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*Full Length Research Paper*

# Medium formulation and impeller design on the biosynthesis of high molecular weight hyaluronic acid by *Streptococcus zooepidemicus* ATCC 39920

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**The effects of medium formulation and impeller design (Rushton turbine and helical ribbon) on hyaluronic acid (HA) biosynthesis by *Streptococcus zooepidemicus* ATCC 39920 were investigated using a 2 L stirred-tank bioreactor. The effect of different glucose concentrations (20, 30, 50 and 60 g/L), nitrogen sources ($(NH_4)_2S_2O_8$, $(NH_4)_2PO_4$, yeast extract, and tryptone) and carbon/nitrogen ratios on the growth of the strain and on HA biosynthesis were initially investigated. Organic nitrogen sources (yeast extract and tryptone) were proven to be favourable in media for HA biosynthesis compared to inorganic nitrogen sources. About 2.442 g/L of HA with a high molecular weight ($4.36 \times 10^6$ Da) was synthesised at an optimal C/N of 2.5:1 (using a mixture of yeast extract and tryptone) in a 2 L stirred-tank bioreactor equipped with a Rushton turbine impeller. When using an optimal medium formulation at equal HA production levels, the helical ribbon impeller resulted in a higher molecular weight of HA ($5.20 \times 10^6$ Da) compared to the Rushton turbine impeller.**

**Key words***: Streptococcus* spp., hyaluronic acid, helical ribbon impeller, carbon/nitrogen.

## INTRODUCTION

Hyaluronic acid (HA) is present in both the dermis and epidermis in humans and about 35% of human HA appears in the muscles/skeleton (Fong et al., 2005). HA is a high molecular mass, unsulphated polysaccharide with an average molecular weight ranging from $10^4$ to $10^7$ Da depending on its source (Armstrong and Johns, 1997). This polymer is composed of D- glucuronic acid and N-acetyl glucosamine residues linked by -1-3 and - 1-4 glycosidic bonds. HA is present in many body tissues and fluids of higher organisms and it is one of the main components of the extracellular matrix (ECM) especially in connective tissues (Pecharki et al., 2008; Asteriou et al., 2001). With its biological functions and unique physicochemical properties such as a high water-holding capacity, mucoadhesion, good viscoelasticity and biocompatibility, HA has been widely used in the areas of drug delivery, ophthalmology, orthopaedics, rheumatology and tissue engineering (Liu et al., 2008). HA with a relatively low molecular weight is also widely used in cosmetic fields as a moisturiser and as a lubricating agent in eye drops (Duan et al., 2008). However, high molecular weight HA is more attractive and appealing to consumers as medical-grade HA posses unique characteristics. HA is isolated and purified from various sources and has a consistent chemical structure. HA is present in rooster combs in high concentrations and is produced by certain bacteria particularly in Lancefield group A and C streptococci and is found in the form of an extracellular capsule (Hynes and Walton, 2000; Liu et al., 2008). The

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use of animal-derived biochemicals for human therapeutics carries the risk of cross-species viral infection; thus, microbial fermentation is gradually replacing extraction as the preferred source of HA which has the advantages of low production costs and more efficient purification (Fong et al., 2005; Liu et al., 2008).

High molecular weight HA is frequently obtained by manipulating microbial culture conditions and using recombinant microorganisms (Im et al., 2009; Zhang et al., 2006). One bacterial producer of HA, *Streptococcus zooepidemicus* grows faster and has a higher biomass yield in the presence of oxygen. The HA yield and average molecular weight in aerobic conditions are higher when compared with anaerobic cultivation (Huang et al., 2006; Duan et al., 2008; Kim et al., 1996). How-ever, HA aerobic fermentation occurs in a high-viscosity culture where HA biosynthesis is limited by low levels of dissolved oxygen due to the high broth viscosity (Hasegawa et al., 1999; Fong et al., 2005). Carbon and energy competition between cell growth and HA synthesis are other limiting factors in HA biosynthesis (Gao et al., 2006) . Much research has been published on the optimisation of culture conditions and various bioreactor strategies for increasing the concentration and molecular weight of HA. Kim et al. (2006) developed a novel multi-stage temperature control strategy to enhance HA biosynthesis. However, this strategy was too complicated to be conveniently applied in industrial HA production. New methods have been extensively studied including the alkaline-stress strategy (Liu et al., 2008), adding lysozyme (Ogrodowski et al., 2005), adding hydrogen peroxide and ascorbate (Liu et al., 2009) and changing the medium composition (Zhang et al., 2006). Recently, studies have focused on producing high molecular weight HA by examining the affect of dissolved oxygen on the yield and molecular weight of HA (Duan et al., 2009). The HA molecular weight increased with impeller speed due to a mass transfer enhancement (Zhang et al., 2010). Group C streptococci exhibited extensive nutrient require-ments with respect to organic nitrogen (Kanchankumar et al., 2009). Organic nitrogen sources are considered essential for good growth and metabolite formation in streptococci because these components also supply a large proportion of carbon for cellular and metabolite biosynthesis (Armstrong et al., 1997).

