption at concentrations higher than 5% compared with the control. The maximum cell concentration increased by 27% at an n-dodecane concentration of 5% compared with the control, and the residual sucrose concentration at 16 h decreased from 8.3±0.1 g/l of the control to 2.3±0.1 g/l at 5% of n-dodecane concentration. Lactic acid production decreased from 60±2 g/l of the control to 42±1 g/l at 5% of n-dodecane concentration owing to the increase of K_La and DO level. HA production increased by 30% at 5% of n-dodecane concentration in comparison with the control owing to the improved K_La and DO level.

Besides HA yield being one of the HA quality indexes, molecular weight and its distribution are also important criteria of HA quality. HA with a high molecular weight has good viscoelasticity and mucoadhesion, allowing it to be applied in the areas of ophthalmology, orthopedics, and wound healing [23], and HA with a relatively low molecular weight is used in cosmetics as a moisturizing factor and in healthcare fields as food ingredients [21]. Therefore, it is important to study the influence of n-dodecane addition on HA molecular weight and its distribution. Fig. 4 shows the influence of n-dodecane addition on HA molecular weight and the polydispersion index of molecular weight. HA molecular weight increased from $(1.3\pm0.1)\times10^6$ Da of the control to $(1.9\pm0.2)\times10^6$ Da at an n-dodecane concentration of 3%, and then decreased to 1.6×10^6 Da at an n-dodecane concentration of 5%. Cleary and Larkin [5] proposed a defensive mechanism where HA capsules protect cells from oxygen in group A, and the protective mechanism is

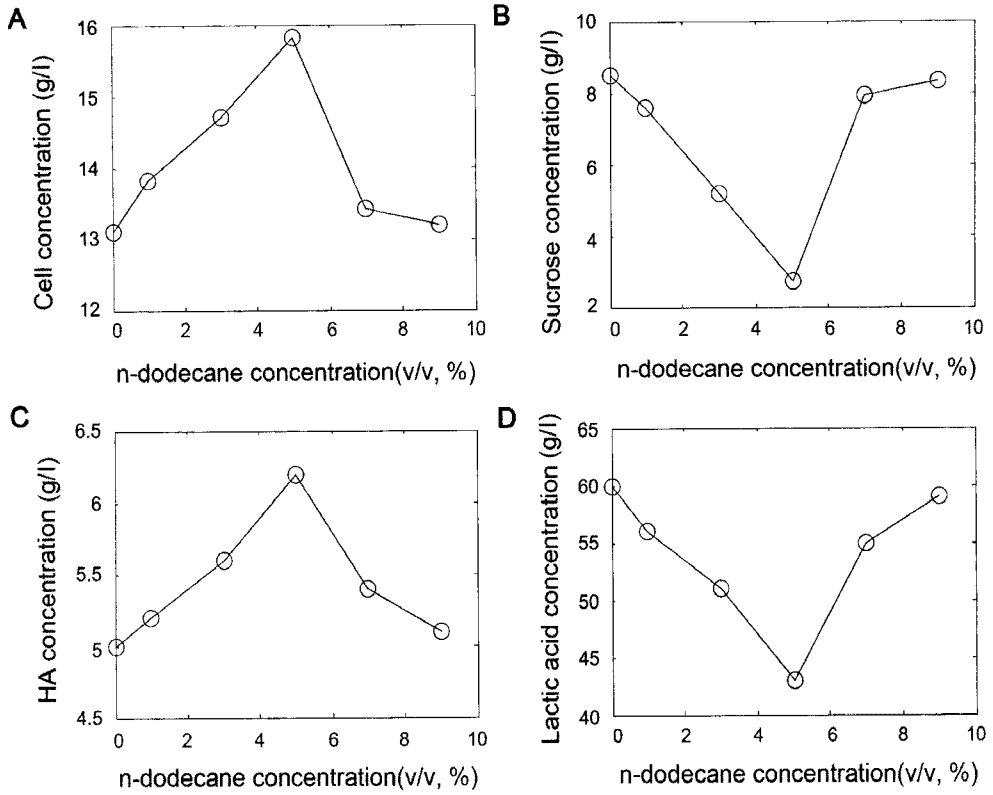

Fig. 3. Influence of n-dodecane addition on the microbial HA production by batch culture of *S. zooepidemicus* **A**. Cell concentration. **B**. Sucrose concentration. **C**. HA production. **D**. Lactic acid.

