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# Application of hydrocarbon and perfluorocarbon oxygen vectors to enhance heterologous production of hyaluronic acid in engineered *Bacillus subtilis*

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## Abstract

In microbial cultivations for hyaluronic acid (HA) production, oxygen can be a limiting substrate due to its poor solubility in aqueous medium and the substantial increase in culture viscosity at relatively low HA titers. Shear stress due to the high agitation and aeration rates required to overcome oxygen limitation may reduce the quality (i.e., molecular weight) of HA, and production costs associated with power consumption and supplemental oxygen may be excessive. Here, we report the application of oxygen vectors to the heterologous production of HA in engineered *Bacillus subtilis*, leading to significantly improved culture performance. We first derived an improved HA-producing strain of *B. subtilis* through engineering of the promoter driving coexpression of *seHas* and *tuaD*, leading to high-level HA production. Out of seven potential oxygen vectors evaluated in a preliminary screening, significant improvements to the HA titer and/or cell density were observed in cultures containing *n*-heptane, *n*-hexadecane, perfluoromethyldecalin, and perfluoro-1,3-dimethylcyclohexane. Adjustments to the vector concentration, timing of vector addition, and the agitation rate resulted in further enhancements, with the HA titer reaching up to 4.5 g/L after only 10 hr cultivation. Moreover, our results indicate that certain vectors may alter the functional expression of Class I hyaluronan synthase (HAS) in *B. subtilis*, and that higher shear rates may drive more carbon flux through the HA biosynthetic pathway without negatively affecting the MW. Our study demonstrates the efficacy of oxygen vectors to enhance heterologous HA production in *B. subtilis*, and provides valuable insight for future bioprocess development in microbial HA production.

**KEYWORDS**
*Bacillus subtilis*, chromosomal engineering, hyaluronan, hyaluronic acid, oxygen vectors

## 1 | INTRODUCTION

Hyaluronic acid (HA) is a linear, unbranched polysaccharide consisting of alternating *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcUA) monomers (Chong, Blank, Mclaughlin, & Nielsen, 2005), and is used in the food, cosmetics, biomedical, and pharmaceutical industries (Widner et al., 2005). At present, the US market for HA is estimated at over $1 billion annually, and is expected to rapidly increase with the demand for viscosupplements and dermal fillers (Liu, Liu, Li, Du, & Chen, 2011). Recently, HA production has shifted from direct extraction from animal and avian sources to microbial platforms in an effort to reduce production costs, increase supply, and avoid the risk of cross-species viral infection (O'Regan, Martini, Crescenzi, De Luca, & Lansing, 1994;

  

Pullman-Mooar, Mooar, Sieck, Clayburne, & Schumacher, 2002). Using attenuated strains of group C streptococci (i.e., *Streptococcus equisimilis* and *Streptococcus zooepidemicus*) is currently the dominant method of industrial HA production (Liu et al., 2011). However, HA produced in streptococcal cultivations may contain endogenous exotoxins (Widner et al., 2005), and the fastidious nature of these organisms can increase manufacturing costs (Vázquez, Montemayor, Fraguas, & Murado, 2010). Accordingly, alternate microbial HA production hosts have received increasing attention in recent years (Liu et al., 2011). In particular, *Bacillus subtilis* is a model Gram-positive bacterium amenable to genetic manipulation, grows well in inexpensive medium, has been granted a "Generally Recognized as Safe (GRAS)" designation by the Food and Drug Administration, USA, and, therefore, has been applied to high-level and large-scale production of HA (Liu et al., 2011; Schallmey, Singh, & Ward, 2004).

Industrial bioprocesses based on microbial platforms are typically aerobic and utilize aqueous growth medium containing salts and other nutrients (Garcia-Ochoa & Gomez, 2009). Oxygen is sparingly soluble in aqueous solutions, a limitation that is exacerbated by factors such as salinity, and culture viscosity (Ming & Zhenhao, 2010; Mueller & Boyle, 1967). High-cell-density cultures are highly viscous, and, therefore, the diffusivity of oxygen becomes limited, causing a steep decline in dissolved oxygen (DO) levels (Garcia-Ochoa & Gomez, 2009; Mueller & Boyle, 1967). The increase in culture viscosity is more severe in bioprocesses in which high molecular weight (MW) biopolymers are secreted into the extracellular environment. For example, a substantial increase in viscosity (up to 500-fold) is observed as HA titers exceed 5 g/L in microbial cultivations (Widner et al., 2005). The DO concentration is an intrinsic factor influencing HA synthesis due to a high demand of ATP for HA-producing cells (Huang, Chen, & Chen, 2006; Widner et al., 2005). Oxygen transfer in microbial cultivations can be improved by increasing agitation and aeration rates, and, if necessary, pure oxygen supplementation. However, shearing due to high agitation and aeration rates may reduce the quality (i.e., MW) of HA (Duan, Yang, Zhang, & Tan, 2008; Gao et al., 2003; Lai, Rahim, Ariff, & Mohamad, 2012), and supplementing pure oxygen can be detrimental to microorganisms as exposure to reactive oxygen species such as $H_2O_2$, $O_2-$, and OH• can result in DNA instability, and protein and lipid denaturation (Farr & Kogoma, 1991). Moreover, recombinant protein expression may be hampered by excessive DO levels due to misfolding, loss of activity, and protease degradation (Dean, Fu, Stocker, & Davies, 1997; Slavica, Dib, & Nidetzky, 2005). Consequently, alternate methods to improve oxygen transfer in microbial cultivations for HA production are of significant interest, and using oxygen vectors is one of them.

Oxygen vectors are water-immiscible compounds possessing a greater capacity to dissolve oxygen compared to water. Hydrocarbons (da Silva et al., 2006; Duan et al., 2008; Quijano, Hernandez, Villaverde, Thalasso, & Muñoz, 2010; Rols, Condoret, Fonade, & Goma, 1991), perfluorocarbons (Elibol, 2001; Liu, Du, Chen, Wang, & Sun, 2009; Rols, Condoret, Fonade, & Goma, 1990), vegetable oil (Rols & Goma, 1991), and silicone oil (Quijano & Hernandez, 2010; Quijano & Rocha-Ríos, 2010) can enhance oxygen transfer in microbial cultivations, while insoluble polymers such as silicone rubber (Littlejohns & Daugulis, 2007; Quijano & Hernandez, 2010), nylon (Littlejohns & Daugulis, 2007), glass beads (Littlejohns & Daugulis, 2007), Hytrel (Quijano & Hernandez, 2010), Kraton (Quijano & Hernandez, 2010), Desmopan (Quijano & Hernandez, 2010; Quijano & Rocha-Ríos, 2010), and Elvax (Quijano & Rocha-Ríos, 2010) have also been investigated as oxygen vectors. Oxygen vectors can enhance oxygen transfer by 1) creating large local concentration gradients in the bulk aqueous phase by shuttling oxygen-rich vector droplets from the gas-liquid interface to the bulk liquid; 2) reducing surface tension at the air-water interface, resulting in reduced bubble coalescence and, in turn, smaller bubble diameter and increased interfacial area for oxygen transfer; and 3) colliding with and breaking air bubbles, in turn, reducing bubble diameter (Chaumat, Billet, & Delmas, 2007; Quijano & Rocha-Ríos, 2010). Oxygen vectors are categorized based on their propensity to form discrete droplets (i.e., non-spreading) or films which spread over gas bubbles (i.e., spreading) in an aqueous phase, and the means by which they affect oxygen transfer may be dictated by their tendency to spread or lack there of (Dumont & Delmas, 2003). The selection of an appropriate oxygen vector can potentially reduce operating costs associated with power consumption from high agitation rates, compressed air delivery, and supplemental oxygen, and can reduce oxidative damage to the producing cell and target metabolite or protein, while also minimizing foaming during high-cell-density cultivations (Farr & Kogoma, 1991; Hristov, Mann, Lossev, Vlaev, & Seichter, 2001; Quijano & Hernandez, 2010).