The carbon source essentially provides the necessary energy for both cell growth and exopolysaccharides (EPS) production. Though much work has been conducted on microbial HA production, there have been few studies on the specific nutritional requirements of *S. zooepidemicus* ATCC 39920 and impeller design, particularly for high molecular weight HA biosynthesis. The present paper highlights the nutritional requirements for growth and high molecular weight HA biosynthesis by *S. zooepidemicus* ATCC 39920, a group C streptococcus with the best impeller design using a 2 L stirred-tank bioreactor.

## MATERIALS AND METHODS

### Microorganism, inoculum and medium

*S. zooepidemicus* ATCC 39920 obtained from the American Type Culture Collection (Rockville, MD) was employed throughout this study. The strain was stored at -20°C in 50% (v/v) glycerol (BDH Laboratory Supplies, England). Solid medium containing Tryptic soy agar (Merck, Germany) and 5% (v/v) horse blood (Clinic of Veterinary, 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). The culture flasks 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 until the optical density reached 0.6 at 600 nm using a spectrophotometer (Genesys 20, Thermo Scientific, USA). To investigate the effect of glucose on HA biosynthesis, experiments were carried out using 10 g/L yeast extract as the sole nitrogen source. The experiment to investigate the feasibility of using different nitrogen sources ($(NH_4)_2S_2O_8$, $(NH_4)_2PO_4$, yeast extract and tryptone) for HA biosynthesis was performed using 10 g/L of each nitrogen source and 50 g/L glucose as the sole carbon source. To determine the effects of carbon/nitrogen on HA yield, *S. zooepidemicus* ATCC 39920 was grown at 37°C for 12 h in fermentation medium supplemented with glucose as a carbon source (50 g/L) and a mixture of yeast extract and tryptone as nitrogen sources at different concentrations which were 15, 20 and 25 g/L. Both nitrogen sources were added equally to the medium.

The same salt compositions ($KH_2PO_4$ 2 g/L, $K_2HPO_4$ 2 g/L, $MgSO_4 \cdot 7\ H_2O$ 0.5 g/L, and $(NH_4)_2PO_4$ 0.5 g/L) were used in all media. The experiments to investigate the influence of the medium formulation in HA biosynthesis were carried out using a 2 L stirred-tank bioreactor equipped with two six-bladed disc Rushton turbine impellers.

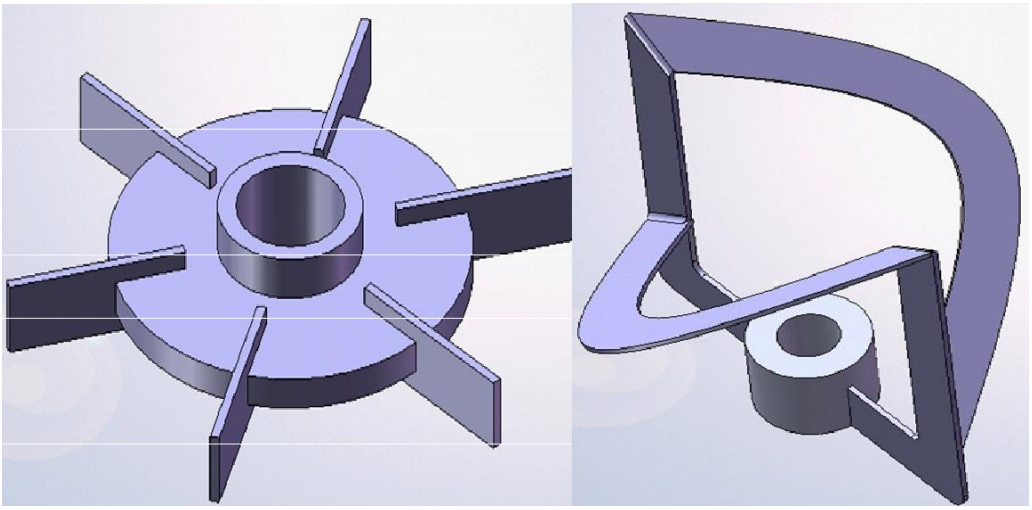
### Batch fermentation

All fermentations were conducted using a 2 L stirred-tank bioreactor (Biostat B., B. Braun Biotech International, Germany) equipped with a temperature, pH and dissolved oxygen controller. A polarographic dissolved oxygen probe (Ingold, Switzerland) was used to measure the dissolved oxygen level and a steam-sterilised glass pH electrode (Ingold) was used to monitor the culture pH. The sterilised bioreactor containing 900 ml of medium was inoculated with 100 ml of the inoculum culture and the culture pH was not controlled throughout 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. Two six-bladed disc Rushton turbine or half-pitched double-blade helical ribbon impellers were used for agitation. The configuration of the bioreactor equipped with different impeller designs is described in Table 1 and Figure 1. The Rushton turbine impeller (Figure 1A) is a type of radial flow impeller which contains six impeller blades that are set at a vertical pitch. The liquid flow from the blades is directed towards the walls of the bioreactor along the radius of the bioreactor. The impeller creates high shear conditions by forming vortices in the wake of the impeller and the high shear is effective in improving the mass transfer rate. The helical ribbon impeller (Figure 1B) is of an axial dispersion type which can be described as a hybrid having a half-pitched helical ribbon jointed to a bottom anchor agitator.