activated in the presence of oxygen. Moreover, capsule HA with a high molecular weight can effectively protect cells from damage [6]. On the other hand, high molecular weight HA could be degraded into low molecular weight HA in the presence of oxygen-derived radicals [28]. In the present study, HA molecular weight was found to be sensitive to the n-dodecane concentration, possibly due to the balance of HA synthesis and oxygen-mediated HA degradation with various DO levels. As such, HA molecular weight increased with an n-dodecane lower than 3% or higher than 5%, at which a lower DO level was reached; whereas HA molecular weight decreased with an n-dodecane concentration between 3% and 5%, at which a higher DO level was reached. The polydispersion index of molecular weight increased with the increase of n-dodecane, possibly because the partial adsorption of n-dodecane on the cells surface disrupted

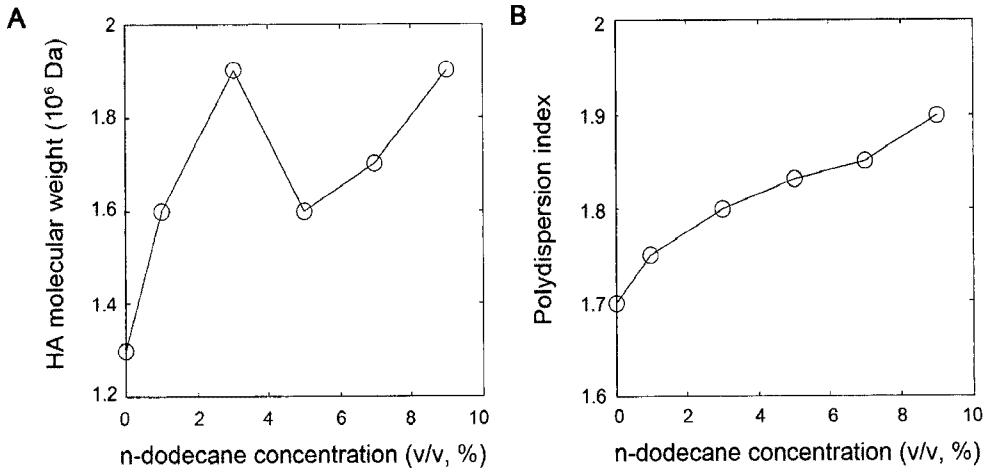

Fig. 4. Influence of n-dodecane addition on the HA molecular weight and the polydispersion index of molecular weight.

