Archives of Razi Institute, Vol. 77, No. 6 (2022) 2295-2305

DOI: 10.22092/ARI.2022.358612.2262

Original Article

Production and Optimization of Hyaluronic Acid Extracted from *Streptococcus thermophilus* Isolates

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Received 7 May 2022; Accepted 8 June 2022
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Abstract

Biopolymers, particularly exopolysaccharides produced by microorganisms such as bacteria, yeasts, and algae, have gained popularity in recent years due to their physical, chemical, and functional properties that are widely useful in food, industrial, cosmetic, and pharmaceutical systems. Hyaluronic acid is one type of these polysaccharide. This study investigated the optimal conditions for producing hyaluronic acid from the *Streptococcus thermophilus* bacterial strain. The isolated *Streptococcus thermophilus* were cultured on MRS broth, Skim milk, and M17 broth with an addition of 1% lactose. The diagnosed bacterial strains were grown in 100 ml of culture media, placed in volumetric flasks of 250 ml capacity, and incubated at 42˚C for 24 hours, pH 6.8, inoculum volume 1%, and a vibrating incubator at 150 rpm. After the end of the fermentation period, the isolation and purification of HA have performed accordingly: proteins were removed using 1% trichloroacetic acid (TCA), and HA in the supernatant was collected by isopropanol precipitation. The collected HA was dialyzed against ultrapure water and lyophilized. The amount of acid produced was estimated. The results show that the best production of hyaluronic acid was from the *S. thermophilus* bacterial strain grown on the alternative medium containing whey at a ratio of 450 ml/L and7.5 g/L yeast extract at 40 ˚C, with a 3% of inoculum volume and 102×10^8 colony-forming units/ml of bacterial cells, in pH 6.8 and agitation speed of 150 rpm for 18 h, which had the most significant effect on the fermentation process and gave the highest value of HA production of 0.598 g/L and biomass of 6.08 g/L. These results showed the best production method for HA to achieve maximal production yelled.

Keywords: Hyaluronic Acid, Optimal Conditions, *S. thermophilus* Bacteria Strain

1. Introduction

Biopolymers, particularly exopolysaccharides produced by microorganisms such as bacteria, yeasts, and algae, have gained popularity in recent years due to their physical, chemical, and functional properties that are widely useful in food, industrial, cosmetic, and pharmaceutical systems Özcan and Öner (1). Hyaluronic acid (HA) is one type of these polysaccharides. It is defined as a linear, negatively charged natural polymer belonging to the heterocyclic polysaccharides called glycosaminoglycans (GAGs) consisting of repeating disaccharide units D-glucuronic acid and N-acetyl-D-glucosamine. It is naturally found in the human body and animals such as rabbits, cows, and roosters, as well as the microorganisms that secrete it as secondary metabolic products, such as *Streptococcus equi*, *Streptococcus zooepidermicus*, *Streptococcus equisimilis*, *Streptococcus uberis*, and yeasts such as *Cryptococcus neoformans* and the algae *Chlorella* sp. (2).

Hyaluronic acid production from microbial sources mainly depends on the fermentation of *Streptococcus*

bacteria. Since some species of this bacteria are pathogenic, studies have focused on producing it from bacterial strains generally recognized as safe (GRAS), such as *Streptococcus thermophilus* (3). The biosynthesis of hyaluronic acid in *S. thermophilus* requires much energy, and bacteria cells compete for the carbon source they use for cell growth. When a small amount of the carbon source is available, the most significant percentage of the carbon source is quickly consumed for growth, which leads to a decrease in acid productivity with a high rate of its molecular weight. However, when bacteria grow in the presence of an abundant amount of carbon source, hyaluronic acid production is observed at a high rate (4). In addition, there is a close relationship between the type of carbon source, the amount of acid produced, and the amount of biomass, as lactose is the preferred carbon source for *S. thermophilus* in the metabolism process. Thus, it produces a more significant amount of polysaccharides, including hyaluronic acid (1, 5). The amount of acid produced is affected by the difference in the nitrogen source and its percentage in the production medium (6).

Previous studies indicated that the amount of acid produced is affected by production conditions such as incubation temperature, inoculum size, pH, incubation period, and incubator vibration speed (7-9). Therefore, the present study was designed to find the best conditions for producing hyaluronic acid from *S. thermophilus* to reduce production costs.

