EFFECTS OF FERMENTATION CONDITIONS FOR HYALURONIC ACID PRODUCTION FROM *Streptococcus thermophilus* TCN03 ISOLATES

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ABSTRACT

Hyaluronic acid consists of repeating units of glucuronic acid and N - acetylglucosamine in a gel form that is hydrated. Hyaluronic acid has garnered significant attention in the scientific community owing to its potential therapeutic use. The purpose of this study is to increase hyaluronic acid production by investigating the effect of suitable fermentation conditions (carbon and nitrogen source, pH, temperature and time) in a culture medium on hyaluronic acid production by the bacterial. Strain *S. thermophilus* TCN03 was studied for suitable conditions for hyaluronic acid biosynthesis. Bacterial strain was fermented for 24 hours on a medium containing the following ingredients: Glucose (20 g/L), yeast extract (5.0 g/L), K_2HPO_4 (g/L) 2.0, NaCl (2.0 g/L), $MgSO_4.7H_2O$ (0.05 g/L) at 36°C, pH 7.0 and shaking at 200 rpm. Under optimal conditions, the maximum hyaluronic acid production achieved was 914.7 mg/l. These findings revealed the most effective hyaluronic acid manufacturing strategy for achieving maximum output.

Keywords: *Hyaluronic acid, optimal conditions, S. thermophilus.*

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1. INTRODUCTION

Hyaluronic acid, a mucopolysaccharide, is generated ubiquitously in human and animal tissues as a hydrated polymer. It is a mucopolysaccharide that is composed of repeating units of glucuronic acid and N - acetyl glucosamine [1]. Due to its distinctive physicochemical attributes like hydrophobicity, lubricating properties and biocompatibility, it finds extensive application in the fields of biomedical, healthcare, food and cosmetics [2]. However, Hyaluronic acid is molecular size determines many of its therapeutic uses and this is the subject of several studies. Traditionally, animal tissues such as rooster combs and cow vitreous humor have been used to extract hyaluronic acid. Nonetheless, limitations in the industrial - scale isolation process and ethical concerns present arguments against its widespread use [3]. Presently, technological advancements have facilitated the production of hyaluronic acid from microbial sources using fermentation methods. There have been several reports of research on the manufacture of hyaluronic acid by microbial fermentation employing organisms such as *S. zooepidemicus* [4] and *S. thermophilus* [5]. Samadi found that glucose content correlated with bacterial growth and led to the highest hyaluronic acid production levels [6]. According to Jagannath, an increase in the initial concentration of glucose and sucrose from 30 to 60 g/L results in a decrease in the specific growth rate of *S. zooepidemicus* [7]. Similarly, other

researchers have demonstrated that a high glucose concentration of 50 g/L inhibits cell multiplication by reducing water activity [8]. Regulating the availability of cellular energy sources, such as glucose, can guide the cellular pathway toward increased hyaluronic acid production and ultimately achieve a higher yield. Biosynthesizing hyaluronic acid in *S. thermophilus* necessitates a substantial amount of energy, while bacterial cells vie for the carbon source essential for their cellular growth. In situations where only a limited quantity of the carbon source is available, the majority of it is swiftly used for bacterial growth, resulting in reduced acid productivity. However, when bacteria thrive in an environment rich in carbon sources, they exhibit a notably higher rate of hyaluronic acid production [9]. In addition, the quantity of acid generated is influenced by variations in the nitrogen source and its concentration in the production medium. In this study, we investigated the appropriate conditions for *S. thermophilus* TCN03 bacteria to improve their ability to synthesize hyaluronic acid. A study on the extraction of hyaluronic acid from microorganisms isolated in Vietnam will be the initial step in developing the technology for producing functional food containing hyaluronic acid for applications in healthcare.

2. MATERIALS AND METHODS

2.1. Microorganism

S. thermophilus TCN03 was isolated from different samples and was used as the hyaluronic acid - producing strain throughout this study. Stock cultures were cultivated for 24 hours in an MRS medium.

Medium for cultivating microorganisms (g/L): Agar 15.0, glucose 20.0, $CaCO_3$ 5.0, Beef extract 10.0, yeast extract 5.0, pepton 10.0, tween 80 1.0, K_2HPO_4 2.0, CH_3COONa 5.0, triamoniumcitrat 2.0, and $MgSO_4$ 0.05.