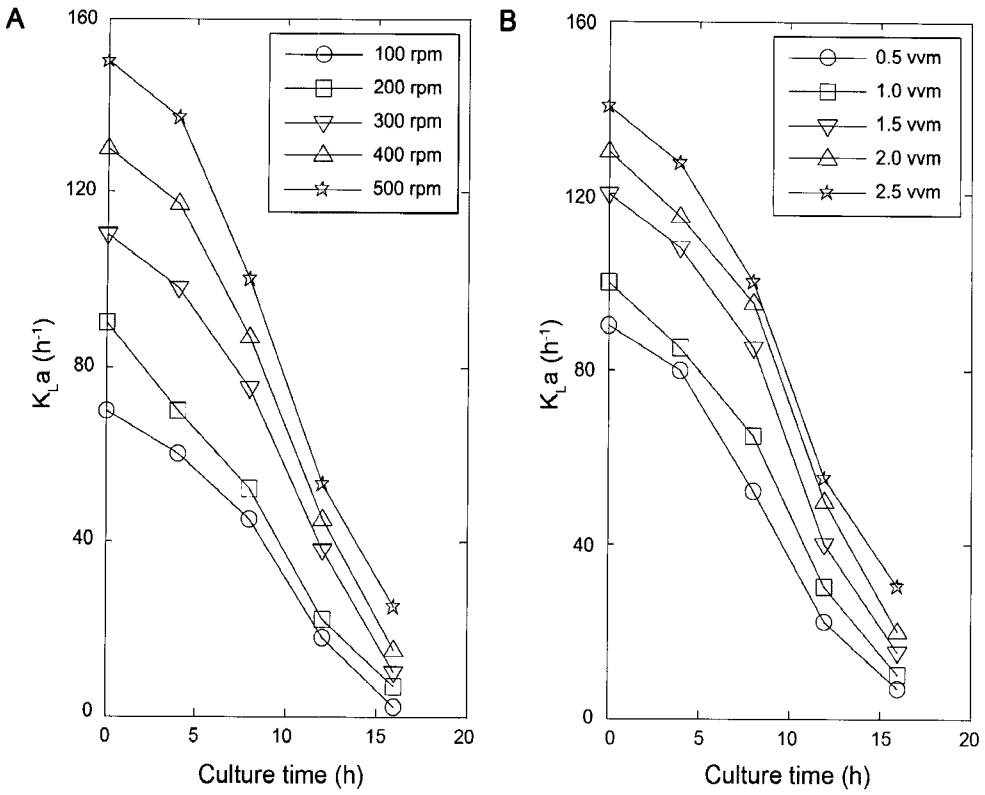

Fig. 5. Influence of agitation speed and aeration rate on K_La in the absence of n-dodecane. **A**. Aeration rate: 0.5 vvm. **B**. Agitation speed: 200 rpm.

HA secretion and widened the distribution of molecular weight.

As one of the most important parameters in aerobic bioprocesses, K_La depends on several factors, such as bioreactor geometry, broth rheology, and operation conditions [12]. In the absence of oxygen vector, there is only a single interface of gas-liquid, and K_La mainly depends on the interface area of gas-liquid. The introduction of an immiscible liquid phase, such as n-dodecane, in the medium, makes the system more complex from an oxygen transfer point of view. There occurred four phases after the addition of oxygen-vectors: the gas phase (air), the aqueous phase (medium), the liquid organic phase (oxygen vector), and the solid phase (biomass). With the formation of new interfacial areas between the gas and liquid phases, five possible routes can achieve the oxygen transfer from air to microorganisms [1]. The oxygen transfer could occur directly to cells, or through oxygen vectors adsorbed or not to the bubble surface. The main resistance to oxygen transfer is due to the diffusion through the aqueous boundary layer from the vector-aqueous phase interface [1]. Therefore, the interface area of the vector-aqueous phase plays a key role for oxygen transfer in the presence of oxygen vector. Considering the significant influence of agitation speed and aeration rate on the interface area of the vector-aqueous phase [18], we further investigated the effects of agitation speed and aeration rate on K_La in the presence of n-dodecane.

Influence of Agitation Speed and Aeration Rate on K_La in the Presence of N-Dodecane

Firstly, the influence of agitation speed and aeration rate on K_La in the absence of n-dodecane was studied. Fig. 5A and Fig. 5B shows that agitation speed and aeration rate had a significant influence on K_La, which increased with the increase of agitation speed or aeration rate. With the same agitation speed and aeration rate, K_La decreased with time owing to the increase of broth viscosity.

As an indicator of oxygen transfer enhancement, an *amplification factor* was proposed to describe the increase of K_La in the presence of oxygen vectors [7]. The amplification factor was defined as the ratio between K_La in the presence of an oxygen vector $(K_La)_v$, and in the absence of an oxygen vector at similar experimental conditions $(K_La)_0$. Here, the amplification factor was introduced to describe the influences of agitation speed and aeration rate on K_La in the presence of n-dodecane. Fig. 6 shows the influences of n-dodecane concentration, agitation speed, and aeration rate on the amplification factor. Fig. 6A shows the changes of amplification factor in the presence of n-dodecane (5%) with 200 rpm of agitation speed and 0.5 vvm of aeration rate. The amplification factor increased from 1.0±0.1 of the control to 4.6±0.2 with the addition of n-dodecane at 8 h, and then decreased owing to the decrease of K_La that resulted from the increased HA production and broth viscosity. Fig. 6B shows the effect of n-dodecane concentration on the amplification factor with