2. Materials and Methods

2.1. Isolation of Bacteria

A series of decadal dilutions of yogurt samples taken from Basrah local markets in 0.1% peptone solution was carried out, and 1 ml of the last three dilutions was taken and spread by L-shape glass diffuser on the surface of M17 agar medium in Petri dishes. The plates were incubated at 42° C for 48 h under aerobic conditions (10).

2.2. Phenotypic Identification of Isolates

2.2.1. DNA Extraction

The Kit Presto™ Mini gDNA Bacteria Extraction Kit supplied by Geneaid Biotech Ltd was used to extract DNA from bacterial isolates. The process of DNA extraction and PCR amplification was carried out according to the manufacturer's instructions.

2.2.2. Primers and PCR Conditions

The following primer, and 0.5 U Taq DNA polymerase (BOIRON), and 500 ng DNA. The reaction comprised of an initial denaturation for 2 min at 95°C; 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at56°C, and elongation for 1 min at 72°C; and final polymerization for 10 min at 72°C; the amplicons were stored at 4°C.

2.3. Culture Media and Fermentations

Three culture media were used to produce MRS broth, Skim milk, and M17 broth, adding 1% lactose (11). The diagnosed bacterial strains were grown in 100 ml of culture media, placed in volumetric flasks of 250 ml capacity, and incubated at 42˚C for 24 hours, pH 6.8, inoculum volume 1%, and a vibrating incubator at 150 rpm (12). Biomass was estimated according Izawa, Serata (13).

2.4. Extraction and Purification of Hyaluronic Acid

After the end of the fermentation period, the isolation and purification of HA were performed by the procedure described previously (14). Briefly, proteins were removed using 1% trichloroacetic acid (TCA), and HA in the supernatant was collected by isopropanol precipitation. The collected HA was dialyzed against ultrapure water and lyophilized. The amount of acid produced was estimated according to the methodSciabica, Tafuro (15).

2.5. Optimal Conditions for HA Production

2.5.1. Carbon and Nitrogen Sources for Hyaluronic Acid Production

Several carbon sources were used to replace it with lactose in the medium of optimal production. The prepared alternatives included date juice according to what was mentioned in Al-Roomi and Al-Sahlany (16), grape juice (17, 18), and whey (19). The total lactose of these substitutes was estimated by the Lane-Eynon method mentioned in Ranganna (20). Lactose constitutes 20 g/L in the medium of optimal production, with equivalents consisting of date juice,

grape juice, and whey with the ratios of 58, 210.5, and 321.15 ml/L ml of medium, respectively.

The remaining ingredients were added, 1% inoculated from the activated bacterial culture, and incubated at 42˚C in a vibrating incubator at a speed of 150 rpm for 24 hours. The hyaluronic acid production was estimated to select the best concentration of whey to be used as a substitute for the carbon source for acid production.

2.5.2. Effect of Various Parameters on HA Production

Effect of various physical parameters on HA production and biomass like incubation temperature (35-42°C), inoculum volume (0.5-10%), pH (5.5-7.8), fermentation period (6-48 hours), and different agitation speed (100-400 rpm) were studied in alternative production medium under shake flask using *S. thermophilus*.

3. Results and Discussion

3.1. PCR Amplification

The 16S rRNA was considered for molecular investigations to identify the most productive bacterial isolates. Figure 1 shows bands with a size of (1500) base pair (bp). These results agreed with Cebeci and Gürakan (21) when the bacterial strains of *S. thermophilus* were isolated from milk with bands at (1500) base pairs.

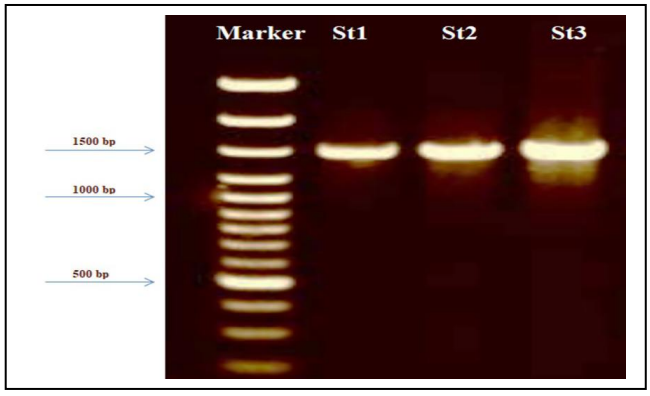

Figure 1. Amplifying16S rRNA of bacterial isolates from local yogurt to produce hyaluronic acid

After analyzing the results in the Gene bank, they showed a (100%) match with *S. thermophilus* JIM 8232, a strain previously registered in the gene bank, as shown in figure 2. The genetic match tree for *the S. thermophilus* strain was given the code ABST and recorded in the Japanese gene bank: http://submit.ncbi.nlm.nih.gov/nucccore/MZ841806.

