Optimization Feed Composition on Hyaluronic Acid Production of in-Batch and Fed-Batch Cultures of *Streptococcus zooepidemicus*

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ABSTRACT: *Hyaluronic acid (HA), as a high-value-added biological product, is one of the most widely used biopolymers in industry and medicine. This study aimed to increase HA production by carefully investigating the effect of glucose concentration, type and concentration of nitrogen source in batch culture, and control of glucose concentration in fed-Batch culture on HA production. The effect of two different nitrogen sources of hydrolyzed casein and tryptone soy broth on HA production was investigated in batch cultures of Streptococcus zooepidemicus. Then, the effect of glucose concentration on HA production was studied in batch and fed-batch cultures. Under optimal conditions, including hydrolyzed casein 20 g/L and an initial glucose concentration of 30 g/L in batch culture, 4.4 g/L HA was obtained. Also, constant feeding of 30 g/L of glucose increased HA production to 5.8 g/L. This amount is one of the highest HA production values ever reported in fed-batch cultures at a constant feeding rate. Weakening of the glycolytic cycle and slowing down the cell growth rate affect the increase of HA production. Also, controlling the concentration of glucose as a source of energy and carbon in the cells by the fed-batch culture can be directed the cell pathway to more hyaluronic acid production and higher yield.*

KEYWORDS: *Hyaluronic acid; Production increasing; Fed-batch culture; Feeding composition; Streptococcus zooepidemicus.*

INTRODUCTION

Hyaluronic Acid (HA) is a non-branched (linear) polysaccharide found in vitreous fluid, synovial fluid of the joints, cartilage, and vertebrate connective tissues. HA has composed of repetitive subunits of glucuronic acid (GlcUA) and N-acetylglucosamine, and its molecular weight varies from 10^4 to 10^7 daltons. HA has significant viscoelastic properties due to its polymeric and polyelectrolytic properties and can absorb water up to 1000 times its volume. In the human body, hyaluronic acid exists in hyaluronate salt, and high concentrations in the skin, umbilical cord, and vitreous fluid [1]. The main uses of hyaluronic acid are the treatment of osteoarthritis, cosmetics, ophthalmology, plastic surgery, wound healing, drug delivery, and tissue engineering [2]. At present, microbial production of HA is an alternative to extracting from animal tissues such as cockroaches, cord blood, and synovial fluid. The microbial approach mainly uses *Streptococcus* bacteria, which produce HA as a protective capsule around the cell wall [3]. Groups A and C *streptococci* synthesize HA as an extracellular capsule.

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1021-9986/2022/8/2728-2734 7/$/5.07

Streptococci are gram-positive bacteria whose main fermentation product is the carbohydrate lactate. Generally, the EPS (Exo Poly Saccharide) is not used as a food by the bacteria producing it, but, *Streptococcus* mutants can degrade and use the produced oligosaccharide by its [4].

In general, the first culture medium for *Streptococcus* proliferation contained animal sources such as sheep blood and BHI (brain-heart injection) for cell growth. These bacteria grow best at high glucose concentrations, pH = 7 and 37 °C, while bacterial growth must be slow to produce more hyaluronic acid. Hemolytic metabolism converts more than 90% of sugars to lactate. However, in some cases, hemolytic metabolism is lost and high amounts of formate, acetate, and ethanol (as mixed acid metabolism) are produced [5]. Controlling the shift from homolactic to mixed acid may be attributed to various factors such as pH [6], glucose concentration [7], aeration, and yeast extract concentration [8]. Lactic acid bacteria, which usually require a large and complex nitrogen source for biomass, convert most of the carbon of glucose into fermentation products and convert only small amounts into biomass [9].