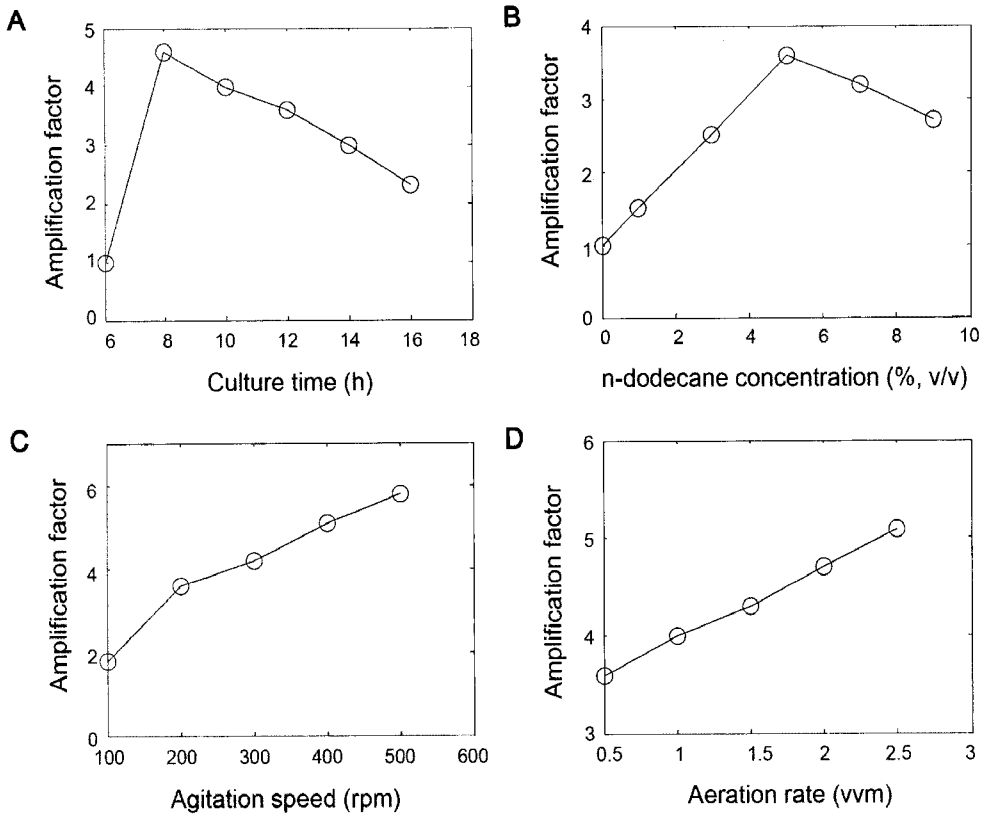

Fig. 6. Influence of n-dodecane concentration, agitation speed, and aeration rate on the amplification factor. **A.** Agitation speed: 200 rpm; aeration rate: 0.5 vvm; n-dodecane concentration: 5%. **B.** Agitation speed: 200 rpm; aeration rate: 0.5 vvm. **C.** Aeration rate: 0.5 vvm; n-dodecane concentration: 5%. **D.** Agitation speed: 200 rpm; n-dodecane concentration: 5%.