2.2. Effect of different carbon and nitrogen sources on hyaluronic acid production

The initial basal medium was composed of glucose (20 g/L), yeast extract (5.0 g/L), K_2HPO_4 (g/L) 2.0, NaCl (2.0 g/L), $MgSO_4.7H_2O$ (0.05 g/L), pH 6.5. To study the effect of carbon sources on hyaluronic acid production, glucose was replaced with sucrose, fructose and lactose. Meanwhile, yeast extract provides a source of nitrogen for bacterial growth from basic culture medium was substituted, and other sources tested including (5 g/L): KNO_3, peptones, and urea. Control samples did not add carbon and nitrogen sources to the culture medium. Sterile medium with an initial volume of 120 mL in 250 mL Erlenmeyer flasks was inoculated with 10 mL stock culture of S. thermophilus TCN03 and cultured under 38°C with occasional shaking for 48 hours. During the process, 1 mL of the culture medium was collected at 12, 18, 24, 30, 36 hours, for analysis of hyaluronic acid and cell growth (OD 530 nm).

2.3. Influence of different factors on the production of hyaluronic acid

The impact of several factors on the synthesis of hyaluronic acid and cell growth was studied, specifically as follows: temperature (32, 34, 36, 38, and 40°C), fermentation time (12, 18, 24, 30, and 36 hours) and pH (6.0, 6.5, 7.0, 7.5, 8.0) in culture medium under shake flask by *S. thermophilus* TCN03.

2.4. Isolation and purification of hyaluronic acid

Purification of hyaluronic acid from *S. thermophilus* TCN03 fermentation was carried out according to the method outlined by Bitter and Muir (1962) [10]. After the fermentation process, the cell was removed using 0.15% w/v sodium dodecyl sulfate for 15 min and centrifuged. The solution from centrifugation was supplemented with 2% activated charcoal and sodium chloride was introduced to reach a concentration of 0.15 M. The mixture was stirred for approximately 5 hours to allow for the adsorption of impurities such as proteins, nucleic acids, endotoxins, etc. Subsequently, it was filtered to separate the adsorbent from the supernatant, which contained hyaluronic acid. To this hyaluronic acid-enriched supernatant, an equal volume of 95% ethanol was

added to induce the precipitation of hyaluronic acid. The resulting precipitate was collected, washed 3 times with 80% ethanol and then freeze - dried to recover the product.

2.5. Determination of hyaluronic acid

The quantitative analysis of hyaluronic acid in the culture is conducted through the detection of N-acetyl-D-glucosamine in hyaluronic acid, using the Reissig method [11]. The collected fermented broth was centrifuged at 9,000 rpm for 15 min. The fermentation solution after centrifugation is alkaline in a borate pH 9.1 environment; at 100°C for 15 minutes. The tube containing 0.25 ml of hyaluronic acid after hydrolysis was added with 0.05 ml of 0.2 M K_3BO_3, boiled at 100°C for 3 minutes, then cooled quickly on ice, added 1.5 ml of DMAB reagent, mixed well and left at 37°C for 20 minutes, then at room temperature after 2 hours. The change in color of the mixture before the reaction and after the reaction from colorless to pink or red (depending on concentration) proves the presence of N-acetyl-D-glucosamine.

2.6. Determination of cell growth

Cell growth was measured using a UV-visible spectrophotometer (Labomed Inc, USA) at 530 nm, with the medium without inoculation used as a reference blank. To obtain reliable measurements, the culture samples were diluted with distilled water to maintain an optical density of OD530 nm (OD < 1).