The impeller is effective at lifting solids from the base of the bioreactor and exhibits low shear properties. The spinning disk helps to create a lower pressure region in the bioreactor and offers more enhancement of mass transfer. During fermentation, the agitation speed (N) was fixed at 300 rev/min (impeller tip

**Table 1.** Sizes and positions of impeller and sparger used in 2 L stirred-tank bioreactor.

| Agitator | Rushton turbine | Helical ribbon |
|---|---|---|
| D (mm) | 130 | 130 |
| d (mm) | 5 | 80 |
| H or H' (mm) | 60 | 65 |
| $d_s$ (mm) | 40 | 40 |
| $h_s$ (mm) | 10 | 10 |



(A) (B)

**Figure 1.** (A) Rushton turbine and (B) Helical ribbon.

speed = 0.785 m/s) using the Rushton turbine impeller and was fixed at 190 rev/min (impeller tip speed = 0.785 m/s) using the helical ribbon impeller.

### Analytical methods

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. Correlation between the dry cell weight (DCW) and OD was estimated from several batch experiments using the equation:

DCW (g/L) = 1.489 x OD

The supernatants were used for HA and glucose determination. After the removal of the cell pellet for cell growth 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 HA. The precipitate was collected by centrifugation at 3,000 x g for 20 min and was re-dissolved with a 2 to 3-fold volume of distilled water. The HA concentration was determined using the carbazole method (Bitter and Muir, 1962) 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. Residual glucose was assayed based on reducing sugars by the DNS method (Miller, 1959).

### Molecular weight of HA

The HA molecular weight ($M_w$) was determined by size-exclusion chromatography on HPLC by means of an 'ultrahydrogel linear column' (7.8 x 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 standard of varying molecular weights. The temperature of the column was maintained at 37°C.

## RESULTS AND DISCUSSION

### Influence of medium formulation on HA biosynthesis

#### *Effect of glucose*

A comparison of the performance and the kinetic parameter values using different glucose concentrations on

**Table 2.** Comparison of kinetic parameter values of HA biosynthesis by *S. zooepidemicus* ATCC 39920 using different concentration of glucose in a 2 L stirred-tank bioreactor.

| Kinetic parameter values | Glucose (g/l) | | | |
|---|---|---|---|---|
| t (h) | 20<br>12 | 30<br>12 | 50<br>10 | 60<br>10 |
| $X_m$ (g cell/L) | 3.650 | 6.349 | 10.671 | 10.126 |
| $P_m$ (g HA/L) | 0.227 | 0.519 | 0.934 | 0.758 |
| $\mu_m$ ($h^{-1}$) | 0.332 | 0.513 | 0.538 | 0.551 |
| $Y_{x/s}$ (g cell/g substrate) | 0.352 | 0.256 | 0.271 | 0.266 |
| $Y_{p/s}$ (g HA /g substrate) | 0.022 | 0.021 | 0.024 | 0.022 |
| $Y_{p/x}$ (g HA/g cell) | 0.0623 | 0.0817 | 0.0876 | 0.0745 |
| $P_r$ (g HA/(L.$h^{-1}$)) | 0.038 | 0.065 | 0.093 | 0.063 |

on cell growth and HA biosynthesis is shown in Table 2. The maximum cell concentration of *S. zooepidemicus* ATCC 39920 increased with an increasing glucose concentration. The highest cell concentration (10.671 g/L) was obtained with 50 g/L glucose and used a fixed 10 g/L yeast extract as the sole nitrogen source. A high glucose consumption rate was observed during the rapid growth phase of the fermentation using 50 g/L glucose with high HA biosynthesis. Although the specific growth rate ($\mu_m$) values in the fermentation using 30 and 50 g/L glucose were similar, the productivity was higher in the 50 g/L glucose fermentation. The low level of HA biosynthesis in the fermentations using glucose concentrations lower than 50 g/L (20 and 30 g/L) may be due to an insufficient amount of carbon for both cell growth and HA biosynthesis. For glucose concentrations higher than 50 g/L, the growth of cells in 60 g/L glucose was slightly lower due to the exhaustion of the limited nitrogen source. Hence, it can be suggested that a balanced carbon and nitrogen sources is required for the growth of the strain and high HA biosynthesis in batch fermentation. The highest HA biosynthesis (0.934 g/L) was obtained in the fermentation using 50 g/L glucose. HA productivity is used as an indicator for assessing the efficiency of HA biosynthesis, and the fermentation of *S. zooepidemicus* ATCC 39920 with 50 g/L glucose gave the maximum productivity ($P_r$) and highest product yield coefficient ( $Y_{p/S}$) values. The addition of glucose at more than 50 g/L showed an inhibition of growth and HA biosynthesis. The excess glucose was converted to other organic acids such as lactic acid and acetic acid which may have inhibited the biosynthesis of HA (Chen et al., 2009). This is similar to the work reported by Mashitah and Noor (2010) in which HA biosynthesis was inhibited as the glucose concentration increased from 50 to 60 g/l. Sugars were used primarily as carbon sources, because the precursors UDP-glucuronic acid and UDP- N-acetyl glucosamine are side products of the glycolytic pathway beginning from glucose-6-phosphate to fructose-6-phosphate. Glucose and sucrose are normally used as the carbon source for HA biosynthesis by *Streptococcus* spp (Cooney et al., 1999; Huang et al., 2006; Johns et al., 1994; Liu et al., 2008; Rangaswamy and Jain, 2008). *Streptococcus* spp. appeared to favour simple sugars for their growth and HA biosynthesis. *S. zooepidemicus* ATCC 39920 cultures grew at high carbon concentrations suggesting that carbon concentration played an important development role.