200 rpm of agitation speed and 0.5 vvm of aeration rate. It seemed that the amplification factor increased with the increase of n-dodecane concentration and reached a maximum value of 3.6±0.2 with 5% of n-dodecane concentration. The amplification factor decreased with the further increase of n-dodecane concentration higher than 5%, due to the increased broth viscosity, as indicated in Fig. 2A and Fig. 2B. Fig. 6C shows the influence of agitation speed on the amplification factor with 5% of n-dodecane concentration and 0.5 vvm of aeration rate. The amplification factor increased with the increase of agitation speed, and increased from 1.8±0.1 at 100 rpm to 5.7±0.3 at 500 rpm. The significant influence of agitation speed on the amplification factor indicated the marked impact of agitation speed on K_La, in accordance with what shown in Fig. 5A. Fig. 6D shows the influence of aeration rate on the amplification factor at 200 rpm of agitation speed and 5% of n-dodecane concentration. The amplification factor increased with the increase of aeration rate, and increased from 3.6±0.1 at 0.5 vvm to 5.1±0.1 at 2.5 vvm, in accordance with what shown in Fig. 6B. It was obvious that agitation speed had a more significant influence on K_La than aeration rate. Moreover, the higher K_La value at a high aeration rate and agitation speed in the absence of oxygen vector could be reached at a low aeration rate and agitation speed in the presence of oxygen vector (Fig. 5 and Fig. 6). The advantage of using an oxygen vector in microbial cultures is that it increases the oxygen transfer rate from the gas phase to microorganism without the need for extra energy supply [11].

Scale-up of bioprocesses is usually based on correlations of various kinds to predict K_La for specific vessel geometry. K_La has been assumed to be dependent on the agitation speed, the gas superficial velocity, and the apparent viscosity [15].

In the present study, a mathematical correlation that describes the influences of considered parameters on K_La in the absence of n-dodecane was established by means of regression of experimental data. The general expression of the proposed model is

$$K_La=\alpha\cdot\frac{N^{\beta}\cdot V_s^{\gamma}}{\eta^{\delta}},\ s^{-1} \tag{2}$$

where α, β, γ, δ are coefficients of the proposed model, N is the agitation speed (s^{-1}), V_S is the superficial air velocity (m/s), and η is the broth viscosity (mPa·s). The values of α, β, γ, δ were calculated by minimizing Eq. (3) using MATLAB software:

$$E=\left[\sum_{i=1}^{n}\left(\alpha\frac{N_i^{\beta}\cdot V_{si}^{\gamma}}{\eta_i^{\delta}}-K_La_i\right)^2\right]^{\frac{1}{2}} \tag{3}$$

The following correlation was obtained:

$$K_La=1.86\times10^{-2}\cdot\frac{N^{0.45}\cdot V_s^{0.26}}{\eta^{0.05}},\ s^{-1} \quad (4)$$

In the presence of n-dodecane, the following model was proposed to correlate K_La with the parameters including agitation speed, superficial air velocity, apparent broth viscosity, and n-dodecane concentration:

$$K_La=\alpha\cdot\frac{N^{\beta}\cdot V_s^{\gamma}\phi^{\lambda}}{\eta^{\delta}},\ s^{-1} \quad (5)$$

where ϕ is the n-dodecane concentration (%, v/v) and λ is the coefficient of ϕ. By minimizing Eq. (6) with MATLAB software,

$$E=\left[\sum_{i=1}^{n}\left(\alpha\frac{N_i^{\beta}\cdot V_{si}^{\gamma}\,\phi_i^{\lambda}}{\eta_i^{\delta}}-K_La_i\right)^2\right]^{\frac{1}{2}} \quad (6)$$

the following correlation model was obtained:

$$K_La=3.94\times10^{-2}\cdot\frac{N^{0.53}\cdot V_s^{0.38}\phi^{0.16}}{\eta^{0.02}},\ s^{-1} \quad (7)$$

The proposed two correlation models offer a good agreement with the experimental data and have an average deviation of ±9.8%. K_La increased with the increase of n-dodecane concentration ϕ at a low concentration, whereas it decreased with the increase of n-dodecane concentration ϕ at a high concentration, owing to the sharp increase of broth viscosity η. This was in accordance with what was indicated in Fig. 1.

The use of n-dodecane as an oxygen vector, as described in this paper, provides an efficient alternative for the optimization of other aerobic biopolymer productions, where K_La usually is a limiting factor. Furthermore, owing to the fact that n-dodecane is biologically inert, has no toxicity, and is very cheap, it is feasible for the n-dodecane as an oxygen vector to be applied in industrial biopolymers production.

Acknowledgments

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