3. RESULTS AND DISCUSSION

3.1. The effect of carbon and nitrogen sources on hyaluronic acid production

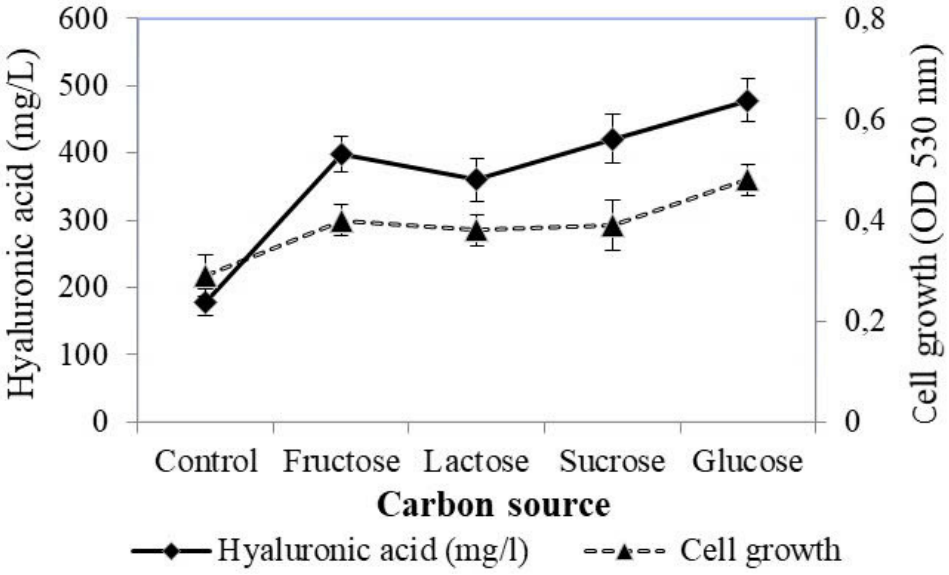

Figure 1. Effect of carbon source on hyaluronic acid production by *S. thermophilus* TCN03

The production of hyaluronic acid using carbon sources is shown in figure 1, *S. thermophilus* TCN03 strain is cultured on media supplemented with different carbon sources after 48 hours of fermentation at 38°C, pH 6.5. The results showed that the strain of bacteria synthesizing hyaluronic acid had higher production compared to the control (178.4 mg/L) on culture mediums with different carbon sources. Hyaluronic acid can be produced from glucose, sucrose, and fructose by *S. thermophilus TCN03*. Using glucose as a carbon source provided the maximum hyaluronic acid content of 478.2 mg/L, while using sucrose, fructose and lactose gave the production yield of 421.5, 398.2, 360.5 mg/L after 48 hours of fermentation, respectively (Figure 1). An increase in the production of hyaluronic acid was shown to be related to a small decrease in the amount of sugar consumed. Glucose molecules were first converted into phosphohexoses, which included glucose-6-phosphate and fructose-6-phosphate. Which were subsequently utilized in

the synthesis of the D-glucuronic acid and N-acetyl glucosamine components of hyaluronic acid [12]. The cell numbers of *S. thermophilus* TCN03 in a biotransformation medium containing different carbon sources at each interval time were presented. Microbial cells rapidly increased, then gradually declined over time. An increase in cell number was correlated with the production yield of hyaluronic acid. When the cell growth decreased within 48 hours for glucose and sucrose and 36 hours for fructose and lactose, the gradual depletion of hyaluronic acid production was observed.

Using sugar as a carbon source in the glycolytic pathway is essential for providing energy and raw materials for both hyaluronic acid synthesis and biomass formation. The molecular weight of the product that the bacteria generated increased when glucose was added to the fermentation medium. Furthermore, the choice of carbon source can influence the efficiency of the glycolytic pathway [13]. Group A *streptococci* were first isolated for hyaluronic acid production, which resulted in a production range of 60 - 140 mg/L [14]. Since that time, several different studies have been carried out to enhance the production of hyaluronic acid. These studies have included the discovery of effective microbial strains, the optimization of growing medium, conditions, and the extraction process, as well as the creation of high molecular weight hyaluronic acid. A range of carbon sources, including starch, lactose, glucose, sucrose, and dextrin, were employed for hyaluronic acid production. Typically, *Streptococci* utilizes glucose as the primary carbon source for hyaluronic acid production. However, being able to utilize carbon sources such as starch, lactose and agricultural by - products remains a key objective in cost - effective hyaluronic acid production [15].

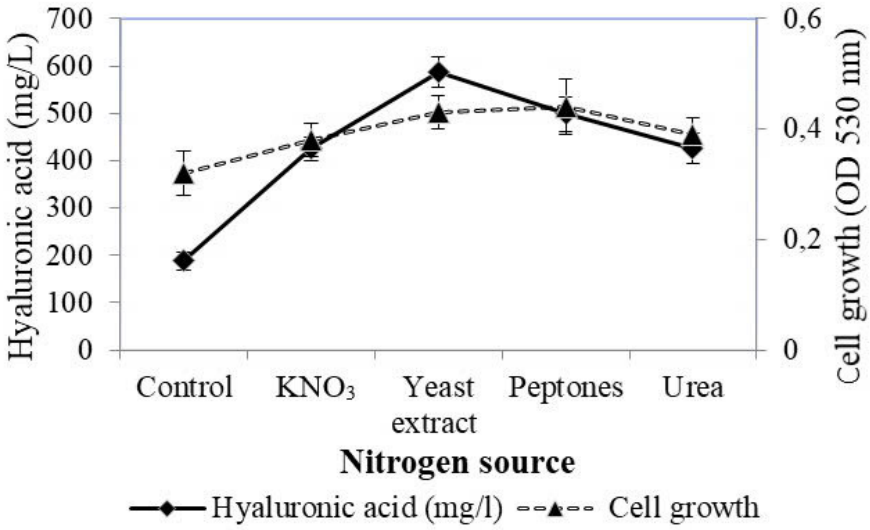

Figure 2. Effect of nitrogen sources on hyaluronic acid production by *S. thermophilus* TCN03