Investigation of the effects of mixing rate, aeration rate, and dissolved oxygen on HA-producing bacteria has shown that the production amount and molecular weight of HA are higher in aerobic conditions [10-13]. Approaching the end of the production phase of HA is accompanied by an increase in viscosity and a gradual decrease in mixing time due to heterogeneous environmental conditions in terms of the mass transfer of available nutrients and oxygen to the cell. To overcome the adverse effects of increasing viscosity and consequently the heterogeneity of the culture medium on the HA production, usually, stirring speed is increased or aeration performed by higher oxygen content. In the competition for carbon to convert to lactic acid or hyaluronic acid, the increased mixing and oxygen available to the cell, produce more energy and consequently more product [14]. Investigations of *Samadi et al.* indicated that glucose correlates with bacterial growth and its concentration was effective in achieving the highest level of HA production[15]. *Cooney et al*. reported that at high glucose concentrations, lactic acid bacteria such as *S. zooepidemicus* produce large amounts of lactate from glucose catabolism (75-85%), thereby inhibiting cell growth [15]. *Jagannath et al.* also reported that when the initial concentration of glucose and sucrose increases from 30 to 60 g/L, the specific growth rate of *S. zooepidemicus* decreases [16]. Other researchers also proved that a high concentration of glucose (50 g/L) inhibits the multiplication of the cells due to the decrease in water activity [9, 17].

There is a competition between hyaluronic acid production and bacterial growth for raw material consumption. Therefore, maybe weakening the glycolytic cycle and slowing down the cell growth rate is effective in the production increase of HA. One study reported that the HA concentration increased in the broth after the cessation of growth due to glucose exhaustion [18]. Using glucose-limited fed-batch culture, the metabolism of lactic acid bacteria can shift from lactic acid to a mixture of formate, acetate, and ethanol (2: 1: 1 molar ratio), as a result, 3 moles of ATP are produced for any mole of glucose consumed instead of 2 moles [3]. Nitrogen levels play a determining role in cell growth that may affect other product characteristics. It may be noted that the yield coefficient $Y_{HA/X}$ is the highest under the nitrogen-limited fed-batch condition and least under the glucose-limited fed-batch condition [19]. Under stress conditions of limited glucose, most of the cell energy (metabolism) is used to produce HA, and by increasing stress, the metabolic pathway fluctuates towards higher cell density. This phenomenon is caused by the cell's reaction to environmental stimuli such as temperature and food stress. One of the factors that increase the production of HA as a capsule around the cell is its production as a cell protector and a cell energy storage source for use in acute conditions such as food shortages. Therefore, controlling the amount of cell energy sources such as glucose available can direct the cellular pathway to more production of hyaluronic acid and achieve higher yield. Batch culture is a simple method for HA production. Although, batch culture is a simple method for HA production but is associated with disadvantages such as substrate and by-product inhibition that limit cell growth and productivity. So fed-batch culture and controlling the feeding is the best choice for HA production enhancement.

Various factors such as the type of culture medium, environmental factors, and genetic characteristics of the bacteria affect the biosynthesis of HA and determine its production amount and molecular weight. In this study, at first, the effect of two complex nitrogen sources of tryptone soy broth and casein hydrolysate medium, and also initial glucose concentration on hyaluronic acid

production was investigated by *S. zooepidemicus* batch culture in the lab-scale fermenter. Then, under the optimal conditions obtained in the batch process, the effect of the glucose concentration of feeding on HA production and cell growth was evaluated in the fed-batch culture of *S. zooepidemicus*. Finally, the constant feed rate fed-batch was applied to justify the residual glucose in the critical concentration and due to the reduction of the oxygen-limiting effect after the HA production and viscosity increasing at the end of the process.

EXPERIMENTAL SECTION

Microorganisms and cultural conditions

The *Streptococcus zooepidemicus* strains mutated from the Tehran Biotechnology Research Center were used to produce HA. The Streptococcus zooepidemicus was mutated by them serial selection exposure to UltraViolet (UV) light and N-methyl-N-nitro-N-nitroguanidine. The cultivation was performed in a 2 liter B. Braun Biostat B bioreactor with two 6-blade Rushton impellers and a blade diameter to container diameter ratio of 0.5. The culture medium contained hydrolyzed casein, tryptone soy broth, glucose, 1.5(w/v)% sodium chloride, and 0.6 (w/v)% magnesium sulfate. The inoculum was prepared in a 200 mL Erlenmeyer flask containing 50 mL from the BHI medium and incubated at 37°C and 150 rpm and added 5 (v/v)% with OD_{600} =0.5 to the production bioreactor. In the fed-batch culture, glucose in different concentrations was fed gradually from the middle of the growth phase (8 h) at a constant rate until the end of the process (20 h). The pH of the culture medium was adjusted at 7 using 5 M NaOH. The temperature was adjusted to 37℃. The minimum oxygen content was controlled on 10-20% saturation air using different amounts of aeration during cultivation.