3.2. Optimal Conditions for Hyaluronic Acid Production

3.2.1. Hyaluronic Acid Production

Figure 3 shows the produced HA and biomass concentration by *S. thermophilus* using three different production media. The results showed that the highest hyaluronic acid production was achieved using the M17 broth. Maximal HA production was 0.334 g/L. The weight of the biomass was 5.32 g/L compared to the production values of the MRS broth and skim milk, which amounted to 0.296, and 0.308 g/L, respectively, due to the difference in the carbon and nitrogen sources and their types, as well as the presence of metal ions that stimulate the production of metabolic substances in the medium And its effect on the concentration of produced HA and biomassChen, Li (22). These results agreed with what was found bySheng, Ling (11) when producing hyaluronic acid from two isolates of genetically modified *Lactococcuslactis* using M17 broth. The amount of acid produced for the bacterial isolates was 0.380 and 0.492 g/L. Saraphanchotiwitthaya and Sripalakit (3) found that when producing hyaluronic acid from *S. thermophilus* bacteria using molasses as a carbon source and incubating for 12 hours, the highest amount of acid produced was 0.213 g/L.

3.3. The Use of Local Substitutes in the Production Medium

3.3.1. Local Alternatives to Carbon Sources

Figure 4 shows the effect of using local alternatives, such as date juice, grape juice, and whey, to substitute the carbon source in the production medium M17 broth on the produced HA and biomass concentration. The whey carbon source gave the highest amount of acid.

The product was 0.359 g/L. The amount of biomass was 5.15 g/L. The amount of acid for date juice and grape juice alternatives was 0.195 and 0.321 g/L, respectively, due to the fact that whey contains a high percentage of lactose, which is the preferred sugar for bacteria in the process of metabolism. Thus, it produces a more significant amount of polysaccharides (1, 5). These results were in agreement with previously published work (5), when they studied the effect of manufacturing wastes of dairy products on the production of dairy products hyaluronic acid from *S. thermophilus*. The amount of acid produced was 0.342 g/L depending on whey as an alternative carbon source in the production medium.

Pires, Macedo (12) used agricultural and industrial waste (degraded soy proteins, whey proteins, apple juice, and decomposed corn) to fortify the industrial production medium consisting of glucose and yeast extract as a carbon and nitrogen source for the production of hyaluronic acid from *Streptococcus. zooepidemicus*. It was found that the apple juice, when used as a carbon source, gave the highest amount of acid, which amounted to 0.890 g/L. The decomposed corn gave 0.84 g/L. The acid production of whey proteins and soy decomposition as nitrogen sources were 0.1 and 0.13 g/L, respectively.

