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Biosynthesis of high molecular weight hyaluronic acid by *Streptococcus zooepidemicus* using oxygen vector and optimum impeller tip speed

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The potential use of *n*-dodecane and *n*-hexadecane as oxygen vectors for enhancing hyaluronic acid (HA) biosynthesis by *Streptococcus zooepidemicus* ATCC 39920 was investigated using a 2-L stirred-tank bioreactor equipped with helical ribbon or Rushton turbine impellers. The volumetric fraction of the oxygen vector influenced the gas–liquid volumetric oxygen transfer coefficient (K_La) positively. Batch HA fermentation with 1% (v/v) *n*-dodecane or 0.5% (v/v) *n*-hexadecane addition was carried out at different impeller tip speeds. Even though cell growth was lower in the fermentation with oxygen vector addition, the HA productivity and molecular weight were higher when compared to the fermentation without oxygen vector at low impeller tip speed. The highest HA concentration (4.25 g HA/l) and molecular weight (1.54×10^7 Da) were obtained when 0.5% (v/v) *n*-hexadecane and 0.785 m/s impeller tip speed of helical ribbon were used.

[**Key words:** Hyaluronic acid; Oxygen vector; *n*-Dodecane; *n*-Hexadecane; K_La; Helical ribbon impeller]

Hyaluronic acid (HA) is a high molecular mass, unsulfated polysaccharide, with an average molecular weight ranging from 10^4 to 10^7 Da depending on its source (1). This polymer composed of D-glucuronic acid and *N*-acetylglucosamine residues linked by β-1-3 and β-1-4 glycosidic bonds. HA belongs to a family of glycosaminoglycan, also known as mucopolysaccharide (2). HA is present in many body tissues and fluids of higher organisms (synovial fluid, vitreous humor of the eye, umbilical cord) and is one of the main components of the extracellular matrix especially in connective tissues (3,4). With its biological functions and unique physicochemical properties, like high water-holding capacity, muco-adhesion, good viscoelasticity and biocompatibility, HA has been widely used in the areas of drug delivery, ophthalmology, orthopedics, rheumatology and tissue engineering (5). The biological function and specific application of HA depend on its molecular weight. High molecular weight HA (around 2 MDa) is responsible for efficient structural space filling, healing damaged cartilage and treating osteoarthritis (6). HA with a relatively low molecular weight is widely used in cosmetics such as moisturizers and as a lubricating agent in eye drops (7).

HA is isolated and purified from various sources but possess chemical identically structure. Many of the high molecular weight HA products used in these applications are extracted from animal sources like rooster comb and bovine vitreous humor. Owing to the fact that the use of animal-derived biochemicals for human therapeutics has the risk of cross-species viral infection and is subjected to stringent regulation, microbial fermentation is gradually replacing extraction as a preferred source of HA with the advantages of low production cost and more efficient purification (5,8). The Lancefield A and C groups of *Streptococci* species, particularly *Streptococcus equi* subsp. *zooepidemicus*, synthesize HA, as a rich capsular material. *S. zooepidemicus* is a catalase-negative, facultative anaerobe, which is also aero-tolerant (9). Under the microscope, these bacteria appear as spherical cells that are typically arranged in pairs or chains surrounded by an extensive extracellular capsule. The capsule may protect the bacteria against reactive oxides released by white cells to fight off the bacteria (10).

To achieve sufficient oxygen supply in large-scale liquid aerobic bacteria fermentation is a major challenge owing to the poor solubility of oxygen in aqueous medium of about 0.25 mM under atmospheric pressure and temperature (11). The broth viscosity increased significantly due to the accumulation of HA during the HA biosynthesis process, and thus higher impeller speed was required for good mixing and oxygen mass transfer in a stirred-tank bioreactor. In order to maintain aerobic conditions during the fermentation, high impeller speed had to be maintained resulting in the increase of power input and high shear stress, which caused mechanical damage to the bacteria resulting decrease in HA molecular weight (7). An alternative to the physical approach of improving the fermentation performance is to generate oxygen chemically by introducing hydrogen peroxide to the medium (12). The use of hydrogen peroxide necessitates the addition of catalase, or the microbes should express and produce catalase themselves (11). However, hydrogen peroxide is toxic to some microbes, causing lower growth rates and biomass yield. It is feasible to

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increase the oxygen transfer rate in microbial fermentation by adding an organic phase with a higher affinity for oxygen known as an oxygen vector (13). The oxygen vector generally used is in liquid form which is insoluble in the fermentation medium. However, the oxygen solubility of *n*-dodecane is 54.90 mg/L at 35°C in contact with air at atmospheric pressure (yielding a distribution coefficient between *n*-dodecane and water at equilibrium of 7.9) (14). Ho et al. (15) found that the solubility of oxygen in *n*-hexadecane is more than 8 times higher than in pure water. The solubility of *n*-hexadecane in water at 298.15 K is reported at 5.57×10^{-9} g/cm^3 (16) and the *n*-dodecane solubility is at 3.70×10^{-9} by weight (17).