Measurement methods

The amount of HA produced (efficiency) was evaluated by the Carbazole method. The basis of this method is to measure the amount of glucuronic acid present in the HA structure. In this method, the HA-containing solution is first boiled in concentrated sulfuric acid to break down the HA into smaller components. Then, the carbazole reagent is added to the medium. This reagent binds to the units of glucuronic acid and produces color by heat. The exact method of measurement has been described in the article by *Zakeri et al.* [20].

The cell concentration was measured from the Optical Density (OD) of the broth at 600 nm using a spectrophotometer; the OD obtained (after adequate dilution) was then correlated with Dry Cell Weight (DCW). The glucose concentration was determined using a glucose oxidase commercial kit (glucose Pars Azmun).

RESULTS AND DISCUSSION

Effect of different concentrations and types of nitrogen sources on the HA production in batch culture

The effect of nitrogen source type and concentration on HA production was investigated using two common nitrogen sources of tryptone soy broth and casein hydrolysis culture medium in the *S. zooepidemicus* batch culture. The initial concentration of glucose was 20 g/L in all cases. The harvest time, which was usually associated with a cessation in production, was 20 h in all experiments.

Fig. 1 shows that the culture medium containing 20 g/L of casein hydrolyzate medium produces more HA. So in the continuing research, it was considered a suitable nitrogen source for HA production enhancement. It may be due to the higher nitrogen content of hydrolyzed casein than the soybean broth medium. This ratio of carbon to nitrogen shows the best results, and the higher amount of the nitrogen source in the medium transfers glucose flux from cell growth synthesis to HA. However, at higher C/N ratios, excess glucose is converted to organic acids instead of biomass or HA which leads to reduced HA productivity, as shown in Fig. 1.

Effect of Initial Concentration of Glucose on the HA production in batch culture

Fig. 2 shows the effect of initial glucose concentration on HA production and final cell density. It is seen, by increasing the initial glucose concentration from 10 to 30 g/L, hyaluronic acid production increases by about 2-fold from 2.4 to 4.4 g/L. However, with more increases in glucose concentration, the HA production does not change. The intensity of growth reduction in higher glucose concentrations was higher than the decrease in production, which can be seen in the product-to-cell yield (Yp/x) in Fig. 2. Increased osmotic pressure is one of the important factors in slowing down the growth of cells. Increasing glucose concentration also slightly increases cell density but decreases with further increasing glucose, possibly

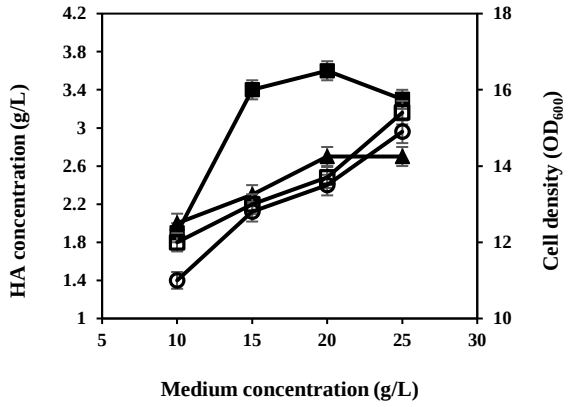

Fig. 1: Effect of different concentrations of two types of media on production rate: Tryptone Soy Broth (▲) and Casein Hydrolysate (■) and cell density: Tryptone Soy Broth (□) and Casein Hydrolysate (○).