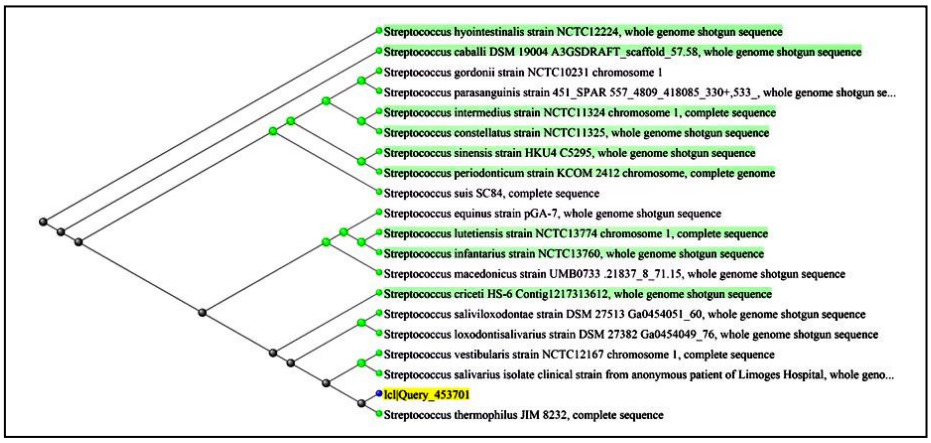

Figure 2. Genetic match tree for *S. thermophiles* bacteria strain

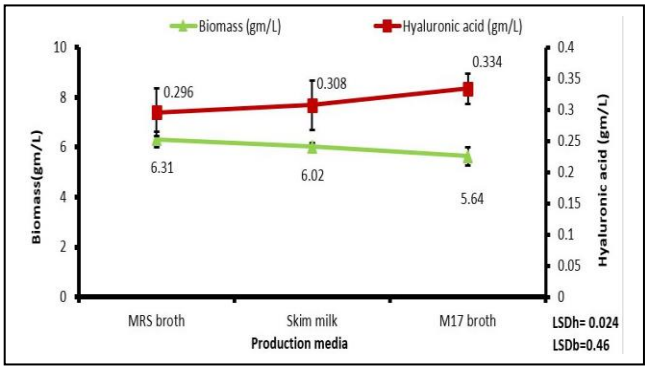

Figure 3. Hyaluronic acid and biomass acid production by *S. thermophilus* at different production media

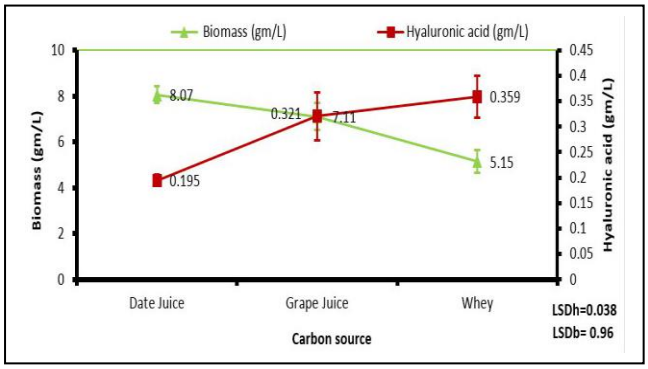

Figure 4. Effect of local alternatives used as carbon sources in the medium concentration of produced HA and biomass

3.4. Using Different Concentrations of Whey in the Production Medium

Different concentrations of whey were used in the production medium of hyaluronic acid, including 200, 250, 300, 321.5, 40, 450, 500, and 550 ml/L of the production medium. The results showed that the acid concentration increases with the increase in whey concentration added to the production medium up to a concentration of 450 ml/L of production medium as shown in figure 5 to decrease the acid concentration after adding 500 and 550 ml/L to the production medium, bringing the amount of hyaluronic acid produced to 0.381 and 0.364 g/L respectively. The decrease in the acid concentration at a high concentration of the carbon source can be explained by the inhibition of the base material (Substrate inhibition), which is a phenomenon that occurs through batch culture. The increase in the proportion of sugars may have a restraining effect on the growth of bacteria.

The best concentration for acid production was 450 ml/L of the production medium. The amount of acid produced was 0.425 g/L, and the amount of biomass was 6.35 g/L. So, this concentration was adopted and used in all subsequent stages of the present study. It was observed that the concentration of the resulting acid decreased at the initial concentrations of whey. The amount of hyaluronic acid produced was 0.188 g/L, which may be due to the insufficient concentration of the carbon source to form a more significant number of the basic building units of hyaluronic acids, such as uranic acid and glucuronic acid.

These results agree with what was mentioned byPrasad, Jayaraman (23) when they studied the production of hyaluronic acid from genetically modified *Lactococcuslactis* bacteria. The production of hyaluronic acid depends on the concentration of the carbon source. The initial concentrations of sugar less than 5 g/L are depleted in the medium for cell growth (biomass) before acid production. At high concentrations of more than 30 g/L, cell growth and biomass increase are caused at the expense of the acid produced.