### *Effect of nitrogen source*

From our observation, *S. zooepidemicus* ATCC 39920 appeared to favour an organic nitrogen source rather than an inorganic nitrogen source in the medium. Very poor growth of the strain and no HA biosynthesis was obtained in a fermentation using $(NH_4)_2S_2O_8$ as a nitrogen source. As shown in Table 3, fermentation using $(NH_4)_2PO_4$ also showed very low HA biosynthesis (0.360 g/L). The maximum cell concentration and HA biosynthesis achieved in the fermentation using organic nitrogen sources were higher than those using inorganic nitrogen sources which was previously reported by Huang et al. (2006). Among all nitrogen sources investigated, the highest cell growth (15.994 g/l) and HA biosynthesis (2.442 g/l) were achieved in the medium containing a mixture of yeast extract and tryptone. This combination of organic nitrogen sources (tryptone and yeast extract) showed highest productivity (0.305 g/l/h) than the use of a single organic nitrogen source. The productivity was higher than the results reported by Huang et al. (2008) who achieved only 0.24 g/L/h in batch fermentation. This result is in agreement with Vazquez et al. (2009) who stated that HA productivity with tryptone were the same as outcomes obtained with peptones from marine wastes. HA biosynthesis was slightly lower with the combination of organic and inorga-nic nitrogen sources than with the combination of

**Table 3.** Comparison of kinetic parameter values of HA biosynthesis by *S. zooepidemicus* ATCC 39920 in batch fermentation using different nitrogen sources in a 2 L stirred-tank bioreactor.

| Kinetic parameter values | Nitrogen sources | | | | |
|---|---|---|---|---|---|
| | Yeast extract | Tryptone | $(NH_4)_2PO_4$ | Yeast extract and Tryptone | Yeast extract and Tryptone plus $(NH_4)_2PO_4$ |
| t (h) | 12 | 10 | 12 | 10 | 10 |
| $X_m$ (g cell/L) | 13.391 | 14.861 | 10.671 | 15.994 | 21.569 |
| $P_m$ (g HA/L) | 0.934 | 2.067 | 0.360 | 2.442 | 2.297 |
| $\mu_m$ ($h^{-1}$) | 0.538 | 0.617 | 0.374 | 0.576 | 0.72 |
| $Y_{x/s}$ (g cell/g substrate) | 0.340 | 0.278 | 0.194 | 0.295 | 0.413 |
| $Y_{p/s}$ (g HA/g substrate) | 0.024 | 0.039 | 0.007 | 0.045 | 0.050 |
| $Y_{p/x}$ (g HA/g cell) | 0.070 | 0.139 | 0.034 | 0.153 | 0.120 |
| $P_r$ (g HA/(L.$h^{-1}$)) | 0.093 | 0.172 | 0.030 | 0.305 | 0.216 |

organic nitrogen sources; the productivity decreased from 0.305 to 0.216 g/L/h. These inorganic nitrogen sources probably contain only the nutrients that satisfy no more than the minimal requirements for growth. It was suggested that certain essential amino acids could not be synthesised from inorganic nitrogen sources. Several enzymes such as glucose-6-phosphate dehydrogenase and hexokinase are involved in HA biosynthesis.

The use of an $NH_4^+$ ion as an inorganic nitrogen source may repress enzymes associated with HA biosynthesis. This result indicated that yeast extract and tryptone were the best nitrogen sources to increase the growth rate of *S. zooepidemicus* and the biosynthesis rate of HA. The specific HA biosynthesis rate is expected to increase with a higher specific growth rate. This statement is supported by Armstrong et al. (1997) who claimed that *Streptococcus* group C needed eleven different amino acids to grow well. Several authors suggested that yeast extract is a good substrate for many microorganisms (Smith et al., 1975; Jackson et al., 1998) because yeast extract is a common source of amino acids and B-complex vitamins in media that stimulate bacterial growth. On the other hand, tryptone seems to balance catabolic and anabolic rates and increase the specific growth rate of streptococci.