The application of hydrocarbon and perfluorocarbon oxygen vectors to microbial cultivations for HA production has been investigated, albeit to a limited extent. The addition of *n*-dodecane (Liu & Yang, 2009) and *n*-hexadecane (Lai et al., 2012) to *S. zooepidemicus* cultivations resulted in improvements to both the HA titer and MW, while perfluorodecalin also increased the HA titer in *S. zooepidemicus* cultivations relative to a three-stage agitation operation in which the agitation speed was increased in a step-wise manner (Liu & Du, 2009). However, these studies investigated only a few compounds as potential oxygen vectors, and no data is currently available on the application of oxygen vectors to heterologous HA production in common microbial hosts. In this study, we evaluated three hydrocarbons (i.e., *n*-heptane, *n*-hexadecane, and 2,2,4-trimethylpentane) and four perfluorocarbons (i.e., *n*-perfluorooctane, perfluorodecalin, perfluoromethyldecalin, and perfluoro-1,3-dimethylcyclohexane) as potential oxygen vectors to enhance heterologous HA production in an engineered strain of *B. subtilis* with a significantly higher HA producing capacity than our previously reported strain (Westbrook, Ren, Moo-Young, & Chou, 2018). Significant improvements to the HA titer and/or cell density were observed in cultures containing *n*-heptane, *n*-hexadecane, perfluoromethyldecalin, and perfluoro-1,3-dimethylcyclohexane, although the MW was marginally affected. We then manipulated the vector concentration and timing of addition, resulting in further improvements to the culture performance. Assessment of oxygen transfer via measuring the overall oxygen mass transfer coefficient $k_La$ revealed that, regardless of

spreading characteristics, hydrocarbon vectors slightly reduced $k_La$ over the low vector concentrations employed in this study, while perfluoromethyldecalin enhanced oxygen transfer. We also investigated culture performance at higher agitation rates, and observed that the addition of *n*-hexadecane or perfluoromethyldecalin further enhanced the HA titer and cell density, although the MW was somewhat reduced when *n*-hexadecane was present, suggesting that functional expression of hyaluronan synthase (HAS) may be affected by *n*-hexadecane exposure in *B. subtilis*. Moreover, our results indicate that higher shear rates may somehow drive more carbon flux through the HA biosynthetic pathway without negatively affecting the MW.

## 2 | MATERIALS AND METHODS

### 2.1 | Bacterial strains and plasmid construction

*Escherichia coli* HI-Control™ 10G chemically competent cells (Lucigen; Wisconsin) were prepared as electrocompetent cells as described previously (Sambrook & Russell, 2001) and used as host for plasmid construction. Strain 1A751 is a derivative of *B. subtilis* 168 (i.e., *B. subtilis* 168 *his npr*R2 *npr*E18 Δ*aprA*3 Δ*eglS*102 Δ*bglT bglS*RV) that has proven to be an effective host for heterologous HA production (Chien & Lee, 2007; Westbrook, Moo-Young, & Chou, 2016; Westbrook et al., 2018). *B. subtilis* and *E. coli* strains were maintained as glycerol stocks at −80 °C. To construct the genomic integration vector pAW009, promoter $P_{grac.UPmod}$ was amplified from pHT01 (Nguyen, Phan, & Schumann, 2007) with primers Pgrac.UPmod,SalI.f (5′-cagattgagtcgac**ggatcactag**aaaatttttttatcttatcacttgaaattgg-3′) and Pgrac.UPmod.BamHI.r (5′-gcacttgaggatcctttcctcctttaattggg-3′), followed by insertion into SalI/BamHI-digested pAW008 (Westbrook et al., 2016) as shown in Figure S1 (restriction sites are underlined in the primer sequences). $P_{grac.UPmod}$ (i.e., promoter P61) is a weaker derivative of promoter $P_{grac}$ (i.e., promoter P01) that contains a modified upstream promoter element (UP) that reduces the relative promoter strength by approximately half (Phan, Nguyen, & Schumann, 2012) (Note that the modified UP was introduced with primer Pgrac.UPmod,SalI.f and the modified sequence is in bold font.). Transformation of pAW009 into *B. subtilis* results in the integration of a $P_{grac.UPmod}$::*seHas*:*tuaD* expression cassette at the *amyE* locus as shown in Figure S1 (*tuaD* encodes native uridine diphosphate [UDP]-glucose-6 dehydrogenase (TuaD) and *seHas* encodes hyaluronan synthase from *S. zooepidemicus* [SeHAS]). Competent cell preparation and transformation of pAW009 was performed as previously described (Westbrook et al., 2016).

### 2.2 | Cultivation medium and conditions

*B. subtilis* strains were plated on non-select LB agar (NaCl, 5 g/L; yeast extract, 5 g/L; tryptone, 10 g/L; agar, 15 g/L), and grown overnight at 37 °C. Shake flask cultures to compare HA production in AW008 and AW009 were performed as previously described (Westbrook et al., 2018). To prepare the seed cultures for bioreactor cultivations, a single colony was used to inoculate 25 ml prewarmed non-select LB, and grown for ~8 hr at 37 °C and 280 revolutions per minute (rpm) to generate the starter culture. The starter culture was subsequently diluted 100-fold into 100 ml prewarmed non-select LB, and grown for ~13 hr at 37 °C and 280 rpm to generate the seed culture. Cultivations were performed in a New Brunswick™ BioFlo® 115 bioreactor (Hamburg, Germany) under the following conditions: 0.7 L working volume (1.3 L vessel), 2 volume per volume per minute (vvm) (i.e., 1.4 L/min) air, 300 rpm, 37 °C, pH 7 (3 M $NH_4OH$). The non-select cultivation medium was of the following composition (per L): sucrose, 30 g; $(NH_4)_2SO_4$, 1 g; $K_2HPO_4 \cdot 3H_2O$, 9.15 g; $KH_2PO_4$, 3 g; trisodium citrate $\cdot 2H_2O$, 1 g; yeast extract, 10 g; $CaCl_2$, 5.5 mg; $FeCl_2 \cdot 6H_2O$, 13.5 mg; $MnCl_2 \cdot 4H_2O$, 1 mg; $ZnCl_2$, 1.7 mg; $CuCl_2 \cdot 2H_2O$, 0.43 mg; $CoCl_2 \cdot 6H_2O$, 0.6 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.6 mg. The bioreactor was inoculated with 10% volume per volume (v/v) of the seed culture. HA purification and analysis was performed as previously described (Westbrook et al., 2016).

### 2.3 | Determination of $k_La$

Experiments to determine $k_La$ were performed under the same cultivation conditions described above without the inoculum. Measurements of $k_La$ were performed using the dynamic "gas out-gas in" method, and the probe response time was neglected due to the relatively low $k_La$ values obtained under our experimental conditions (Van't Riet, 1979). $k_La$ values were obtained from the mass balance equation:

$$\ln\left(\frac{DO^* - DO(t)}{DO^* - DO(t_o)}\right) = -k_La(t - t_0)$$

where $DO^*$ is the saturation reading of the probe. ANOVA was performed to determine the significance between $k_La$ measurements, and the results of the analysis are presented in Table S1 of the Supplementary Materials.

### 2.4 | Oxygen vectors

n-heptane (>99%), *n*-hexadecane (>99%), *n*-perfluorooctane (98%), perfluorodecalin (95%), and perfluoro-1,3-dimethylcyclohexane (80%) were obtained from MilliporeSigma (MO). Perfluoromethyldecalin (80%), a mixture of perfluoro-1-methyldecalin and perfluoro-2-methyldecalin, was also obtained from MilliporeSigma. *n*-Heptane (>99%) was obtained from J.T. Baker (NJ). The physical properties of oxygen vectors used in this study are summarized in Table 1.