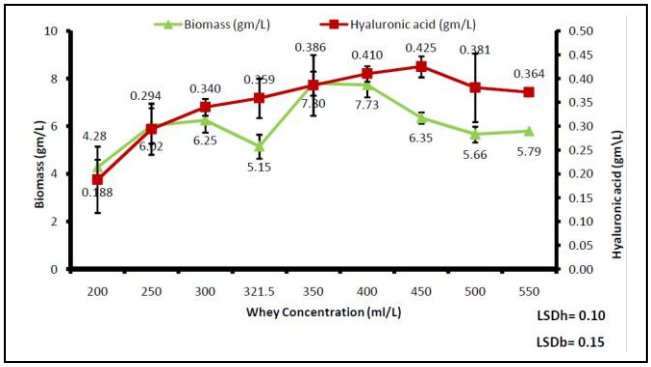

Figure 5. Effect of whey concentration on HA production and biomass

3.5. Alternatives of nitrogen source

Figure 6 shows the effect of using different sources of nitrogen source yeast extract, tryptone, urea, peptone, and casamino acid on the production medium of hyaluronic acid at a rate of 17.5 g/L using the most productive bacterial strain and whey carbon source with an amount of 450 ml/L of the production medium. The results showed that the values of the produced acid varied according to the nitrogen source. The nitrogen source gave the yeast extract the highest amount of acid, 0.462 g/L. The amount of biomass was 6.8 g/L. The remaining nitrogen sources were tryptone, peptone, urea, and casamino acid. The hyaluronic acid produced is 0.415, 0.436, 0.418, and 0.098 g/L, respectively. This may be due to the fact that these bacteria need complex nutritional requirements for their growth that can be provided by organic nitrogen sources of amino acids and peptides that stimulate metabolic activities, compared to inorganic nitrogen sources such as Urea (6). The decrease in the amount of acid produced when using urea as a nitrogen source could be due to the lack of *S. thermophilus*, which degrades the enzyme and water. Thus, it is impossible to benefit from the nitrogen source in acid production. These results agree with what was mentioned byLee, Ha (24), who studied the effect of nitrogen sources on the amount of hyaluronic acid produced by *S. zooepidemicus*. They

found that the yeast extract gave the highest amount of acid, amounting to 0.410 g/L. The amount of acid produced was less than that obtained byChen, Chen (25), who produced hyaluronic acid from *S. zooepidemicus*. The amount of hyaluronic acid was 2.5 g/L using yeast extract as a nitrogen source, which is 10 g/L of the production medium.

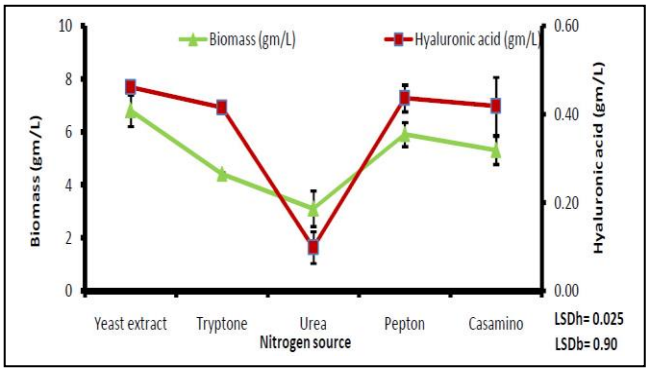

Figure 6. Effect of nitrogen sources on HA production and biomass

3.6. Effect of using Different Concentrations of Yeast Extract in the Production Medium

Figures 3-7 shows the effect of using different percentages of the optimal nitrogen source for yeast extract 5, 7.5, 10, 15, 17.5, and 20 g/L, as the results showed the best percentage of the optimal nitrogen source for yeast extract was 7.5 g/L. The acid was 0.496 g/L, and the amount of biomass was 5.84 g/L using the *S. thermophilus* and whey as a carbon source with an amount of 450 ml /L from the production medium. The mentioned percentage was adopted for the nitrogen source in all subsequent stages of the study, while the amount of hyaluronic acid was for proportions of 5, 10, 15, 17.5, and 20 g/L, which amounted to 0.312, 0.489, 0.482, 0.457, 0.351 g/L. Perhaps due to the amount of the nitrogen source that helped achieve a balance in the ratio of carbon and nitrogen (C/N) that affects the amount of biomass and the amount of acid. The resulting hyaluronic may be because streptococcus aureus generally depends on the nitrogen source in biomass production. In contrast, the carbon source is mainly used in the production of hyaluronic acid, which led to an increase in the production of hyaluronic acid. However, with the increase in the carbon source, bacteria tend to produce organic acids hyaluronic acid instead of hyaluronic acid (26).

The increase in the ratio of the nitrogen source stimulates the bacteria to produce biomass to provide the amino acids and proteins needed by cells in metabolism to multiply and increase their biomass.