### *Effect of the C/N ratio*

In this experiment, glucose was used as the sole carbon source due to its ability to produce a high amount of HA. Using a fixed glucose concentration (50 g/L), supplemented yeast extract and tryptone were fixed at 15, 20 and 25 g/L to obtain a C/N ratio of 2:1, 2.5:1 and 3:1 respectively. Figure 2A shows the sigmoidal growth trend of *S. zooepidemicus* ATCC 39920 in which the strain grew very slowly in the first 2 h during the initial lag phase. This is in agreement with the results of Huang et al. (2007) who reported that the growth and formation of HA in the culture broth were found to be very low during the lag phase due to the adaptation of bacterial cells to the new environmental conditions. As illustrated in Figure 2A, the lowest cell concentration (7.496 g/L) was obtained with C/N 3:1; it was slightly higher at a ratio of 2:1 (11.689 g/L) and the highest cell concentration (15.994 g/L) was observed at a C/N of 2.5:1. The highest amount of HA biosynthesis was achieved at a ratio of 2.5:1 (2.442 g/L) which is about 50% higher than a C/N of 2:1 and 3:1. In general, the observed HA yields were the highest at the end of the rapid growth phase (Figure 2B). At a low concentration of glucose, the carbon source was preferentially fully utilised for cell growth, and thus, a negative relationship was observed between the cell concentrations and HA biosynthesis. At a C/N ratio of 3:1, excess glucose was converted to organic acids rather than used for cell growth and HA biosynthesis; this is demonstrated by the yield coefficient value of HA produced based on the glucose consumed ($Y_{p/S}$) which decreased gradually with increasing glucose concentrations. The carbon to nitrogen ratio is an important parameter that affects the cell growth and HA biosynthesis of *Streptococcus* spp. in submerged fermentation. An optimal balance between the carbon and nitrogen sources is absolutely necessary to achieve a high HA yield. This may be explained by the fact that *S. zooepidemicus* strains are dependent on the nitrogen source for cell synthesis, whereas the carbon source is mainly utilised for energy generation.

The maximum specific cell growth rate ($\mu_m$) decreased from 0.576 to 0.363 $h^{-1}$ when the carbon to nitrogen ratio was higher than 2.5:1. Higher HA biosynthesis was achieved in a $\mu_m$ range between 0.53 and 0.62 $h^{-1}$. The HA biosynthesis became lower when $\mu_m$ was lower or higher than this range. This result indicated that an excessive $\mu_m$ caused the strain to produce more by-products, especially lactic acid; an optimum specific growth rate is required for the high biosynthesis of HA. The microbial specific growth rate can be manipulated by fed-batch or continuous operation. The optimum C/N ratio for HA biosynthesis was further confirmed using a fixed

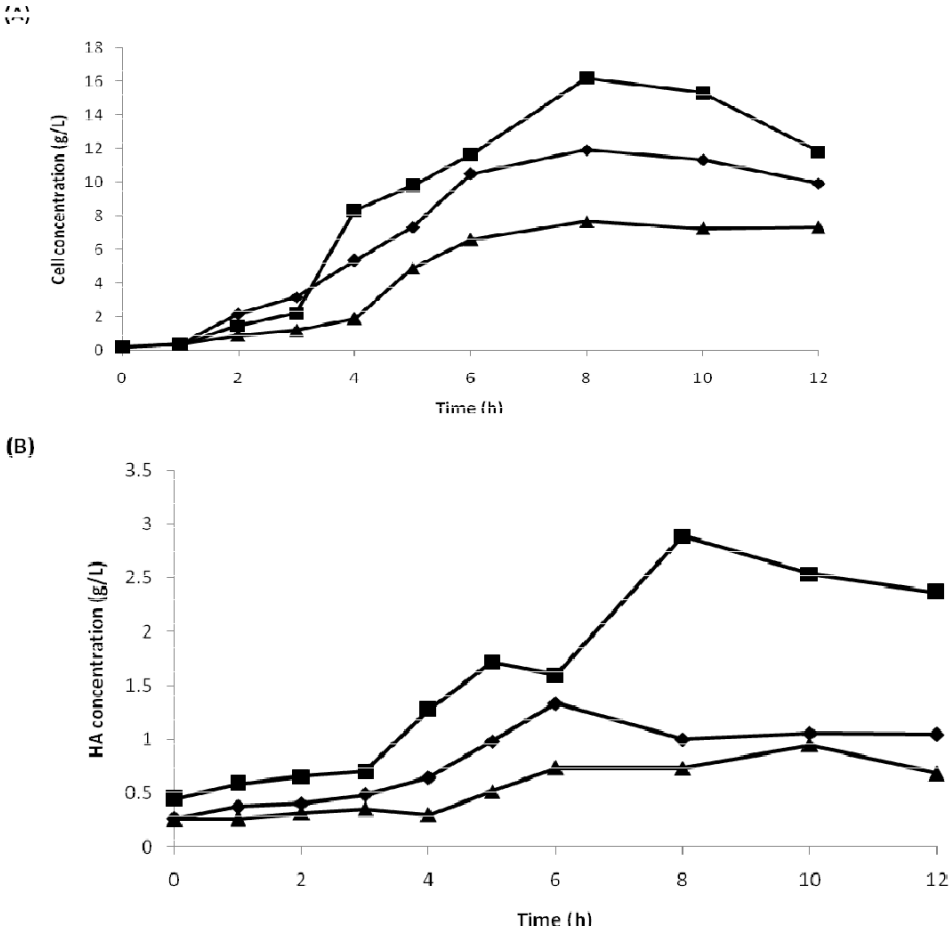



**Figure 2.** Batch HA fermentation by *S. zooepidemicus* ATCC 39920 in a 2 L stirred-tank bioreactor using different C/N ratio. (A) Cell concentration and (B) HA concentration. ( ) C/N 2.5:1; ( ) C/N 2:1 and ( ) C/N 3:1.