### 2.5 | Side metabolite analysis

The titers of the side metabolites lactate, acetate, and 2,3-butanediol (2,3-BDO) were analyzed via high-performance liquid chromatography (HPLC). The system consists of a Shimadzu (Kyoto, Japan) LC-10AT solvent delivery unit, Shimadzu RID-10A refractive index detector, Shimadzu CTO-20A column oven, and an Aminex HPX-87H chromatographic

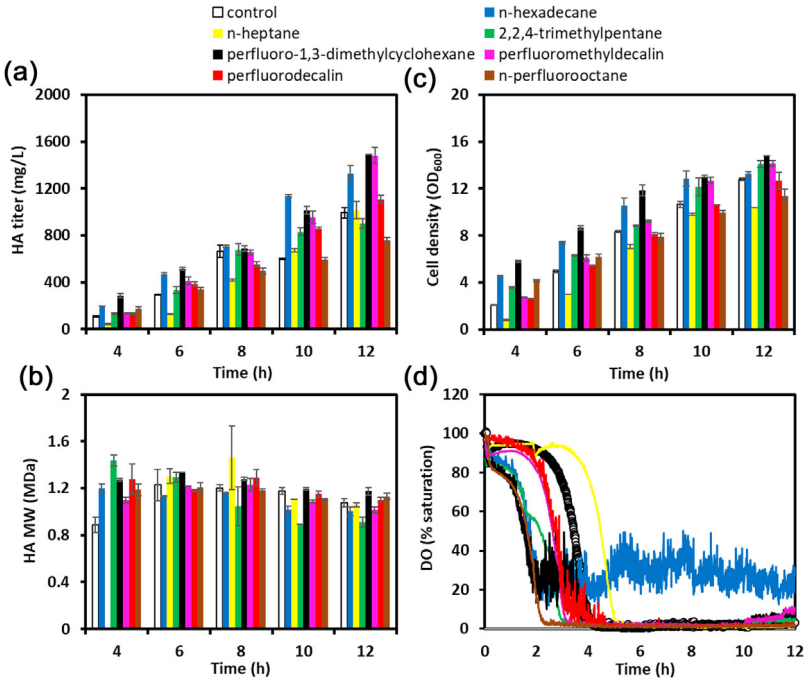


**FIGURE 1** Time profiles of (a) HA titer, (b) HA MW, (c) cell density, and (d) DO in cultures of AW009 with no vector, or in which *n*-hexadecane, *n*-heptane, 2,2,4-trimethylpentane, perfluoro-1,3-dimethylcyclohexane, perfluoromethyldecalin, perfluorodecalin, or *n*-perfluorooctane was added 2 hr after inoculation at a final concentration of 1% v/v. SD of duplicate samples shown in Panels a, b, and c

column (Bio-Rad Laboratories, CA). The column temperature was maintained at 65 °C, and the flow rate of the mobile phase (i.e., 5 mM $H_2SO_4$) was 0.6 ml/min. Signal acquisition and data processing was performed with Clarity Lite (DataApex, Prague, Czech Republic).

## 3 | RESULTS

### 3.1 | Preliminary evaluation of potential oxygen vectors

Three hydrocarbons and four perfluorocarbons were evaluated for their effects on HA production in batch cultivations of strain AW009 in a bench-scale stirred-tank reactor (STR). AW009 is a derivative of a strain previously constructed by us, i.e., AW008 (Westbrook et al., 2018), and strain construction and culture performance are presented in Figures S1 and S2, respectively, of the Supplementary Materials. Potential oxygen vectors were selected based on their volatility (i.e., a minimum normal boiling point of ~100 °C) and relative oxygen solubility (i.e., at least 8-fold higher than water). Compounds such as *n*-heptane, *n*-hexadecane, and perfluorodecalin were preferred as they had been successfully applied to either microbial HA production (Lai et al., 2012; Liu & Du, 2009) or biopolymer production in *B. subtilis* (Zhang, Feng, Li, Chen, & Xu, 2012). In the preliminary evaluation, each compound was added to the cultivation medium at a concentration of 1% v/v 2 hr after inoculation. A relatively low agitation rate at 300 rpm was used to minimize potential HA degradation resulting from polymer shearing (Duan et al., 2008).

AW009 produced 992 mg/L of HA after 12 hr (Figure 1a) with a peak MW of 1.2 MDa at 8 hr, (Figure 1b), and final cell density of $OD_{600}$ 12.8 (Figure 1c). The DO fell below 5% saturation after 4 hr where it remained for the rest of the cultivation (Figure 1d). The addition of *n*-hexadecane resulted in a 34% increase to the final HA titer (1,327 mg/L; Figure 1a), although the MW was not significantly affected (Figure 1b). Note that ANOVA was performed on all measurements of the HA titer, MW, and cell density for all data sets presented herein, and a summary of the results of the analysis is presented in Table S1 of the Supplementary Materials. Moreover, the cell growth rate was improved such that the final cell density ($OD_{600}$ 13.3) was obtained at 10 hr, compared to 12 hr with no vector (Figure 1c). Notably, *n*-hexadecane was the only compound tested that provided a substantial increase in DO levels, which remained above 10% saturation throughout the cultivation (Figure 1d). While the addition of *n*-heptane did not markedly affect the volumetric HA titer, it did improve the specific HA titer by 26%, i.e., 97.6 mg/(L · $OD_{600}$) for *n*-heptane compared to 77.5 mg/(L · $OD_{600}$) with no vector. Moreover, the addition of *n*-heptane prolonged the oxygen limitation by 1 hr compared to the cultivation with no vector (Figure 1d).

Two of the four perfluorocarbons tested, i.e., perfluoro-1,3-dimethylcyclohexane and perfluoromethyldecalin, significantly improved culture performance. The addition of perfluoro-1,3-dimethylcyclohexane and perfluoromethyldecalin resulted in 50% (1,490 mg/L) and 49% (1,481 mg/L) increases to the final HA titer (Figure 1a), respectively, although the MW was not significantly affected (Figure 1b). Moreover, the respective final cell densities increased by 16% ($OD_{600}$ 14.8) and 11% ($OD_{600}$ 14.2) relative to the cultivation

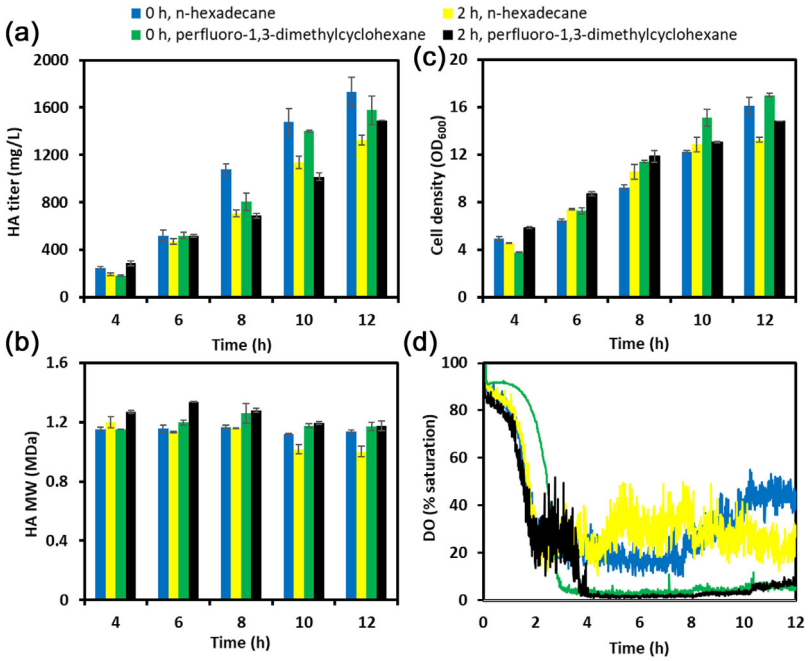


**FIGURE 2** Time profiles of (a) HA titer, (b) HA MW, (c) cell density, and (d) DO in cultures of AW009 in which *n*-hexadecane or perfluoro-1,3-dimethylcyclohexane was added at the time of inoculation (0 hr) or 2 hr after inoculation at a final concentration of 1% v/v. SD of duplicate samples shown in Panels a, b, and c

with no vector (Figure 1c) although DO levels were marginally improved (Figure 1d). Conversely, the addition of *n*-perfluorooctane resulted in a 23% decrease to the HA titer (761 mg/L; Figure 1a), and an 11% decrease in the final cell density ($OD_{600}$ 11.4; Figure 1c). The specific HA titer was highest for the cultivation with perfluoromethyldecalin (104.7 mg/(L · $OD_{600}$)), compared to all other vectors, and was 35% higher relative to the cultivation with no vector.