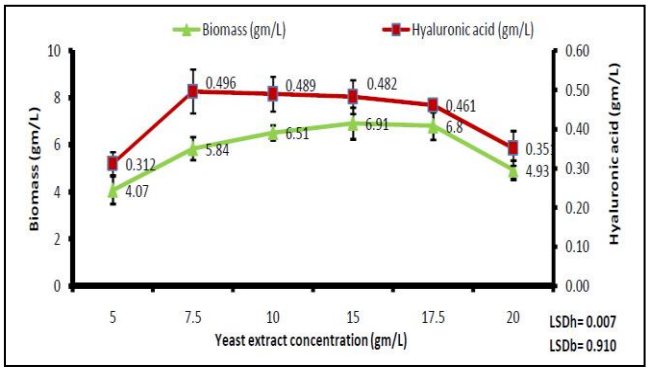

Figure 7. Effect of yeast extract concentrations on HA production and biomass

This study agrees with what was found byIm, Song (27) when producing hyaluronic acid produced by *Streptococcus* sp. bacteria, which indicated a superiority of 0.75% of yeast extract as a nitrogen source. The hyaluronic acid produced was 1.56 - 2.24 g/L, among several concentrations of 0.25, 0.5, and 1%.

3.7. Effect of Incubation Temperature on HA Production

There is an effect of different temperatures on the production of hyaluronic acid from the *S. thermophilus* bacterial strain. The results shown in figure 8 reveal that the highest amount of hyaluronic acid produced was achieved using the incubation temperature of 40°C. The amount of acid was 0.519 g/L, and the amount of biomass was 6.59 g/L. The amounts of the obtained hyaluronic acid were 0.044, 0.167, 0.489, 0.368, and 0.312 g/L for temperatures of 35, 37, 42, 45, and 50°C, respectively.

Several studies showed the effect of incubation temperature on the amount of hyaluronic acid produced. It was reported by Izawa, Hanamizu (28) that the best temperature for the production of hyaluronic acid from *S. thermophilus* was between 33-40°C. After

studying the effect of incubation temperature within a range of (30-45 °C), the results differed from what was found by Tu and Trang (9), who indicated that the best incubation temperature was 37 °C when producing hyaluronic acid from *S. thermophilus* using different thermal ranges of 34, 37, and 40 °C.

The rate of polysaccharide production increases with the increase in the incubation temperature until the optimum incubation temperature is reached, which depends on the type of bacteria used in the study. The incubation temperature is a critical factor in the biosynthesis of polysaccharides. It affects the rate of biomass, the activity of enzymes inside the cell, and the time required to build the main sugar units of polysaccharides (29).

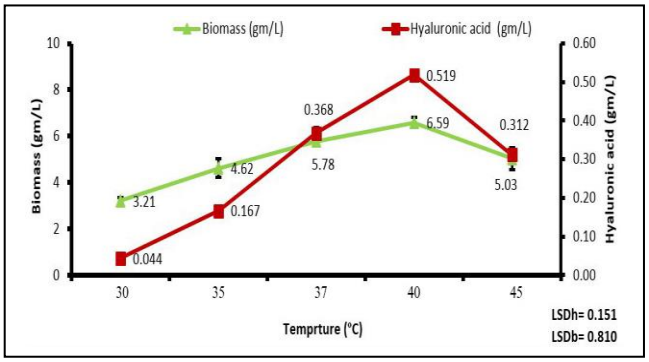

Figure 8. Effect of Incubation temperatures on HA production and biomass

3.8. Effect of the Inoculum Volume on HA Production

Figure 9 shows the effect of the inoculum volumes used in producing hyaluronic acid and biomass formed from the alternative medium. The results showed that the best inoculum volume in the production of hyaluronic acid was 3%, and the number of bacterial cells was (102 x 10^8) CFU/ml. The amount of acid produced was (0.578) g/L, and the amount of biomass was 6.63 g/L. The amount of hyaluronic acid produced was 0.288, 0.519, 0.537, 0.578, 0.469, and 0.304 g/L for the pollen volumes of 0.5, 1, 2, 5, and 10% respectively.

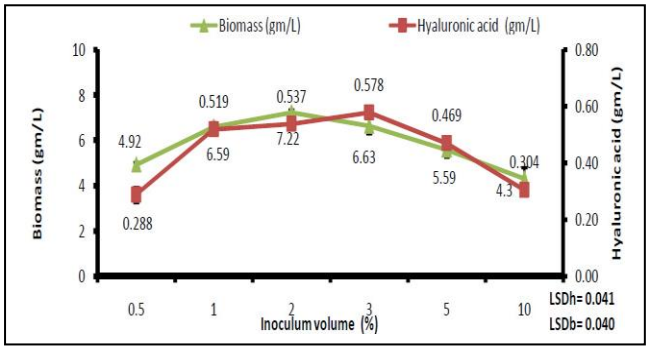

Figure 9. Effect of inoculum volumes on HA production and biomass

The success of the industrial fermentation process requires providing an appropriate inoculum volume free of contaminants to produce the desired products. The less the inoculum volume, the longer it requires to adapt, known as the logarithmic phase, in which the bacterial cells must still be in an active state in the metabolism process. On the other hand, the increase in the inoculum volume causes the consumption of oxygen and the consumption of components of the medium, and the increase in the acidity in the medium very quickly without producing the product in sufficient quantity.