concentration (20 g/L) of the organic nitrogen source (yeast extract and tryptone) mixture. The glucose concentration was varied accordingly. As illustrated in Table 4, the highest cell efficiency coefficient value ($Y_{p/x}$) was produced with a C/N ratio of 2.5:1. Cheng et al. (1991) suggested that yeast extract contains a specific peptide and provides convenient growth factors that increase the growth rate of *S. zooepidemicus* ATCC 39920 and HA biosynthesis. This implies that a balanced medium is necessary for HA biosynthesis.

## Influence of medium formulation on HA molecular weight

The carbon and energy competition during cell growth, by-product production and HA biosynthesis limited the overproduction of HA. Among the three C/N investigated, the HA concentration and molecular weight at a ratio of 2.5:1 was enhanced by about 60 and 38%, respectively when compared to the other two C/N. As can be seen in Figure 3C, the HA molecular weight reached its maximum value of 4.36 x $10^6$ Da at a C/N of 2.5:1, then decreased with a ratio of above C/N 2.5:1. A similar observation was made by Armstrong and Johns (1997) who reported that the molecular weight of HA increased by 17 to 21% when the initial glucose level was doubled and was reduced as the glucose level increased to 60 g/L. These authors achieved an HA biosynthesis of 4.20 g/L with a molecular weight of 3.1 x $10^6$ Da. Conditions which reduced the molecular weight of HA in the pre-sence of excess glucose could be due to the availability and channelling of common resources into other competing metabolic pathways. The most common reports of HA molecular weight were in the range of 1 to 2.5 x $10^6$ Da with an initial glucose concentration of 10 to 60 g/L in a batch culture. Duan et al. (2008) obtained 3.65 g/L HA with a molecular weight of 2.00 x $10^6$ Da, and Liu et al. (2008) obtained an HA concentration of 5 g/L with a molecular weight of 1.30 x $10^6$ Da. The molecular weight of HA is considered high if it is above this range of values.

**Table 4.** Comparison of kinetic parameter values of HA biosynthesis by *S. zooepidemicus* ATCC 39920 in batch fermentation using different C/N ratio in a 2 L stirred-tank bioreactor.

| Kinetic parameter values | C/N | | |
|---|---|---|---|
| | 2:1 | 2.5:1 | 3:1 |
| t (h) | 10 | 10 | 8 |
| $X_m$ (g cell/L) | 11.689 | 15.994 | 7.496 |
| $P_m$ (g HA/L) | 1.333 | 2.442 | 0.946 |
| $\mu_m$ ($h^{-1}$) | 0.532 | 0.576 | 0.363 |
| $Y_{x/s}$ (g cell/g substrate) | 0.222 | 0.295 | 0.211 |
| $Y_{p/s}$ (g HA/g substrate) | 0.025 | 0.045 | 0.027 |
| $Y_{p/x}$ (g HA/g cell) | 0.114 | 0.153 | 0.126 |
| $P_r$ ( g HA/(L.$h^{-1}$)) | 0.222 | 0.244 | 0.095 |

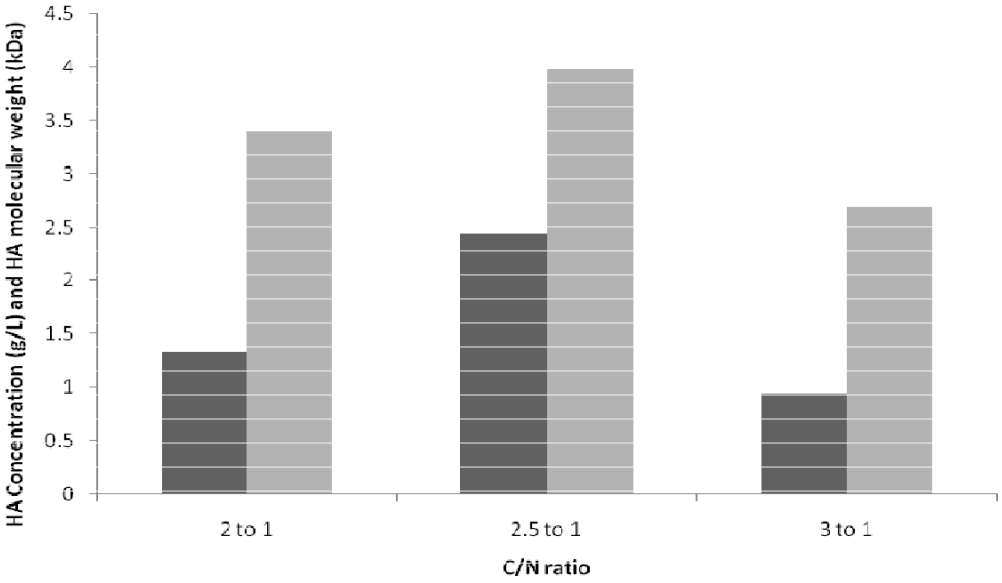



**Figure 3.** Effect of C/N on maximum of HA concentration and molecular weight by *S. zooepidemicus* ATCC 39920 in a 2 L stirred-tank bioreactor using Rushton turbine impeller.