For initial experiments with *n*-hexadecane and perfluoro-1,3-dimethylcyclohexane, the oxygen vectors were added at the time of seed culture inoculation. However, the addition of 2,2,4-trimethylpentane and perfluoromethyldecalin at the time of inoculation completely inhibited growth, such that in subsequent experiments all vectors were added 2 hr after inoculation when the exponential growth phase was reached. This bioprocessing strategy was developed based on the prior observation that 2,2,4-trimethylpentane is toxic to *E. coli* in the stationary phase, but is not during exponential growth (Kang & Chang, 2012). Adding *n*-hexadecane at the time of inoculation resulted in a 30% increase in the final HA titer (1,731 mg/L; Figure 2a), a 14% increase in the final MW (1.14 MDa; Figure 2b), and a 22% increase in the final cell density ($OD_{600}$ 16.1; Figure 2c), although DO levels were lower between 5 hr and 8 hr (Figure 2d), compared to the addition of *n*-hexadecane at 2 hr. The addition of perfluoro-1,3-dimethylcyclohexane at the time of inoculation did not significantly increase the final HA titer or MW, although the titer after 10 hr was similar to the final titer when the vector

**TABLE 1** Physical properties of oxygen vectors used in this study measured at 25 °C and atmospheric pressure unless otherwise indicated

| Compound | Molecular formula/ structure | Dynamic viscosity (mPa·s) | Density (g/mL) | Boiling point (°C) | Relative oxygen solubility* | References |
|---|---|---|---|---|---|---|
| *n*-hexadecane | $C_{16}H_{34}$ (linear) | 3.0 | 0.77 | 287 | 8.2 | Aralaguppi, Aminabhavi, Balundgi, & Joshi, 1991, Ju and Ho (1989) |
| *n*-heptane | $C_7H_{16}$ (linear) | 0.39 | 0.68 | 98.4 | 10.8 | McKeown and Hibbard (1956), Michailidou, Assael, Huber, Abdulagatov, & Perkins, 2014 |
| 2,2,4-trimethylpentane | $C_8H_{18}$ (branched) | 3.19 | 0.77 | 99 | 14.0 | Hesse, Battino, Scharlin, & Wilhelm, 1999, Pádua et al. (1996) |
| perfluoro-1,3-dimethylcyclohexane | $C_8F_{16}$ (cyclic, branched) | 1.92 | 1.83 | 102 | 16.1[a] | Deschamps, Menz, Padua, & Gomes, 2006, Weber et al. (1998) |
| perfluoromethyldecalin | $C_{11}F_{20}$ (bicyclic, branched) | 6.47 | 1.95 | 137-160 | 12.7 | Jeng, Yang, Wolfson, & Shaffer, 2003, Refojo et al. (1985) |
| perfluorodecalin | $C_{10}F_{18}$ (bicyclic) | 5.41 | 1.91 | 142 | 13.9[b] | Deschamps et al. (2006), Freire et al. (2008) |
| n-perfluorooctane | $C_8F_{18}$ (linear) | 1.26 | 1.77 | 103 | 14.0[c] | Dias et al. (2004); Freire et al. (2008) |

*Expressed as the fold increase in oxygen solubility relative to water. Measured at 16 °C[a], 38 °C[b], and 36 °C[c].

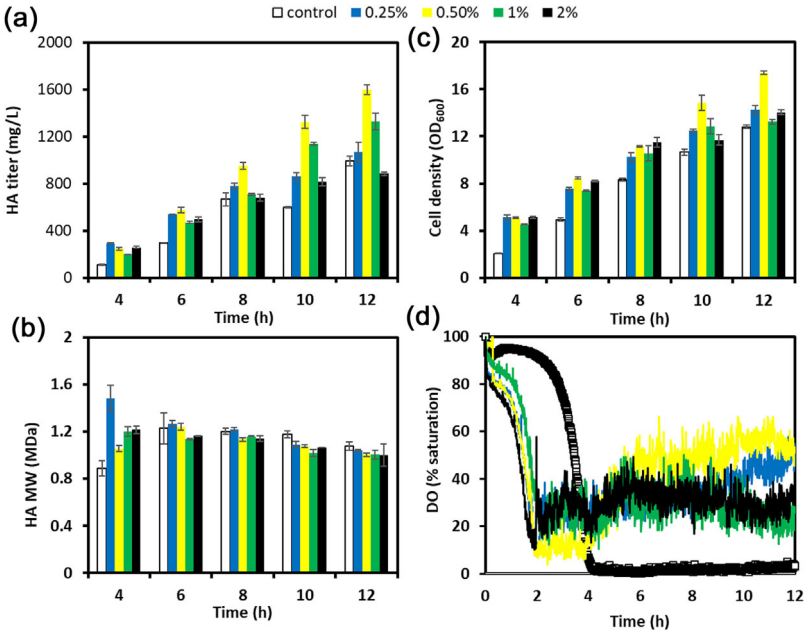


**FIGURE 3** Time profiles of (a) HA titer, (b) HA MW, (c) cell density, and (d) DO in cultures of AW009 with no vector, or in which *n*-hexadecane was added 2 hr after inoculation at a final concentration of 0.25, 0.5, 1, or 2% v/v. SD of duplicate samples shown in Panels a, b, and c

was added at 2 hr (Figure 2a). Moreover, the final cell density increased by 15% ($OD_{600}$ 17; Figure 2c), although the DO profiles were not significantly different (Figure 2d).

## 3.2 | Vector concentration affects HA production

Based on the results from the preliminary evaluation, *n*-hexadecane provided the greatest enhancements to DO levels and HA production. Accordingly, we attempted to further improve culture performance by manipulating the *n*-hexadecane concentration. Reducing the *n*-hexadecane concentration to 0.5% v/v increased the final HA titer by 20% (1,596 mg/L; Figure 3a), without significantly affecting the MW (Figure 3b). The final cell density also increased by 22% ($OD_{600}$ 17.4; Figure 3c), which may be attributed, in part, to the increase in DO levels after 6 hr (Figure 3d). Our results are similar to an earlier report of enhanced HA production in batch cultures of *S. zooepidemicus* upon

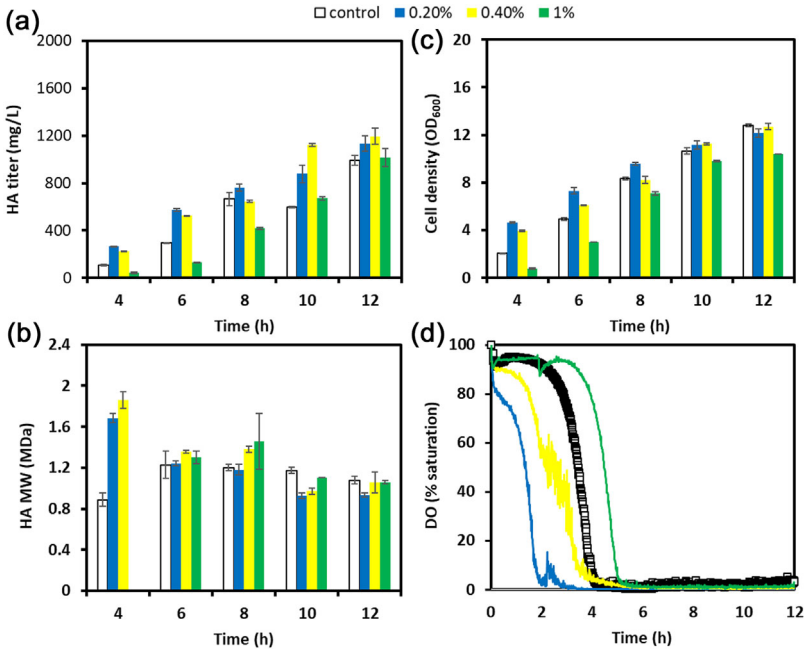


**FIGURE 4** Time profiles of (a) HA titer, (b) HA MW, (c) cell density, and (d) DO in cultures of AW009 with no vector, or in which *n*-heptane was added 2 hr after inoculation at a final concentration of 0.2, 0.4, or 1% v/v. SD of duplicate samples shown in Panels a, b, and c

the addition of *n*-hexadecane to 0.5% v/v, which maximized $k_La$ (Lai et al., 2012). In contrast, adding *n*-hexadecane to 1% v/v was found to be optimal for poly (γ-glutamic acid) (PGA) production in *B. subtilis* (Zhang et al., 2012). The application of *n*-heptane as an oxygen vector to enhance PGA production in cultures of *B. subtilis* resulted in greater improvements to both the titer and MW, compared to cultures supplemented with *n*-hexadecane, and the optimal concentration of *n*-heptane (0.3% v/v) was significantly lower compared to *n*-hexadecane (1% v/v) (Zhang et al., 2012). On the other hand, we obtained significantly higher HA titers with *n*-hexadecane compared to *n*-heptane (Figure 1a), albeit with a slight reduction in MW (Figure 1b). Given that our preliminary evaluation was conducted with a vector concentration of 1% v/v, we considered that reducing the *n*-heptane concentration might improve culture performance. Reducing the *n*-heptane concentration to 0.4% v/v resulted in a substantially higher HA titer after 10 hr cultivation (1,123 mg/L), compared to the cultivations containing either 1% v/v *n*-heptane (672 mg/L), or no vector (599 mg/L; Figure 4a). However, the final HA titer (Figure 4a) and MW profile (Figure 4b) were not significantly improved by reducing the *n*-heptane concentration. The DO levels declined quickly prior to the addition of 0.2% *n*-heptane, compared to the cultivation with no vector, and were only temporarily rescued upon adding *n*-heptane, before dropping to approximately 0% saturation at around 3 hr (Figure 4d). Similarly, the addition of 0.4% v/v *n*-heptane did not delay oxygen limitation, compared to the cultivation with no vector, such that in both cultivations the DO level fell below 5% saturation after approximately 4 hr (Figure 4d). This is in contrast to the cultivation containing 1% v/v *n*-heptane in which oxygen limitation was postponed by approximately 1 hr.