The inoculum volumes varied in the production of hyaluronic acid using different microorganisms.Aroskar, Kamat (30) stated that the best inoculum volume was 3% when producing hyaluronic acid from *S. zooepidemicus*, and there were no significant differences between the inoculum volume of 3 and 5% in contrast with the inoculum volume of 10%, which showed lower productivity due to the increased consumption of the carbon source in the initial stages of growth, which leads to a decrease in pH due to the production of lactic acid as well as a high rate of biomass at the expense of the rate of production of hyaluronic acid.

3.9. The Effect of the Initial pH on HA Production

Figure 10 shows the effect of the different pH of the hyaluronic acid production medium using the *S. thermophilus* bacterial strain. The best initial pH of the

production medium was 6.8. The amount of hyaluronic acid produced was 0.578 g/L, and the amount of biomass was 6.63 g/L. The amount of acid produced had pH levels of 5.5, 6, 7.3, and 7.8, which amounted to 0.251, 0.283, 0.448, and 0.321 g/L, respectively. The neutral pH is the optimum for bacterial growth (biomass) and acid production in a more significant amount (31). However, the greater effect of pH on the production of hyaluronic acid compared to the growth of cells (biomass) mainly affects the enzymes responsible for acid production by bacteria (29). The results agree with Izawa, Hanamizu (28), who studied the effect of different pH ranges of (4.8, 5.3, 5.8, 6.3, 6.8, 7.3, and 7.8). The best pH for acid production was (6.8) as the amount of hyaluronic acid was (0.208) g/L.

Amado, Vázquez (7) showed that the best pH for producing hyaluronic acid from *S. zooepidemicus* by using agricultural residues molasses and corn decomposer as alternatives in the production medium was (6.7). The amount of acid produced was (3.48) g/L.

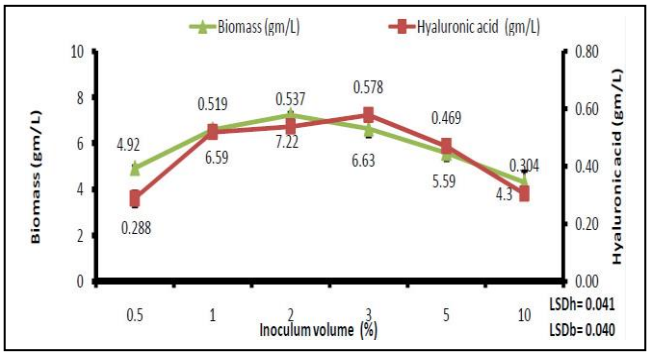

Figure 10. Effect of pH on HA production and biomass

3.10. Effect of Incubation Periods on HA Production

The effect of incubation times of 6, 12, 18, 24, 30, 36, and 48 hours on the production of hyaluronic acid and the amount of biomass formed from the alternative medium using the *S. thermophilus* strain was studied. The results showed that the best production was after 18 hours of fermentation. The amount of hyaluronic acid was 0.598 g/L. The amount of biomass was 6.08 g/L. The amounts of hyaluronic acid produced were 0.305, 0.425, 0.578, 0.277, 0.175, and 0.034 g/L for incubation periods of 6, 12, 24, 30, 36, and 48 hours, respectively (Figure 11).

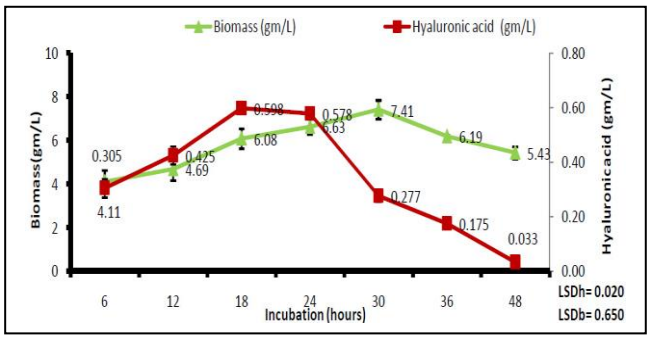

Figure 11. Effect of different incubation times on HA production and biomass

The amount of hyaluronic acid production begins to decrease after 18 hours of the fermentation process due to the depletion of nutrients from the production medium over time and the direction of cells towards the stage of death, which causes an increase in toxins produced in the production medium.