To determine the effect of nitrogen source on hyaluronic acid production, yeast extract was replaced with three different nitrogen sources (KNO_3; peptones and urea). The glucose used provides a carbon source for bacterial growth. Yeast extract was found to be the best effective nitrogen source and the yield was 587.2 mg/L, while using KNO_3, peptones; urea gave the production yield of 425.7, 498.6, 427.1 mg/L after 48 hours of fermentation, respectively in our study. The cell number of *S. thermophilus* TCN03 in a medium containing different nitrogen sources correlated with the production yield of hyaluronic acid (Figure 2). The study by Im *et al.* (2009) [16] yielded similar results and found that the yeast extract-casein peptone mixture produced the highest hyaluronic acid yield. Other nitrogen sources, such as tryptone, peptone, urea and

casamino acid, produced hyaluronic acid levels of 0.415, 0.436, 0.418 and 0.098 g/L, respectively. This phenomenon may be related to bacteria's need for complicated nutritional demands for growth, which may be satisfied by organic nitrogen sources such as amino acids and peptides, which promote metabolic activities, as opposed to inorganic nitrogen sources such as urea. The reduction in acid production when utilizing urea as a nitrogen source can be ascribed to the absence of *S. thermophilus*, an enzyme - degrading microorganism that contributes to water formation. Consequently, it becomes impossible to harness the nitrogen source for acid production [17]. Pan *et al.* (2015) [18] demonstrated that an increase in the concentration of yeast extract led to a concomitant surge in the synthesis of hyaluronic acid and lactate. Besides, Khue and Vo (2013) [19] conducted an assessment to quantify the level effects of various substances, including manganese sulfate, potassium phosphate, sodium acetate, ammonium citrate, meat extract and yeast extract, in addition to glucose and Tween - 80. The yeast extract exhibited the most pronounced influence on the yield of hyaluronic acid.

Previous studies focusing on hyaluronic acid production through microbial fermentation have explored the utilization of diverse carbon and nitrogen sources derived from agricultural products and their by - products. For instance, *S. zooepidemicus* ATCC 39920 demonstrated successful hyaluronic acid production using agricultural resources such as soy protein concentrate, whey protein concentrates and cashew apple juice, with the highest hyaluronic acid yield recorded at 0.89 g/L from the cashew apple juice [20]. In a separate study, *S. zooepidemicus* achieved an hyaluronic acid productivity rate of 0.28 mg/g when utilizing cashew apple bagasse in solid-state fermentation [21]. Additionally, Izawa *et al.* (2009) [22] successfully generated hyaluronic acid through the cultivation of isolated *S. thermophilus* YIT 2084, a generally recognized safe bacterium, in skimmed milk, resulting in an hyaluronic acid productivity of approximately 8.0 mg/L. These findings align with the research conducted by Lee *et al.* (2009) [23], who investigated the impact of nitrogen sources on the production of hyaluronic acid by *S. zooepidemicus*. Their study revealed that yeast extract yielded the highest acid production (0.410 g/L).