Due to the high specific HA titer observed in the cultivation containing perfluoromethyldecalin, and its lower cost compared to perfluoro-1,3-dimethylcyclohexane, we further evaluated the effect of perfluoromethyldecalin concentration on HA production. Reducing the perfluoromethyldecalin concentration to 0.5% v/v resulted in culture performance similar to the cultivation with no vector (Figure 5). Moreover, further increasing the perfluoromethyldecalin concentration to 2% v/v did not significantly improve the culture performance compared to 1% v/v (Figure 5). In spite of the delay in reaching oxygen limitation by 2 hr upon adding perfluoromethyldecalin to 3% v/v, the final HA titer and cell density were not improved, compared to 2% v/v, and the MW declined potentially due to an increase in viscosity with increasing perfluoromethyldecalin concentration (Tlapak-Simmons, Baron, & Weigel, 2004).

## 3.3 | The effect of oxygen vectors on oxygen mass transfer

In an attempt to understand how oxygen vectors can enhance HA production in *B. subtilis*, we assessed oxygen mass transfer in our cultivation system. The addition of 0.25% v/v *n*-hexadecane resulted in a modest decrease in $k_La$, although increasing the concentration to 2% v/v had essentially no further effect on $k_La$ (Figure 6a). The magnitude and trend of our $k_La$ estimates with increasing *n*-hexadecane concentration are consistent with previous work in which $k_La$ decreased modestly for concentrations up to 2.5% v/v during the operation at 288 rpm and 1 vvm air-purging (Lai et al., 2012). In contrast to our results, an initial increase in $k_La$ was observed at low *n*-hexadecane concentrations (i.e., < 2.5% v/v), followed by a decline in $k_La$ at higher *n*-hexadecane concentrations until phase inversion occurred (i.e., the culture becomes a continuous phase when the vector concentration is high enough) (Ngo & Schumpe, 2012). However, the latter study employed surface aeration in a STR operated at 1,000 rpm such that direct comparison of results is difficult. As was the case for *n*-hexadecane, the addition of *n*-heptane at 0.2% v/v resulted in a modest decrease to $k_La$, and increasing the concentration to 1% v/v did not appreciably affect $k_La$ (Figure 6b). In contrast to our results, the addition of *n*-heptane at 0.1% v/v in a STR operated at 300 rpm and 0.5 vvm resulted in a 15% increase in $k_La$, and increasing the *n*-heptane concentration to 5% v/v increased $k_La$ by 175% (Alves & Pinho, 2013). On the other hand, simultaneously increasing the agitation and aeration rates to 400 rpm and 1 vvm, respectively, resulted in an initial 50% reduction in $k_La$ for *n*-heptane concentrations up to 1% v/v, followed by a gradual increase in $k_La$ with a further increase in the *n*-heptane concentration (Alves & Pinho, 2013). Accordingly, the effect of *n*-heptane on $k_La$ in aerated STRs appears to vary considerably with agitation and aeration rates, such that our observation of a slight reduction in $k_La$ was considered reasonable. Finally, *n*-heptane can dramatically increase $k_La$ in bubble column reactors as both vector concentration and superficial gas velocity are increased (Kundu, Dumont, Duquenne, & Delmas, 2003). In general, non-spreading hydrocarbons (e.g. *n*-hexadecane) negatively or hardly affect $k_La$ (Dumont & Delmas, 2003), although at lower vector concentrations $k_La$ may increase due to the shuttling effect, through which vector droplets briefly contact the air bubble surface and subsequently transfer oxygen to the bulk aqueous phase (Alves & Pinho, 2013; Quijano & Hernandez, 2010). Alternatively, spreading hydrocarbons (e.g., *n*-heptane) generally induce an initial decrease in $k_La$ at low concentrations, followed by a gradual increase in $k_La$ with increasing concentration (Alves & Pinho, 2013; Cents, Brilman, & Versteeg, 2001; Linek & Beneš, 1976; Yoshida, Yamane, & Miyamoto, 1970). It was hypothesized that the initial decrease in $k_La$ resulted from increased liquid phase resistance due to the accumulation of surface active contaminants at the gas-water interface, and that the gas bubble size decreased with an increasing vector concentration as the system approached phase inversion (Linek & Beneš, 1976; Yoshida et al., 1970). An alternate/complementary explanation is that the vector film forming around air bubbles becomes unstable with increasing thickness as the vector concentration increases, resulting in the release of oxygen-rich droplets into the bulk aqueous phase (Alves & Pinho, 2013).

While *n*-hexadecane and *n*-heptane slightly reduced $k_La$, perfluoromethyldecalin had the opposite effect such that $k_La$ increased with increasing concentration up to 2% v/v, resulting in a maximum increase of 35% in $k_La$ compared to the control with no vector (Figure 6c). While $k_La$ measurements for perfluoromethyldecalin are not available in the literature, perfluorodecalin, a structurally similar

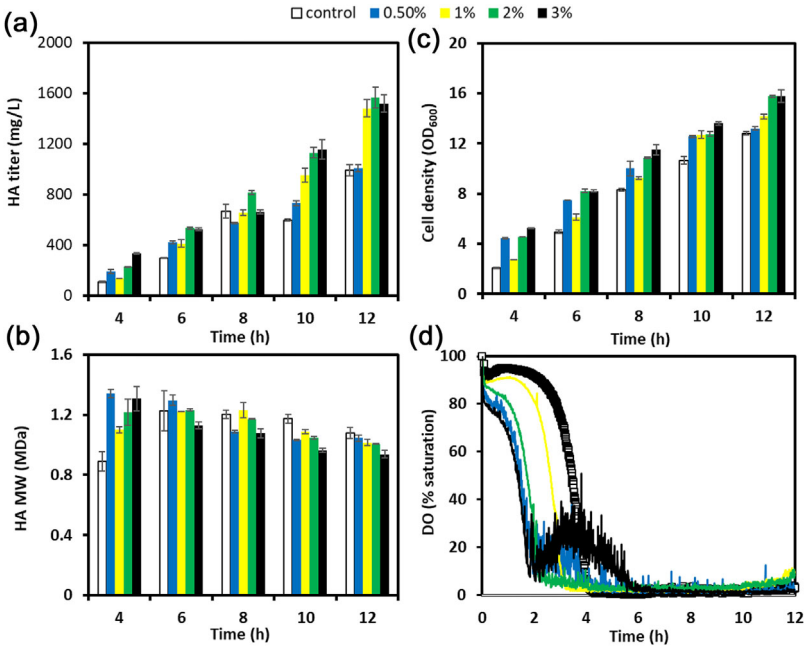


**FIGURE 5** Time profiles of (a) HA titer, (b) HA MW, (c) cell density, and (d) DO in cultures of AW009 with no vector, or in which perfluoromethyldecalin was added 2 hr after inoculation at a final concentration of 0.5, 1, 2%, or 3% v/v. SD of duplicate samples shown in Panels a, b, and c

vector, was observed to increase $k_La$ in a STR at concentrations up to 20% v/v, using the same agitation rate and lower aeration (1.1 vvm) compared to our study, resulting in a maximum increase of ~70% in $k_La$ (Elibol & Mavituna, 1999). Similarly, the addition of perfluorodecalin to 5% v/v provided a slight increase in $k_La$ in a STR operated at 300 rpm and 0.5 vvm air-purging, while increasing the concentration to 10% v/v resulted in a 40% increase in $k_La$ (Elibol, 1999). The addition of forane F66E, an unsaturated perfluorocarbon, to *Aerobacter aerogenes* cultures operated at 400 rpm and 0.21 vvm air-purging, increased $k_La$ for concentrations up to 23% v/v, resulting in a maximum increase