These results agree with the finding of Gedikli, Güngör (8), who studied the effect of incubation times on hyaluronic acid production. The production of hyaluronic acid using *Streptococcus equisimilus* was 0.592 g/L after 18 hours of fermentation. Kumar, Janakiraman (32) studied the optimal conditions for producing hyaluronic acid from *S. equisimilus* and stated that the best incubation period was 28 hours as the amount of hyaluronic acid was 0.68 g/L.

3.11. Effect of Agitation Speed on HA Production

Figure 12 shows the effect of different agitation speeds on HA production and biomass using *S. thermophilus*. The highest amount of hyaluronic acid at a speed of 150 rpm was 0.598 g/L, and the biomass was 6.08 g/L. The amount of hyaluronic acid at the velocity of vibration 0, 100, 200, 300, and 400 rpm was 0.086, 0.438, 0.525, 0.251, and 0.115 g/L, respectively. Most previous studies indicate that the speed of vibration increases the amount of Hyaluronic acid under aerobic and anaerobic conditions (33-35). This is because the speed of vibration works to transfer biomass, prevents its agglomeration, and reduces the viscosity of the

medium due to the production of hyaluronic acid (35). Increasing the high vibration speed reduces the production of hyaluronic acid due to the increase in the percentage of dissolved oxygen in the production medium (36).

The vibration speed of the incubator used to produce hyaluronic acid varied for different microorganisms. Pires, Macedo (12) used a vibration speed of 150 rpm when producing hyaluronic acid from *S. zooepidemicus* using agricultural residues as alternatives in the production medium. The amount of hyaluronic acid was 0.89 g/L. Zakeri and Rasaee (37) found that the best vibration speed for producing hyaluronic acid from *S. zooepidemicus* was 300 rpm. The amount of acid was 5.3 g/L. Sunguroğlu, Sezgin (38) was also able to produce hyaluronic acid using genetically modified *Lactococcuslactis* at a vibration speed of 150 rpm. The amount of hyaluronic acid produced was 6.09 g/L. Duffeck, Pan (39) did not notice any differences in the amount of acid produced when producing hyaluronic acid using *S. zooepidemicus* when using a vibration speed that ranged between 100-300 rpm, which amounted to 2.55 g/L.

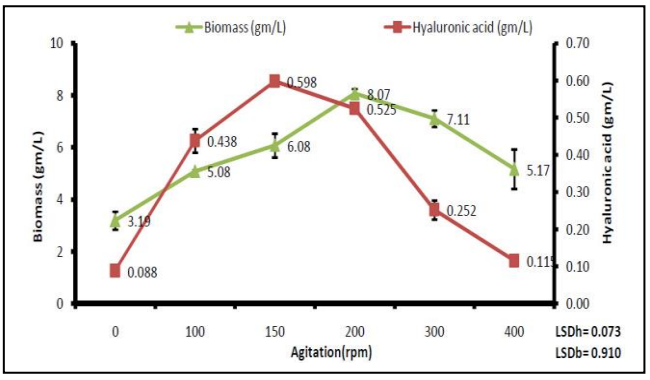

Figure 12. Effect of agitation speed on HA production and biomass

Despite numerous research on GRAS bacteria for the generation of hyaluronic acid (HA) utilizing metabolic techniques, *Streptococcus*, which is regarded as a safe and probiotic bacterium, has received less attention. *S. thermophilus* is a unique bacterium that has been cultural media modified to generate more HA. Then, because the HA synthesis route competed with the glycolytic pathway and cell wall biosynthesis, choosing influential variables for shifting precursors toward HA synthesis impacted the quantity of output. Finally, the highest amount of HA at optimal conditions was 0.598 g/L.

Authors' Contribution

Study concept and design: A. K. N.
Acquisition of data: A. A. M.
Analysis and interpretation of data: A. A. M.
Drafting of the manuscript: A. K. N.
Critical revision of the manuscript for important intellectual content: A. K. N.
Statistical analysis: A. A. M.
Administrative, technical, and material support: A. A. M.

Conflict of Interest

The authors declare that they have no conflict of interest.

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