3.2. Effect of incubation temperature on hyaluronic acid production

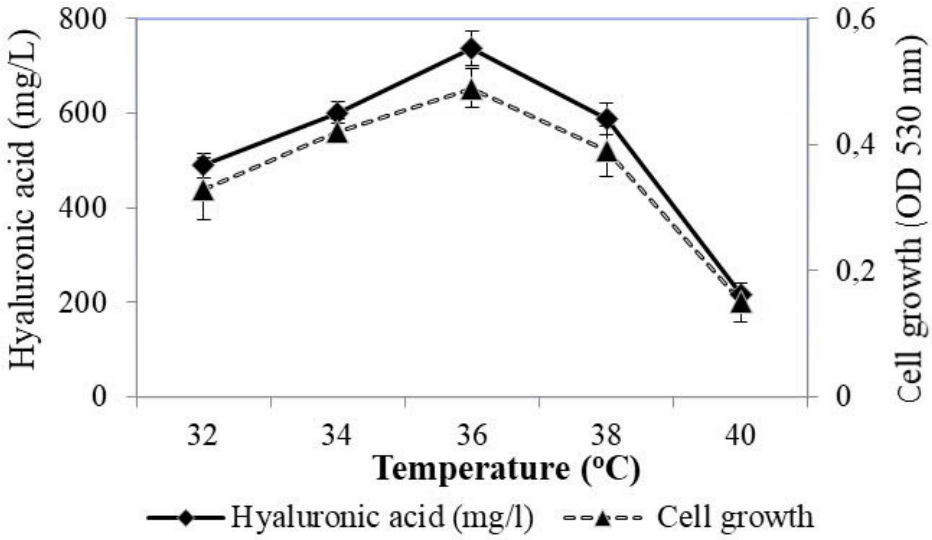

Figure 3. Effect of temperature on hyaluronic acid production by *S. thermophilus* TCN03

S. thermophilus TCN03 was fermented on a liquid medium supplemented with carbon source from glucose (20 g/L), nitrogen source from yeast extract (5 g/L), pH 6.5, fermentation time of 48 hours with different temperatures (32, 34, 36, 38, 40°C). The generation of hyaluronic acid by the *S. thermophilus* bacterial strain is affected by temperature. Figure 3 shows that the highest hyaluronic acid was generated when the incubation temperature was 36°C. It was observed that the highest hyaluronic acid production (738 mg/L) at 36°C, while the cell growth indicated by OD530 was 0.49. For temperatures of 32, 34, 38, and 40°C, the amounts of hyaluronic acid produced were 490.5, 601.3, 587.2, 215.7 mg/L, respectively. Hyaluronic acid production was less when the temperature was lower than < 34°C and higher than 38°C. Then, the hyaluronic acid concentration was 601.3 mg/L and 587.2 mg/L, respectively. Then, there was a correlation between an increase in cell number and the amount of hyaluronic acid that was produced. Several investigations demonstrated that the quantity of hyaluronic acid synthesized was influenced by the incubation temperature. Izawa *et al.* (2010) [24] observed that 33 - 40°C was optimal for hyaluronic acid synthesis by *S. thermophilus*. The findings presented align with those in the study by Tu and Trang (2013) [25], which suggested that the optimal incubation temperature for hyaluronic acid production from *S. thermophilus* at 37°C. This conclusion was drawn after exploring various temperature ranges, including 34°C, 37°C and 40°C.

3.3. Effect of pH on hyaluronic acid production

After 48 hours of fermentation, *S. thermophilus* TCN03 was cultured on a liquid medium supplemented with suitable nutritional components, pH 6.5 at 36°C. The maximum hyaluronic acid production (887.4 mg/L) was obtained at pH 7.0, while the cell growth indicated by OD530 was 0.48. For the pH range of 6.0, 6.5, 7.5, 8.0, the amounts of hyaluronic acid produced were 530.8, 738.1, 638.9, 325.3 mg/L, respectively (Figure 4).

pH and temperature are two of the most important parameters that influence cell proliferation and whether or not an organism can make metabolites, the impact of these two variables on the culture conditions of *S. zooepidemicus* 3523 - 7 was studied to determine which was more favorable for the formation of hyaluronic acid. The pH of the culture had a significant impact on the amount of hyaluronic acid produced and the development of the cells. Changes in culture pH further lowered HA production, which is consistent with the findings of Johns *et al.* (1994) [26], who detected a comparable drop in hyaluronic acid output at pH 6.0 and pH 7.9. This contradicts the findings of Patil *et al.* (2009) [27], who indicated that the optimal pH for hyaluronic acid synthesis is 7.5.