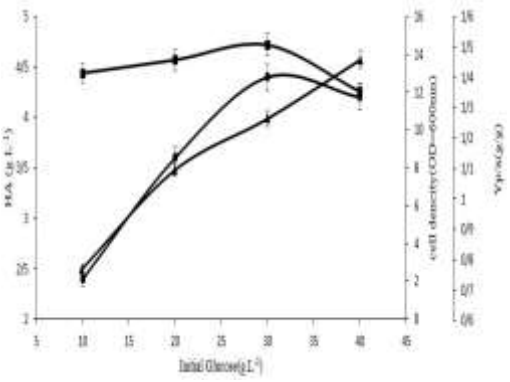

Fig. 2: Effect of initial glucose concentration on the concentration of hyaluronic acid (●)cell density(■) and Yp/x(▲) in the batch culture of S. zooepidemicus.

due to the maximum tolerable concentration obtained for *streptococci* being 30-40 g/L [21,22]. In studies on different amounts of primary glucose from 10 to 60 g/L, the maximum amount of HA produced reported has not been more than 3 g/L [21,23,24]. In another study with initial glucose ranging from 5 to 90 g/L, more than 1.2 g of HA didn't produce [7], and in 70 g/L initial glucose, the most product reached 4.75 g/L [25].

Effect of Different Concentrations of feeding glucose in fed-batch cultivation

There is a competition between hyaluronic acid production and bacterial growth for raw material consumption. Therefore, weakening the glycolytic cycle and slowing down cell growth rate influence increasing HA production. Examination of the cell growth at different concentrations of feed glucose in the fed-Batch culture of *S. zooepidemicus* shows that by increasing glucose concentration and creating stress conditions on the bacterial cells, more cellular energy (metabolism) is uptaken to produce HA [17,22]. According to the remaining amount of glucose, its complete consumption, and the limitation of using the maximum initial concentration of glucose, the effect of a constant feeding rate of 100 mL of sugar solution with different concentrations (10-40 g/L) on HA production enhancement was investigated. The results show that HA production yield to biomass first increases with increasing glucose concentration from 10 to 30 g/L but decreases with a further increase in glucose concentration. Probably because of increasing glucose concentration, cell energy is more consumed to produce biomass, and preferably glucose is used for cell growth instead of HA synthesis.

Fig. 4 shows the changes in cell growth and residual glucose concentration at feeding with different glucose concentrations. Also, it is seen that the cell growth rate does not change significantly by increasing the glucose concentration of feeding to 30 g/L, but by a further increase, the cell density increases. Taking a closer look at the results obtained in the final hours shown in Fig.4 and Table 1, it is found that the proliferation of cells coincides with the increase of residual glucose concentration. As the feed glucose concentration increases to 30 g/L, the remaining glucose at the end of the process increases to a maximum of 1 g/L. Cell density remains constant despite this amount of glucose, but with increasing concentrations of residual glucose due to feeding with higher glucose concentrations, cell mass also increases. Fig. 5 shows the time profile of hyaluronic acid concentration at feeding with different glucose concentrations in the fed-batch cultivation of *S. Zooepidemicus.* Hyaluronic acid is a growth-associated product and according to Table 1, under the stress of glucose restriction, HA production enhances by increasing the feed glucose concentration. With a further increase in feed glucose concentration and consequently a decrease in stress due to glucose concentration limit, cell density increases, and hyaluronic acid production decreases. Under glucose-limiting conditions, lactic acid bacteria resort to heterofermentative glucose catabolism to compensate for the decrease in

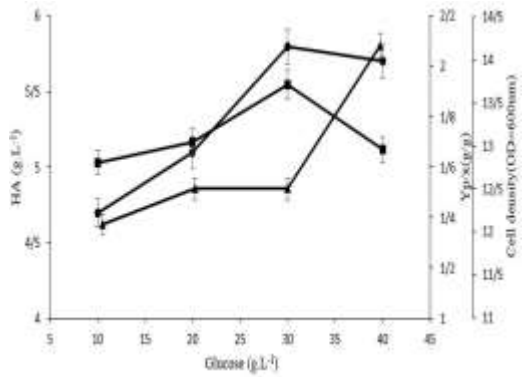

Fig. 3: Effect of feeding glucose concentration on HA yield(●) and Yp/x(■)cell density(▲) in the fed-batch cultivation of S. zooepidemicus.