The supply of oxygen to the aqueous phase from the gas stream may be supplemented by equilibrium partitioning of dissolved oxygen from the organic phase to the aqueous phase. Their utilization in an emulsified form can significantly increase the oxygen transfer coefficient between the gas and aqueous phases. A mechanism for oxygen transfer within four phases after the addition of the oxygen vector occurred thus: the gas phase (air), the aqueous phase (medium), the liquid organic phase (oxygen vector), and solid phase (biomass). New interfacial areas will exist, and the transfer of oxygen from gas bubble to cells may be achieved by five possible routes (18). The vector acts as an active intermediate in the oxygen transport from gas bubbles to the aqueous phase. In aerobic fermentation, the oxygen supply rate to the aqueous phase is the limiting factor due to its low solubility in water.

Several research groups have applied oxygen vectors to enhance oxygen supply and, as a consequence, to increase the biomass build-up in different culture systems. Perfluorocarbons and hydrocarbons are generally used as oxygen vectors. The oxygen solubility in these compounds is about 15–20 times higher than in water (19). Oxygen vectors have no toxicity to the cultured bacteria, and they could be used as supplementary sources of energy and carbon (20). However, perfluorocarbons have been indicated to be environmentally hazardous chemicals (21). Rols et al. (14) have elucidated the mechanism of enhanced oxygen transfer using oxygen vectors. About 30% (w/v) increase in HA biosynthesis was obtained by adding 5% (v/v) *n*-dodecane (5) and 3% (v/v) perfluorodecalin (22).

Although oxygen supply has been recognized as a major limiting factor for HA biosynthesis by *S. zooepidemicus*, little effort has been made to explore effective chemical and physical approaches for oxygen transfer enhancement in the culture process. Most researchers focused on improving HA production through the conventional approach related to increasing agitation or aeration rates. Hence, the present study aimed to identify the beneficial oxygen vector for *S. zooepidemicus* culture, and to evaluate its use for improving oxygen transfer and high molecular weight HA biosynthesis in batch fermentation. The performances of batch HA fermentations using 2-L stirred-tank bioreactors equipped with different impeller designs (helical ribbon and Rushton turbine) were also compared.

MATERIALS AND METHODS

Microorganism, inoculum and medium *S. zooepidemicus* ATCC 39920 obtained from the American Type Culture Collection (Rockville, MD, USA) was utilized throughout this study. The strain was stored at −30°C in 50% (v/v) glycerol (BDH Laboratory Supplies, UK). Solid medium containing Tryptic soy agar (Merck, Germany) and 5% (v/v) horse blood (Veterinary Clinic, Faculty of Veterinary Medicine, UPM) incubated for 24 h at 37°C was used for the stock culture. For the preparation of the inoculum, the stock culture was subcultured for 10 h in a 500 ml Erlenmeyer flask containing 100 ml Tryptic soy broth (Merck, Germany). Flask cultures were incubated in a rotary shaker (B. Braun Biotech International, Germany) agitated at a rate of 250 rev/min and a temperature of 37°C in order to obtain an optical density of cell within 0.1–0.6 read at 600 nm using a spectrophotometer (model GENESYS 20, Thermo Scientific, USA). The fermentation medium contained (in g/l) glucose 50, tryptone 15, yeast extract 5, KH_2PO_4 2, K_2HPO_4 2, $MgSO_4 \cdot 7H_2O$ 0.5. The culture medium was sterilized at 121°C for 15 min. Fermentations were carried out in duplicates and the mean value of each experiment was obtained.