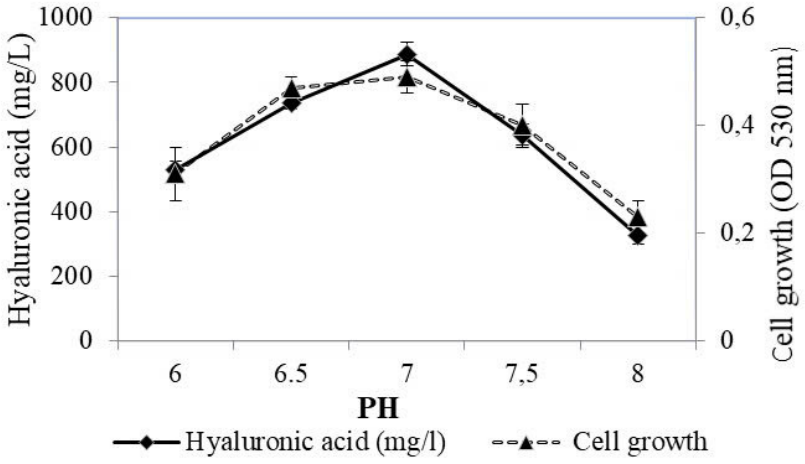

Figure 4. Effect of pH on hyaluronic acid production

3.4. Effect of fermentation time on hyaluronic acid production

HA production was significantly impacted by growth time, with the highest concentration (914 mg/L) detected at 20th of fermentation. The impact of different incubation durations (12, 18, 24, 30, 36 hours) on hyaluronic acid production and cell growth from an alternative medium using the *S. thermophilus* strain was investigated. The results revealed that the optimal hyaluronic acid production occurred at the 24-hour fermentation, yielding a quantity of 914.7 mg/L. Concurrently, cell growth, as indicated by an OD530, reached 0.46. Notably, the quantities of hyaluronic acid produced for incubation durations of 12, 18, 30, 36 hours were 426.7, 758.2, 908.4, 900.3 mg/L, respectively (Figure 5).

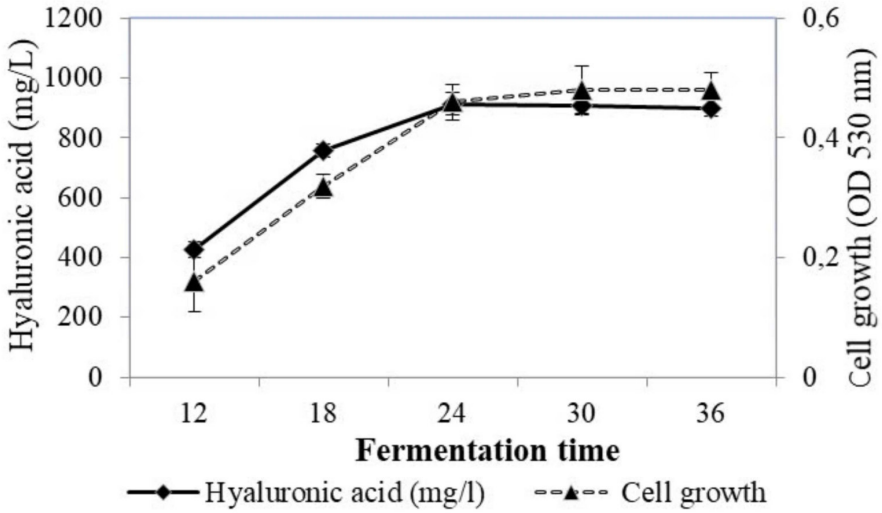

Figure 5. Effect of fermentation time on hyaluronic acid production

After a 24 - hour fermentation period, hyaluronic acid production begins to diminish due to the gradual depletion of nutrients within the growth medium and the advancing cell population entering a state of decline. This decline in cell viability results in an increased release of toxins into the production medium. These findings are consistent with Gedikli's study [28], which examined the impact of incubation periods on hyaluronic acid production. In their study, after 18 hours of fermentation, *Streptococcus equisimilus* yielded 0.592 g/L of hyaluronic acid. Similarly, Kumar *et al.* (2021) [29] conducted investigations to optimize hyaluronic acid production from *S. equisimilus* and determined that the most favorable incubation duration was 28 hours, resulting in a hyaluronic acid concentration of 0.68 g/L.

4. CONCLUSION

In this study, strain *S. thermophilus* TCN03 was studied for suitable conditions for hyaluronic acid biosynthesis. Bacterial strain were fermented for 24 hours on medium containing the following ingredients: Glucose (20 g/L); yeast extract (5.0 g/L), K_2HPO_4 (g/L) 2.0, NaCl (2.0 g/L), $MgSO_4.7H_2O$ (0.05 g/L), pH 7.0 at 36°C under the condition of continuous shaking at 200 rpm, The maximum hyaluronic acid production achieved was 914.7 mg/L. The bacterial strain mentioned has a high potential for synthesizing hyaluronic acid and is used for manufacturing products in the fields of healthcare, medical, food, and cosmetics.

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