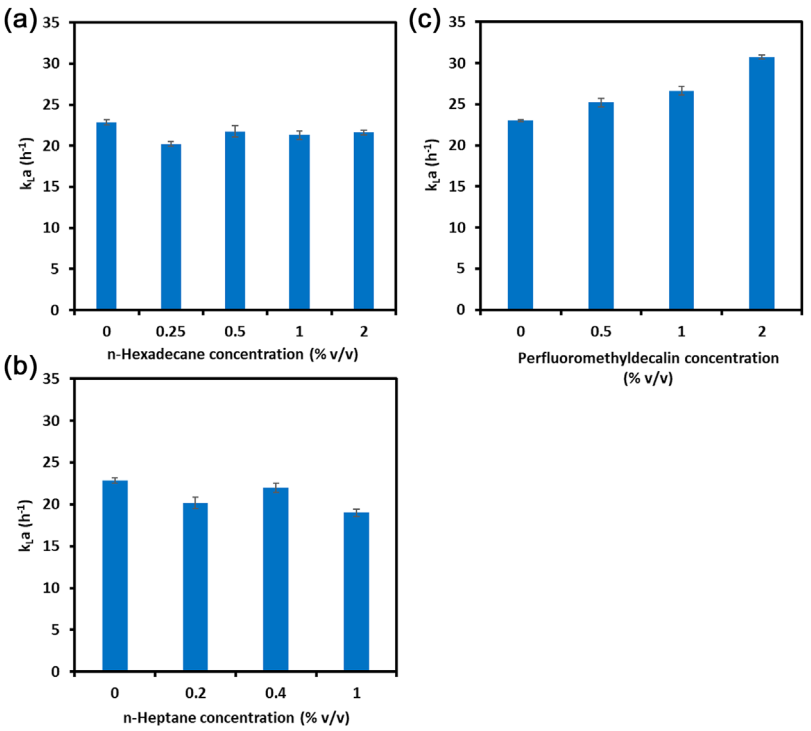


**FIGURE 6** $k_La$ determined for different concentrations of (a) *n*-hexadecane, (b) *n*-heptane, and (c) perfluoromethyldecalin. Experimental conditions described in M & M. SD of three experiments shown

of ~250% in $k_La$ (Rols et al., 1990). Accordingly, a consensus seems to exist regarding the positive influence of perfluorocarbons on oxygen transfer in biological cultivation systems, and this is in line with our observations.

## 4 | DISCUSSION

Simultaneously achieving a high MW and titer in cultivations for HA production is a challenging task. Generally, it requires a high carbon flux through both branches of the HA biosynthetic pathway to ensure a sufficient and balanced monomer supply of UDP-GlcNAc and UDP-GlcUA (Chen, Marcellin, Hung, & Nielsen, 2009). Moreover, an adequate supply of oxygen is essential to generate the substantial amount of ATP consumed during HA biosynthesis, and this is particularly challenging due to the rapid increase in culture viscosity with increasing HA titer (Liu & Du, 2009; Widner et al., 2005). To further complicate matters, the functional expression of HAS to maximize HA production is dependent on its specific orientation with certain lipids in the cell membrane (Weigel, Kyossev, & Torres, 2006; Westbrook et al., 2018). Here we have shown that hydrocarbon and perfluorocarbon oxygen vectors can significantly increase the HA titer in *B. subtilis* cultures. However, certain vectors may reduce the efficacy with which streptococcal Class I HAS can extend the growing HA chain, compromising the MW of the produced HA in *B. subtilis*.

Maintaining the DO above a critical level of ~5% saturation was essential to maximize the HA yield in *S. zooepidemicus* cultivations (Huang et al., 2006), and this is expected given that three mol ATP are required to produce one mol HA disaccharide (Widner et al., 2005). While evidence exists to suggest that *B. subtilis* is a facultative anaerobe which uses nitrate as an electron acceptor (Hoffmann, Troup, Szabo, Hungerer, & Jahn, 1995; Ramos et al., 1995), our attempts to grow 1A751 anaerobically were unsuccessful (unpublished data), indicating that this strain may require a more oxygenic condition to achieve optimal growth. In our study, only supplementation of *n*-hexadecane resulted in DO levels that exceeded 5% saturation throughout the cultivation. Nonetheless, supplementation of *n*-hexadecane, *n*-heptane, perfluoro-1,3,-dimethylcyclohexane, and perfluoromethyldecalin resulted in improvements to the HA titer. To gain further insight into the improved culture performance upon the addition of *n*-hexadecane, we analyzed the side metabolite distribution in cultures with no vector or 0.5% v/v *n*-hexadecane. No ethanol or succinate was detected in cultures with or without *n*-hexadecane, and lactate (Figure 7a) and acetate (Figure 7b) accumulation were not significantly different between cultures as determined by ANOVA (Table 1). Conversely, the 2,3-BDO titer was 2-fold higher in cultures with no vector compared to those with *n*-hexadecane, i.e., 5,385 mg/L with no vector compared to 2,694 mg/L for *n*-hexadecane (Figure 7c). Exogenous supplementation of 2,3-BDO at a concentration of 10 g/L significantly reduced the PGA titer in *B. subtilis* cultures (Zhu et al., 2013), such that 2,3-BDO accumulation in cultures with no vector may have negatively affected HA production in our study. Moreover, reduced carbon flux toward 2,3-BDO production due to improved aerobicity may, in part, explain the improved HA titers in cultures containing *n*-hexadecane compared to those with no vector. Interestingly, the HA titer was lower in cultures containing 1% v/v *n*-hexadecane, compared to 1% v/v perfluoro-1,3,-dimethylcyclohexane or perfluoromethyldecalin, in spite of significantly higher DO levels when *n*-hexadecane was present. Manipulation of the *n*-hexadecane concentration led to further increases to the DO level, particularly between 6 and 12 hr, although the final HA titer was not significantly higher than that obtained in cultivations containing perfluoro-1,3-dimethylcyclohexane or perfluoromethyldecalin. As a result, we cultivated AW009 under a higher agitation rate of 600 rpm with or without *n*-hexadecane to investigate the individual effects of increased DO levels and *n*-hexadecane addition. The HA titer increased by 245% (3,423 mg/L; Figure 8a) after 10 hr and the peak MW at 6 hr increased by 58% (1.94 MDa; Figure 8b) upon increasing the agitation rate from 300 to 600 rpm with no vector. Cultivation with 0.5% v/v *n*-hexadecane at 600 rpm further increased the HA titer by 30% (4,462 mg/L; Figure 8a), although the MW was 12% lower (1.71 MDa; Figure 8b), compared to the cultivation at 600 rpm with no vector. As was the case at 300 rpm, the final cell density was improved upon the addition of *n*-hexadecane, i.e., $OD_{600}$ 27 for 0.5% v/v *n*-hexadecane vs. $OD_{600}$ 22.1 for the control with no vector (Figure 8c), although the DO profiles were not much different (Figure 8d). Moreover, increasing the *n*-hexadecane concentration to 1% v/v during cultivation at 600 rpm reduced the MW by an additional 30%, compared to 0.5% v/v *n*-hexadecane, although DO levels were markedly increased and the final cell density was slightly higher (data not shown).

While our results show that *n*-hexadecane does not impede cell growth in the concentration range up to 2% v/v, the effects of *n*-hexadecane on HA production may be somewhat polar in nature. Streptococcal HAS is an integral membrane protein for autonomous HA synthesis, which is a process requiring as many as seven different functions for initiation, elongation, and secretion of the HA chain (Weigel, 2002). The relatively small size and few transmembrane domains of streptococcal HAS suggest that they cannot facilitate pore formation for translocation of the growing HA chain (Weigel, 2002), and cardiolipin or other lipids may assist HAS in creating an internal pore for HA translocation (Tlapak-Simmons, Kempner, Baggenstoss, & Weigel, 1998; Westbrook et al., 2018). Moreover, interactions between cardiolipin and HA may add to the net retention force that keeps the HA-UDP/HAS complex together during polymerization until a sufficient opposing force exceeds the net retention force, resulting in the release of the growing HA chain (Tlapak-Simmons, Baggenstoss, Clyne, & Weigel, 1999; Weigel & Baggenstoss, 2012). The use of *n*-hexadecane as an oxygen vector resulted in significant increases to the HA titer in this work, and in a prior study in which HA was produced in the native production host *S. zooepidemicus* (Lai et al., 2012). Accordingly, the enhanced HA production upon supplementation of *n*-hexadecane may not be associated with the catalytic activities of HAS, but the increased ATP generation resulting from increased oxygen transfer (Yoshimura, Shibata, Hamano, & Yamanaka, 2015). On the other hand, the lower MW of HA produced in cultures containing *n*-hexadecane suggests premature release of the HA chain. Longer