Rangaswamy and Jain (2008) obtained an HA concentration of 6.00 g/L with a molecular weight of 4.00 x $10^6$ Da. In this study, although the enhancement of the HA concentration was not extremely high, the enhancement of the average molecular weight was very high and it was the highest in the reported literature using this *S. zooepidemicus* strain.

## Influence of impeller design on HA biosynthesis

Another important factor limiting microbial HA biosynthesis and molecular weight is the low oxygen mass transfer efficiency resulting from the high broth viscosity in batch fermentation. The viscosity of an aqueous HA solution increases 1000-fold when the HA concentration rises from 2 to 10 g/l (Akasaka and Yamaguchi, 1986). The HA production yield by using Maxblend® impeller was over 20% higher than the conventional - type of turbine blade under the operating condition of high aeration rate and low vessel pressure since the viscosity of both viscosity increased (Hasegawa et al., 1999). Helical ribbon impellers have been established in numerous industrial applications for mixing highly viscous fluids (Delaplace et al., 2006). Figure 4 shows the concentra-tion of cells and HA biosynthesis using Rushton turbine and helical ribbon impellers. The cell concentration obtained using a helical ribbon impeller is lower than

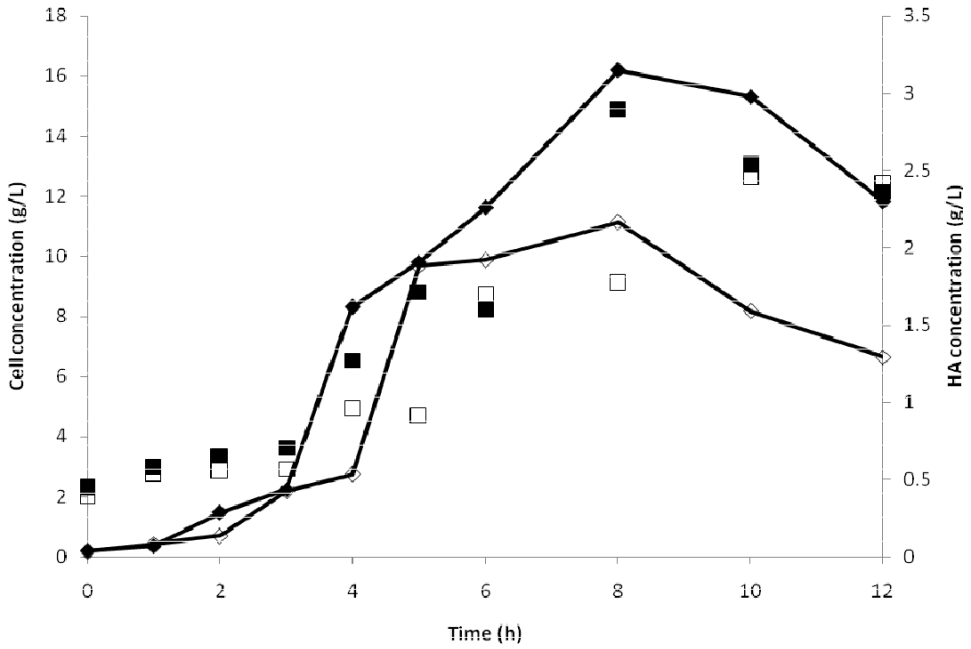



**Figure 4.** Batch HA fermentation by *S. zooepidemicus* ATCC 39920 in a 2 L stirred-tank bioreactor using helical ribbon impeller (open symbol) and Rushton turbine impeller (close symbol). ( ) Cell concentration and ( ) HA concentration.

that obtained by using a Rushton turbine impeller; however, the $\mu_m$ and $P_m$ are higher. In our preliminary experiment, excess impeller tip speed resulted in a remarkable decrease in HA biosynthesis. The poor performance at higher impeller tip speeds may be due to the high shear rate of bacterial cells at the tip of the impeller (Stephenie et al., 2007). In addition, increased agitation in the bioreactor creates shear forces which can cause mechanical damage to microorganisms and can also result in higher rates of energy consumption which subsequently increases the production cost (Giavasis et al., 2006). Thus, the impeller tip speed was maintained at 0.785 m/s throughout fermentation to investigate the effect of the impeller design on HA biosynthesis and molecular weight. The cell concentration was decreased from 15.994 to 13.412 g/l and HA biosynthesis was maintained at approximately the same values which were 2.442 g/l for the turbine impeller and 2.453 g/l for the helical ribbon impeller (Table 5). The $\mu_m$ value for the strain was also increased from 0.576 to 0.66 $h^{-1}$.