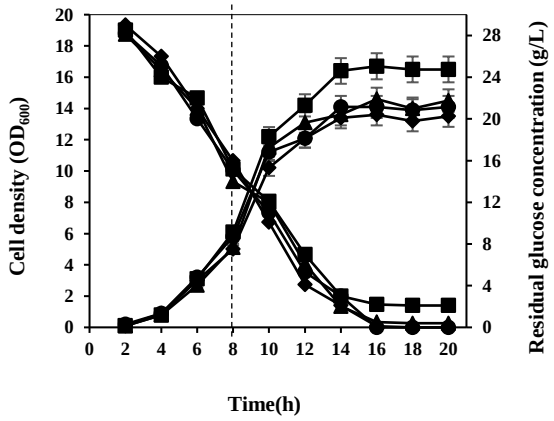

Fig. 4: Time courses of cell growth and residual glucose curve at different concentrations of feeding glucose (g/L)(dashed line): 10 (♦) 20 (●) 30 (▲) 40 (■)in the fed-batch cultivation of S. Zooepidemicus.

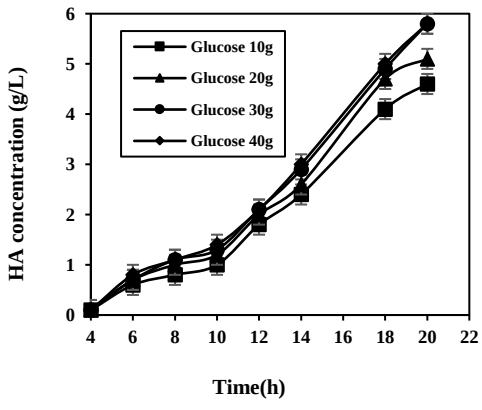

Fig. 5: Time courses of the hyaluronic acid curve at different concentrations of feeding glucose (g/L): 10 (♦) 20 (▲) 30 (●) 40 (■)in the fed-batch cultivation of S. Zooepidemicus.

Table 1: Time courses of residual glucose concentration in the fed-batch culture of S. zooepidemicus by different concentrations of feeding glucose.

Time (h)	Feeding glucose concentrations (g/L)			
0	10	20	30	40
12	4.1	5.3	6.1	7
14	2.1	3	2	3
16	0.1	0	0.5	2.2
18	0	0	0.4	2.1
20	0	0	0.4	2.1

cellular energy production (ATP) due to lower glucose uptake, and extra mole of ATP during the conversion of the glycolytic intermediate, pyruvate, changed to acetate, instead of lactate. Another effect of glucose restriction is slowing growth because the total cellular energy produced is reduced.

A balanced medium with an optimum carbon-to-nitrogen ratio of about 2:1 should be prepared to maximize the capacity of HA synthesis. Investigation of the effect of glucose to nitrogen ratio on fed-batch cultivation in another study did not produce more than 3.5 g/L [8]. In another study, by fed-batch culture and controlling the concentration of reducing sugars above 10 g/L with repeated addition of glucose, a maximum of 4.85 g/L HA was obtained [26].

CONCLUSIONS

The main contribution of this paper is the demonstration that adjusting glucose concentration in the specified range can enhance the production of HA. The cell's response to environmental conditions, such as the amount of glucose remaining, has a significant effect on HA production that can be carefully controlled to achieve higher production.

EPS plays a vital role in the protection of microbes from adverse conditions such as nutrient shortage [27]. Environmental stressors such as glucose availability, mineral availability, and pH have been described as influential aspects of HA production [22,28]. There is a competition between cell growth and HA synthesis, and turning this competition in favor of producing more HA can be an effective strategy to increase yield. By closely controlling glucose feeding during fed-batch culture and stabilizing the residual glucose concentration within the specified range, it can increase cellular metabolism

competition in favor of HA production due to the cell's response to environmental stress conditions such as starvation. Therefore, keeping glucose concentration within a desirable range can be effective on HA production. In this study, carefully examining the glucose remaining during Fed-batch culture revealed that 1) controlling glucose concentration in the amount of nearly zero stimulates the cell to produce more storage (product), 2) more reduction of reducing sugar decrease HA production and cell density simultaneously, 3) with more glucose concentration, more energy is consumed for increasing cell density and lactic acid production.