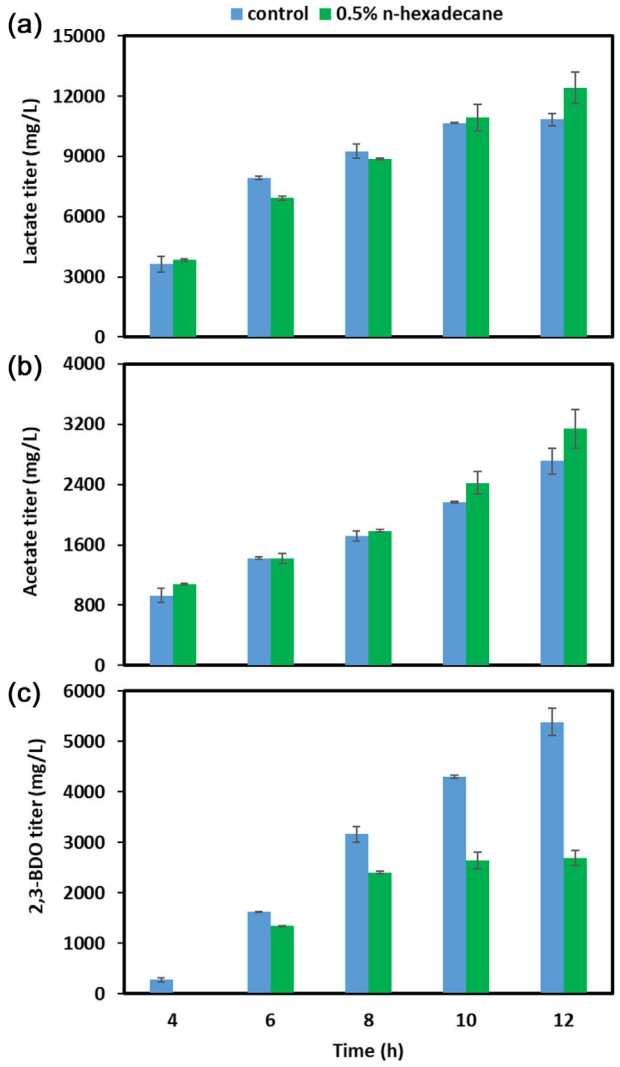


**FIGURE 7** Time profiles of (a) lactate titer, (b) acetate titer, and (c) 2,3-BDO titer in cultures of AW009 with no vector, or in which *n*-hexadecane was added 2 hr after inoculation at a final concentration of 0.5% v/v. SD of duplicate samples shown in Panels a, b, and c

alkanes such as *n*-hexadecane can align parallel to the acyl chains in the lipid bilayer, and, therefore, affect acyl chain packing and tilt, resulting in a reduction in the projected area occupied per lipid molecule and an overall increase in the degree of ordering of the lipid bilayer (McIntosh, Simon, & MacDonald, 1980; Sikkema, De Bont, & Poolman, 1995). In our study, penetration of *n*-hexadecane through the cell membrane may result in the reordering of lipid acyl chains in the lipid bilayer, in turn, altering their interaction with SeHAS. As a result, the HA-binding regions that retain and coordinate translocation of the growing HA chain, and the pore-like structure through which the chain is extruded may be altered, resulting in lower MW HA.

In addition to the reordering of lipids in the bilayer, exposure to organic solvents can alter the fatty acid and lipid composition of the cell membrane. For example, insertion of long chain alcohols (>C5) into a lipid bilayer can increase membrane fluidity (Grisham & Barnett, 1973; Paterson et al., 1972), and an increase in the abundance of saturated fatty acids has been observed in the cell membrane of *E. coli* upon exposure to C5-C8 alcohols (Ingram, 1976). Conversely, exposure to ethanol resulted in an increase in unsaturated fatty acids, presumably to compensate for a reduction in membrane fluidity in *E. coli* (Ingram, 1976). Moreover, an increase in either saturated or unsaturated fatty acids has been reported for *E. coli* exposed to a number of other organic solvents (Ingram, 1977). In some cases, the shift in cellular fatty acid composition coincided with changes in the ratio of the major lipids phosphatidylglycerol and phosphatidylethanolamine, while certain solvents caused a dramatic increase in the cardiolipin content (Ingram, 1977). Considering that the functional expression of HAS is dependent on its orientation with certain lipids in the cell membrane (i.e., a strong preference for cardiolipin has been observed in vitro) (Tlapak-Simmons et al., 1999), it is possible that alterations to the lipid content of the cell membrane of AW009 via exposure to various oxygen vectors could modulate the functional expression of SeHAS. Furthermore, the composition of cardiolipin acyl chains affected SeHAS activity in vitro (Tlapak-Simmons et al., 2004; Weigel et al., 2006), such that a shift in acyl chain composition of lipids in the cell membrane may also affect the functional expression of SeHAS and, in turn, HA production.

The spreading coefficient ($S_{vw}$) is an indicator of the spreading characteristics of a given compound, and spreading behavior is anticipated when $S_{vw}>0$ (Rols et al., 1990). While measurements of $S_{vw}$ tend to vary, there is agreement that $S_{vw}$ decreases with increasing size and branching or methylation of the molecule (Hassan & Robinson, 1977; Mori, Tsui, & Kiyomiya, 1984; Pinho & Alves, 2010). *n*-Heptane is reportedly a spreading vector (Ngo & Schumpe, 2012; Pinho & Alves, 2010), while *n*-hexadecane may be a spreading (Ngo & Schumpe, 2012), or non-spreading (Hassan & Robinson, 1977) vector. Given the significant molecular size difference between the two vectors, *n*-heptane is more prone to spreading than *n*-hexadecane, such that oxygen transfer was more likely to improve upon the addition of *n*-hexadecane at the low concentrations employed in our study. However, our estimates of $k_La$ suggest that the specific rate of oxygen transfer was nearly identical at the respective optimal concentration of each vector, that is 21.8 $h^{-1}$ for 0.5% v/v *n*-hexadecane (Figure 6a) versus 22 $h^{-1}$ for 0.4% v/v *n*-heptane (Figure 6b), both of which are slightly lower than $k_La$ with no vector (22.9 $h^{-1}$; Figure 6). The reduced $k_La$ in the presence of *n*-hexadecane could be due to an increased time needed to reach oxygen saturation in the bulk liquid, and this was observed during the application of silicone rubber, a compound with a high affinity for oxygen, as a solid oxygen vector (Littlejohns & Daugulis, 2007). In any case, the specific rate of oxygen transfer is not the sole determinant of culture performance for HA production, given that the HA titers and cell densities were higher in cultures containing the optimal concentration of *n*-hexadecane, compared to perfluoromethyldecalin, at both 300 and 600 rpm. Furthermore, the relative affinities for oxygen between vectors do not correspond to culture performance, as the respective oxygen solubilities of *n*-heptane, and perfluoromethyldecalin are ~80% and 100% higher than *n*-hexadecane (McKeown & Hibbard, 1956; Refojo, Friend, & Leong, 1985). Consequently, alternate factors specific to cell

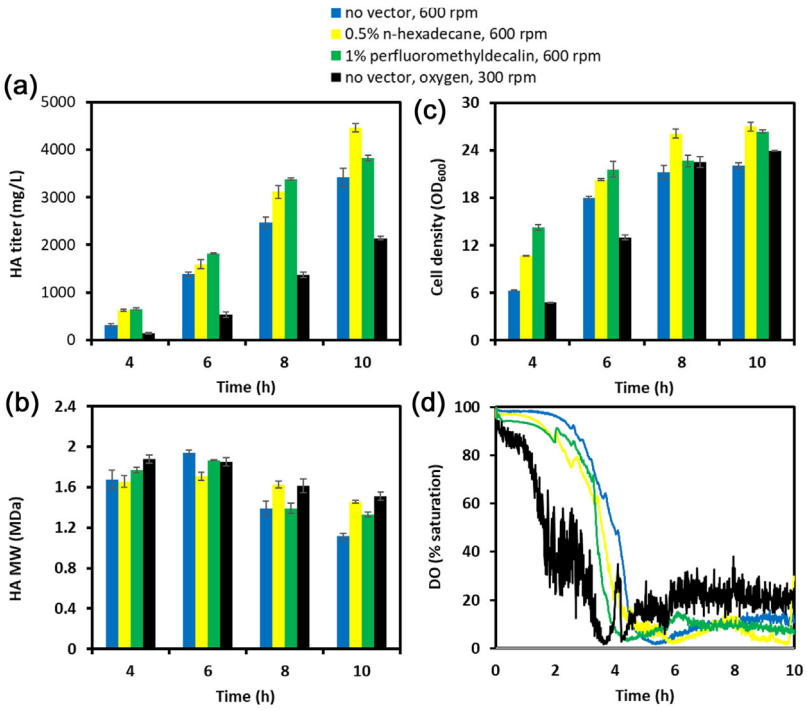


**FIGURE 8** Time profiles of (a) HA titer, (b) HA MW, (c) cell density, and (d) DO in cultures of AW009. Experimental conditions: no vector and 600 rpm; 0.5% v/v *n*-hexadecane and 600 rpm; 1% v/v perfluoromethyldecalin and 600 rpm; or no vector, 300 rpm, and supplemental oxygen to maintain the DO concentration at 20 ± 5% saturation. Oxygen vectors were added 2 hr after inoculation. SD of duplicate samples shown in Panels a, b, and c