Batch HA fermentation All experiments were conducted using a 2-L stirred-tank bioreactor (Biostat B., B. Braun Biotech International, Germany) equipped with temperature, pH and dissolved oxygen controllers. A polarographic dissolved oxygen probe (Ingold, Switzerland) was used to measure the dissolved oxygen level, and a steam-sterilized glass pH electrode (Ingold) was used to monitor the culture pH. It was assumed that the response of the oxygen probe to a change in the dissolved oxygen concentration was sufficiently fast. The sterilized bioreactor containing 900 ml of medium was inoculated with 100 ml of the inoculum culture, and the culture pH was not controlled throughout the fermentation. The initial pH of the culture was adjusted to 7 using 3 M NaOH, and the temperature within the bioreactor was maintained at 37°C. All experiments were carried out at 1 vvm aeration. The agitation speed of the batch HA fermentation was controlled according to the requirement of the experiment. Either a two six-bladed disc Rushton turbine or a half-pitched double-blade helical ribbon impeller with a diameter of 52 mm mounted on the agitator shaft was used for the agitation (23). Two liquid hydrocarbons, *n*-dodecane and *n*-hexadecane were used as the oxygen vectors in the experiments. The oxygen vectors were sterilized by filtration through 0.22 μm syringe filters. A volume of 10 ml of *n*-dodecane or 5 ml of *n*-hexadecane was added aseptically to the culture medium prior to the start of the fermentation. For the investigations on the effects of volumetric fractions of oxygen vector on the gas–liquid volumetric oxygen transfer coefficient (K_La), a volume of 1 L of distilled water was used to replace the culture medium and the temperature within the bioreactor was maintained at 37°C.

Analytical methods, K_La measurement The gas-liquid volumetric oxygen transfer coefficient, K_La was measured according to the dynamic gassing-out method (24). This method was performed by sparging nitrogen until the dissolved oxygen concentration fell to zero and then monitoring the dissolved oxygen concentration (C) after the start of the aeration using atmospheric air. The K_La value can be calculated as the slope of the linear Eq. 1:

$$\ln (1 - E) = -K_La \cdot t \tag{1}$$

where,

$$E = \frac{C}{C^*} \tag{2}$$

where, C^* is the equilibrium dissolved oxygen concentration and t is the time.

The K_La was measured at different impeller tips speeds of 0.524, 0.785 and 1.047 m/s. It was assumed that the response of the oxygen electrode to the variations in oxygen concentration is fast enough and does not affect the accuracy of the determination (20).

Broth rheology Broth rheology is usually described in terms of the Ostwald-de Waele (Power-law) model, as shown in Eq. 3:

$$\tau = K \cdot \gamma^n \tag{3}$$

where τ is the shear stress (Pa), γ is the shear rate (s^{-1}), K is the consistency index (Pa·s^n), and n is the flow behavior index and is dependent on the properties of the culture broth. For Newtonian fluid, $n = 1$; and for non-Newtonian fluid, $n < 1$, the increase of K means the increase of broth viscosity, while the decrease of n indicates the increase of broth pseudoplasticity.

Cell, HA and glucose concentrations During fermentation, samples were withdrawn at various time intervals for analysis. Cell growth was observed by measuring the optical density of the culture broth at 600 nm using a spectrophotometer. The correlation between dry cell weight (DCW) and OD was estimated from several batch experiments using the equation,

$$\text{g DCW/1} = 1.489 \times \text{OD} \tag{4}$$

The supernatants were used for HA and glucose determination. After the removal of the cell pellets for cell concentration determination, two volumes of ethanol were added to one volume of the supernatant in a 15 ml centrifuge tube, and the solution was refrigerated at 4°C for 1 h to precipitate the HA. The precipitate was collected by centrifugation at 3000 ×*g* for 20 min and was re-dissolved with a 2–3-fold volume of distilled water. The HA concentration was determined using the carbazole method (25) and the optical density was measured at 530 nm. The HA concentration was calculated using a standard curve prepared at different concentrations of HA standards (Sigma–Aldrich, Malaysia). The supernatant was collected for the analysis of residual glucose. The residual glucose was determined enzymatically by glucose oxidase using SIGMA Diagnostics Glucose (Trinder) reagent (catalog number 315-100); the absorbance was measured at 505 nm.

Molecular weight of HA The HA molecular weight was determined by size-exclusion chromatography on HPLC by means of an Ultrahydrogel Linear column (7.8 mm × 300 mm, Waters Corporation, Milford, MA, USA) equipped with a refractive index detector and a gel permeation chromatography program. Briefly, the solution was filtered with a 0.2-μm pore size syringe membrane filter (National Scientific, USA). For the mobile phase, 0.1 M $NaNO_3$ at a flow rate of 0.6 ml/min was used. The column was calibrated with pullulan (Shodex, Japan) as a reference