Received : Sep. 27, 2021 ; Accepted : Jan. 31, 2022

REFERENCES

[1] Kogan G., Soltes L., Stern R., Gemeiner R., Hyaluronic Acid: A Natural Biopolymer with a Broad Range of Biomedical and Industrial Applications, *Biotechnol Lett.*, **29**:17- 25 (2007).

[2] Moscovici M., Present and Future Medical Applications of Microbial Exopolysaccharides, *Front Microbiol.*, **6**: 1012 (2015).

[3] Chong B.F, Blank L.M., Mclaughlin R., Nielsen L.K., Microbial Hyaluronic Acid Production, *Appl. Microbiol. Biotechnol*., **66**: 341–351 (2005).

[4] Patel S., Majumder A., Goyal A., Potentials of Exopolysaccharides from Lactic Acid Bacteria, *Indian J. Microbiol.*, **52(1)**: 3-12 (2012).

[5] Garrigues C., Mercade M., Cocaignbousquet M., Lindleyn N.D., Loubiere P., Regulation of Pyruvate Metabolism in *Lactococcus lactis* Depends on the Imbalance between Catabolism and Anabolism, *Biotechnol. Bioeng.*, **74**: 108-115 (2001).

[6] Liu L., Wang M., Du G., Chen J., Enhanced Hyaluronic Acid Production of *Streptococcus zooepidemicus* by an Intermittent Alkaline-Stress Strategy, *Lett. Appl. Microbiol.*, **46**: 383-388 (2008).

[7] Pires A.M.B, Santana M.H.A., Metabolic Effects of the Initial Glucose Concentration on Microbial Production of Hyaluronic Acid, *Appl. Biochem. Biotechnol*., **162**: 1751-1761 (2010).

[8] Chen S.J., Chen J.L., Huang W.C., Chen H.L., Fermentation process Development for Hyaluronic Acid Production by *Streptococcus zooepidemicus* ATCC 39920, *Korean J. Chem. Eng.*, **26**: 428-432, (2009).

[9] Chong B.F., Blank L.M, Mclaughlin R., Nielsen L.K. Microbial Hyaluronic Acid Production, *Appl. Microbiol. Biotechnol*., **66**: 341-351 (2005).

[10] Liu L., Du G.C., Chen J., Wang M., Sun J., Comparative Study on The Influence of Dissolved Oxygen Control Approaches on the Microbial Hyaluronic Acid Production of *Streptococcus zooepidemicus*, *Bioprocess Biosyst. Eng.*, **32**: 755-763, (2009).

[11] Huang W.C, Chen S.J, Chen T.L., The Role of Dissolved Oxygen and Function of Agitation in Hyaluronic Acid Fermentation, *Biochem. Eng.*, **32**: 239-243 (2006).

[12] Chong F.B., Nielsen L.K., Aerobic Cultivation of *Streptococcus zooepidemicus* and the Role of NADH Oxidase, *Biochem. Eng.*, **16**: 153-162 (2003).

[13] Duan X.J., Niu H.X., Tan W.S., Zhang X., Mechanism Analysis of Effect of Oxygen on Molecular Weight of Hyaluronic Acid Produced by *Streptococcus zooepidemicus*, *J. Microbiol. Biotechnol.*, **19(3)**: 299-306 (2009).

[14] Saharkhiz S., Babaeipour V., The Dilution Effect of Media Culture on Mixing Time, Kla O_2, and Hyaluronic Acid Production in *S. zooepidemicus* Fed-batch Culture, *Biotechnol. Lett.*, **43(12)**:2217-2222 (2021).