The Rushton turbine is known to generate a radial flow field acclaimed for gas dispersion and it is able to provide high-shear conditions. When the culture broth containing HA was agitated with turbine impellers, the culture broth appeared difficult to mix well due to its non- Newtonian high viscosity, and controlling the pH and dissolved oxygen (DO) concentration were not effective in maintaining high HA productivity. This impeller design also decreased the ability of gas handling and caused a high gas power drop due to the effect of large gas cavities (Nurashikin et al., 2010). On the other hand, helical ribbon impellers generate tangential and axial flow which is preferred for increasing the mixing efficiency. Helical ribbon impellers often operate at lower speed than radial flow impellers and for condition under equal Reynolds number. Nevertheless, the net energy required to attain a specific degree of homogenization is significantly lower since mixing operation with helical ribbon usually yield shorter mixing times (Szulc and Kuncewicz, 2006). This impeller design can maintain low shear conditions for culturing fragile, higher eukaryotic cells and can homogenise a highly dense and viscous broth of extracellular microbial polysaccharides by capitalising on the agitator's bulk handling and pumping capacity (Mohamed et al., 2009).

## Influence of impeller design on HA molecular weight

Even though the HA biosynthesis was at approximately at the same level using the two impeller designs, the molecular weight of HA in the culture broth was greatly influenced by the impeller type. In terms of quality indicator, the final product possessing high molecular weight points to superior performance as gentle helical ribbon impeller mixing protected biopolymer molecules from shear damage or mechanical degradation. The molecular weight of HA produced by the helical ribbon impeller (5.20 x $10^6$ Da) was much higher than that produced by the Rushton turbine impeller (4.36 ×$10^6$ Da).

**Table 5.** Comparison of kinetic parameter values of HA biosynthesis by *S. zooepidemicus* ATCC 39920 in batch fermentation using different impeller designs.

| Kinetic parameter values | Helical ribbon | Rushton turbine |
|---|---|---|
| t (h) | 10 | 10 |
| $X_m$ (g cell/L) | 13.412 | 15.994 |
| $P_m$ (g HA/L) | 2.453 | 2.442 |
| $\mu_m$ ($h^{-1}$) | 0.660 | 0.576 |
| $Y_{x/s}$ (g cell/g substrate) | 0.240 | 0.295 |
| $Y_{p/s}$ (g HA/g substrate) | 0.044 | 0.045 |
| $Y_{p/x}$ (g HA/g cell) | 0.183 | 0.153 |
| $P_r$ ( g HA/(L.$h^{-1}$)) | 0.245 | 0.244 |

These results are higher than those of Kim et al. (1996) who were able to produce 3.90 x $10^6$ Da of HA using a Rushton-type impeller and 4.80 x $10^6$ Da of HA using an Intermig-type impeller. Under the shear thinning fluid scenario, the helical ribbon impeller which has an axial flow pattern was predicted to fare better in mixing a viscous fluid than the two radial flow patterns of the Rushton turbine impeller. With the power exceeding 800 W $m^{-3}$, the helical ribbon impeller managed to produce 15 to 20% of a volumetric oxygen transfer rate, $K_La$, that was higher than the two- turbines model (Mohamed et al., 2009). Nevertheless, in the two-turbine system the power uptake range applied might still be insufficient to penetrate the viscous boundary layers which led to low oxygen transfer in a mixing vessel and thus a poorer overall $K_La$ performance for the Rushton turbine compared with the helical ribbon impeller.

For the helical ribbon impeller, the spinning disk helped to create a lower pressure region in the bioreactor, resulting in decreased power consumption and a subsequent reduction in production costs. A different impeller design exerts a notable effect on the oxygen mass transfer and thus influences the HA molecular weight. Further work is being carried out to clarify the effective viscosity of the broth and flow pattern induced by the motion of the impeller to enhance the HA biosynthesis and molecular weight. The medium formulation and impeller design clearly affected the growth and HA biosynthesis of *S. zoopedimicus* ATCC 39920 using a stirred-tank bioreactor. A mixture of organic nitrogen sources was preferred at a balance C/N 2.5:1 which exhibited a high concentration and molecular weight of HA. HA biosynthesis and the molecular weight of HA were increased by 60 and 38% respectively, using an optimal medium formulation and a helical ribbon impeller as a mixing device in a bioreactor as compared to non-optimal conditions.

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**Abbreviations: t,** time at the maximum cell concentration (h); **$X_m$,** maximum cell concentration (g cell/L); **$P_m$,** maximum HA concentration (g HA/L); **$\mu_m$,** maximum specific growth rate ($h^{-1}$); **$Y_{x/s}$,** growth yield coefficient (g cell/g substrate); **$Y_{p/s}$,** HA yield based on the substrate utilised coefficient (g HA/g substrate); **$Y_{p/x}$,** HA biosynthesis per cell (g HA/g cell); **$P_r$,** HA productivity (g HA/(L·$h^{-1}$)); **D**, diameter of stirred tank bioreactor (mm); **d**, diameter of impeller (mm); **H,** height of impeller (mm); **H',** distance between two turbine impellers (mm); **$d_s$** , diameter of ring sparger (mm); **$h_s$**, distance between the stirred tank bioreactor bottom and ring sparger (mm).

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