physiology may contribute to the net effects of oxygen vectors on culture performance, and this is underlined by the observation that adding *n*-hexadecane to cultures in different growth phases, i.e., stationary at 0 hr and exponential at 2 hr, has a dramatic effect on culture performance (Figure 2). As previously mentioned, longer alkanes such as *n*-hexadecane can align parallel to the acyl chains in the lipid bilayer, affecting acyl chain packing, and tilt for an overall increase in the ordering of the lipid bilayer (McIntosh et al., 1980; Sikkema et al., 1995). On the other hand, the interactions between smaller alkanes (e.g., *n*-heptane) and acyl chains disturb the interactions between acyl chains and, in turn, reduce the ordering of the lipid bilayer (Sikkema et al., 1995). The net effect on the structure of the lipid bilayer of AW009 may partially explain the difference in culture performance, which seems to be largely dependent on cell growth, based on the highly similar specific HA titers of 91.7 mg/(L · $OD_{600}$) and 93.9 mg/(L · $OD_{600}$) obtained in cultivations containing optimal concentrations of *n*-hexadecane and *n*-heptane, respectively. In the case of perfluorocarbons, membrane uptake of them is slow compared to hydrocarbons due to their relatively high lipophobicity (Lehmler, 2008), such that partitioning of perfluoromethyldecalin into the lipid bilayer may be less extensive compared to hydrocarbon vectors. Accordingly, increased oxygen transfer may be the most significant factor enhancing culture performance with the presence of perfluoromethyldecalin and perfluoro-1,3-dimethylcyclohexane.

Evidence suggests that excessive shear stress due to high agitation rates can be detrimental to the MW of HA produced in *S. zooepidemicus* cultures (Duan et al., 2008; Gao et al., 2003; Lai et al., 2012). The MW increased from 1.69 MDa to 2.01 MDa as the agitation rate increased from 150 rpm to 450 rpm, while at 700 rpm the MW declined to 1.84 MDa (Duan et al., 2008). However, the DO was not held constant during respective experiments at different agitation rates, and it was hypothesized that the lower oxygen transfer rate at 150 rpm negatively affected the MW (Duan et al., 2008). Similarly, we observed a 58% increase in the MW of HA produced by AW009 as the agitation rate increased from 300 rpm to 600 rpm. To distinguish between the effects of shear stress and enhanced oxygen transfer, AW009 was cultivated at 300 rpm with supplemental oxygen to maintain the DO concentration at 20 ± 5% saturation (with no vector) to ensure operation above the critical DO level. The peak MW was within 3% (1.88 MDa; Figure 8b) and the final cell density was 9% higher ($OD_{600}$ 24; Figure 8c), although the titer was 37% lower (2141 mg/L; Figure 8a) compared to the cultivation at 600 rpm with no vector. Based on these observations, it appears that a higher shear rate is not detrimental to the MW of HA produced by *B. subtilis* in batch cultivations operated up to 600 rpm and, in fact, may be beneficial in maximizing the HA titer. Maximum growth rates corresponding to optimal agitation rates have been observed in cultures of various microorganisms (Bronnenmeier & Märkl, 1982; Toma et al., 1991), although the maximum growth rate also corresponded to a maximum oxygen uptake rate in *Brevibacterium flavum* (Toma et al., 1991). In our study, oxygen uptake is not limiting in cultivations operated at 300 rpm with supplemental oxygen, suggesting that either a

higher shear rate improved flux through the HA biosynthetic pathway, or excess DO levels were detrimental to the functional expression of SeHAS or carbon flux for HA production. An increase in the specific growth rate, cell density, and expression of certain extracellular enzymes was observed with increasing shear rates in *B. subtilis* cultures (Sahoo et al., 2003), indicating that a higher agitation rate could contribute to the higher growth rate and increased HA synthesis observed during operation at 600 rpm with no vector. On the other hand, excess DO can reduce the yields of certain recombinant proteins via oxidation of Met and Cys residues (Farr & Kogoma, 1991), resulting in misfolding, loss of activity, and protease degradation (Dean et al., 1997; Slavica et al., 2005). Throughout much of the cultivation in our study, pure oxygen was needed to maintain the desired DO level. However, the peak MW of HA did not decrease when operating at 300 rpm with supplemental oxygen. While the functional expression of SeHAS could still be affected by pure oxygen feeding, the control of chain length and polymerizing activity are independent functions of SeHAS that are not coupled to one another (Weigel & Baggenstoss, 2012). Accordingly, further investigation is required to elucidate the effects of shear stress and DO levels in cultivations of HA-producing *B. subtilis*. In addition, our results suggest that operating at agitation rates in excess of 300 rpm can enhance the MW and HA titer, and that oxygen vectors can further enhance culture performance under these conditions.

Few reports exist on the application of oxygen vectors to microbial HA production or biopolymer production in *B. subtilis*. The application of 0.5% v/v *n*-hexadecane to cultivations of *S. zooepidemicus* for HA production resulted in a similar increase to the HA titer as observed in our work (Lai et al., 2012). However, the highest reported MW of 15.4 MDa in the cited study is far in excess of any reported MWs to date, such that comparison of MW data may be not be reasonable. *n*-Dodecane has also been applied to cultivations of *S. zooepidemicus* for enhanced HA production (Liu & Yang, 2009). The addition of 5% v/v *n*-dodecane resulted in a 25% increase in the HA titer, although the maximum increase in MW of 46% was obtained at a concentration of 3% v/v *n*-dodecane, with the peak MW reaching 1.9 MDa (Liu & Yang, 2009). Perfluorodecalin has also been used to enhance HA production in cultures of *S. zooepidemicus*, although the MW was not assessed (Liu & Du, 2009). The addition of perfluorodecalin to 3% v/v resulted in a 32% improvement to the HA titer, compared to a three phase agitation strategy in which the agitation speed was increased from 200 rpm to 600 rpm, and a further increase in the perfluorodecalin concentration reduced DO levels presumably due to an increase in culture viscosity (Liu & Du, 2009). While the application of oxygen vectors to cultivations of *S. zooepidemicus* result in relative increases in the HA titer similar to our cultivations with *B. subtilis*, the improvements to the MW suggest that the functional expression of native HAS may be less sensitive to perturbations that may affect cell membrane ordering and fluidity. *n*-Heptane and *n*-hexadecane have also been applied as oxygen vectors to PGA production in cultures of *B. subtilis* (Zhang et al., 2012), resulting in respective increases in the titer of 25% and 20%, as well as respective increases in the MWs of 25% and 20%. PgsB and PgsC, which are the capsular PGA synthetase and capsular PGA amide ligase/translocase subunit, respectively, polymerize PGA, which is presumably translocated out of the cell by CapA and PgsE (whose respective functions have not been formally elucidated) with assistance from PgsC (Candela & Fouet, 2006). Accordingly, at least four proteins are involved in the synthesis and translocation of PGA in *B. subtilis*, and this is in contrast to HA production for which a single enzyme (i.e., HAS) is responsible (Weigel, 2002). Due to the autonomous functionality of SeHAS, which is dependent on its orientation with lipids in the cell membrane (Tlapak-Simmons et al., 1998; Westbrook et al., 2018), reordering or alteration of lipids in the cell membrane may have a more pronounced impact on the functional expression of SeHAS, but not the elaborate PGA-producing enzyme complex. This potentially results in markedly different effects on the MW of different biopolymers.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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