[15] Samadi M., Khodabandeh Shahraky M., Tabandeh F., Aminzadeh S., Dina M., Enhanced Hyaluronic Acid Production in *Streptococcus zooepidemicus* by an Optimized Culture Medium Containing Hyaluronidase Inhibitor, *Prep. Biochem. Biotechnol.*, 1-11 (2021).

[16] Cooney M.J., Goh L.T., Lee P.L., Johns M.R., Structured Model- Based Analysis and Control of the Hyaluronic Acid Fermentation by *Streptococcus zooepidemicus*: Physiological Implications of Glucose and Complex- Nitrogen- Limited Growth, *Biotechnol. Prog.*, **15(5)**: 898-910 (1999).

[17] Jagannath S, Ramachandran K., Influence of Competing Metabolic Processes on the Molecular Weight of Hyaluronic Acid Synthesized by *Streptococcus zooepidemicus*, *Biochem Eng.*, **48**: 148-158 (2010).

[18] Armstrong D.C., Johns M.R., Culture Conditions Affect the Molecular Weight Properties of Hyaluronic Acid Produced by *Streptococcus zooepidemicus*, *Appl. Environ. Microbiol.*, **63**: 2759-2764 (1997).

[19] Navin C.V., George P., Modeling and Simulation of a Structured Model for Analysis of Hyaluronic Acid Fermentation by *Streptococcus zooepidemicus* under Aerobic and Anaerobic Batch Conditions, *Int. J. Curr. Res.*, **1**: 1-10 (2011).

[20] Zakeri A., Rasaee M.J., Pourzardosht N., Enhanced Hyaluronic Acid Production in *Streptococcus zooepidemicus* by over Expressing HasA and Molecular Weight Control with Niscin and Glucose, *Biotechnol. Rep.*, **16**: 65-70 (2017).

[21] Mohan N., Pavan S., Achar A., Swaminathan N., Sivaprakasam S., Calorespirometric Investigation of *Streptococcus zooepidemicus* Metabolism: Thermodynamics of Anabolic Payload Contribution by Growth and Hyaluronic Acid Synthesis, *Biochem. Eng.*, **152**: 107367 (2019).

[22] Don M.M., Shoparwe N.F., Kinetics of Hyaluronic Acid Production by *Streptococcus zooepidemicus* Considering the Effect of Glucose, *Biochem. Eng.*, **49(1)**: 95-103 (2010).

[23] Lai Z.W., Rahim R.A., Ariff A., Mohamad R., Medium Formulation And Impeller Design on the Biosynthesis of High Molecular Weight Hyaluronic Acid by *Streptococcus zooepidemicus* ATCC 39920, *Afr. J. Microbiol.*, **15**: 2114-2123 (2011).

[24] Pan N.C., Vignoli J.A., Baldo C., Pereira H.C.B., Silva R.S., Celligoi M. A. Effect of Fermentation Conditions on the Production of Hyaluronic Acid by *Streptococcus zooepidemicus* ATCC 39920, *Acta. Biol. Sci.*, **37(4)**: 411-417 (2015).

[25] Liu J., Wang Y., Li Z., Ren Y., Zhao Y., Zhao G., Efficient Production of High-Molecular- Weight Hyaluronic Acid with a Two-Stage Fermentation, *RSC Advances*, **8(63)**: 36167-36171(2018).

[26] Vázquez J.A., Montemayor M.I., Fraguas J., Murado M.A., High Production of Hyaluronic and Lactic Acids by *Streptococcus zooepidemicus* in Fed-Batch Culture Using Commercial and Marine Peptones from Fishing By-Products, *Biochem. Eng.*, **44**: 125-130 (2009).

[27] Looijesteijn P.J., Trapet L., vries E., Abee T., Hugenholtz., Physiological Function of Exopolysaccharides Produced by *Lactococcus lactis*, *Int. J. Food Microbiol.*, **64**: 71–80 (2001).

[28] Pires A.M., Eguchi S.Y., Santana M.H., The Influence of Mineral Ions on the Microbial Production and Molecular Weight of Hyaluronic Acid, *Appl. Biochem. Biotechnol.*, **162(8)**: 2125-35, (2010).