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Screening and Enhancement of Hyaluronic Acid Production in Yogurt via Modification of Temperature and Nutrient Composition

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HIGHLIGHTS

- Maximum HA production was achieved at 37°C with glucose (15 g/L), yeast extract (20 g/L), urea (0.6 g/L), and lactose (35 g/L).
- LAB isolates derived from sample T15 produced 1.75 times more HA than its baseline level under optimized conditions.
- The optimized yogurt exhibited reduced syneresis and maintained favorable sensory acceptability during storage.
- Temperature and nutrient composition significantly influenced HA production within the yogurt matrix.

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Abbreviations
ANOVA= Analysis of Variance
HA= Hyaluronic Acid
LAB= Lactic Acid Bacteria
MRS= De Man, Rogosa and Sharpe
OFAT= One-Factor-at-a-Time

ABSTRACT

Background: Hyaluronic acid (HA) is a valuable bioactive polysaccharide produced by lactic acid bacteria (LAB) that plays a role in tissue hydration, joint lubrication, and tissue repair.

Methods: Twenty yogurt samples (15 traditional, T1–T15; 5 commercial, C1–C5) were collected from Zanjan Province, Iran, between May and September 2023. LAB were isolated using De Man, Rogosa and Sharpe (MRS) agar and M17 media and identified via Gram staining, cellular morphology, and catalase/oxidase tests. The four samples with the highest HA production (C1, T2, T14, T15) were selected. HA was extracted using trichloroacetic acid (TCA) precipitation and quantified via the carbazole assay. The effects of fermentation temperature, carbon source, and nitrogen source on HA production were evaluated using a One-Factor-at-a-Time (OFAT) approach. Yogurts were assessed for syneresis and sensory characteristics over 21 days of refrigerated storage. Data were analyzed using one-way Analysis of Variance (ANOVA) and Tukey's HSD post-hoc test in IBM SPSS Statistics software (version 20; IBM Corporation, Armonk, NY, USA).

Results: Among the 20 samples, T15 showed the highest initial HA production (0.71 g/L). Optimization identified 37°C, 15 g/L glucose, and 20 g/L yeast extract as the most favorable conditions, increasing HA production by T15 1.75-fold. The optimized yogurt exhibited a 37.2% reduction in syneresis compared with T14 on day 21 and significantly higher sensory scores at several time points ($p < 0.05$).

Conclusion: Optimizing fermentation temperature and nutrient composition significantly enhanced HA production in yogurt while improving water-holding capacity and sensory quality. These findings confirm the feasibility of in situ HA production by LAB for functional dairy products.

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Introduction

Hyaluronic acid (HA) is a naturally occurring, linear, nonsulfated glycosaminoglycan composed of repeating disaccharide units of N-acetylglucosamine and glucuronic acid linked through alternating β-(1→4) and β-(1→3) glycosidic bonds. Its high water-binding capacity and viscoelastic properties are associated with important structural and functional roles in biological systems (Zhai et al., 2020). HA is widely investigated for applications in pharmaceutical, biomedical, cosmetic, and food-related products because of its hydration, rheological, and tissue-associated properties.

Conventional HA production has historically relied on extraction from animal tissues such as rooster combs and bovine tissues. These approaches may involve relatively high downstream purification requirements and concerns related to protein and nucleic acid contamination and the potential transmission of animal-derived contaminants (Cavalcanti et al., 2020; Liu et al., 2023). Microbial production therefore represents an attractive alternative because it can provide a more controlled and non-animal-based production platform.

Among microorganisms capable of producing extracellular polysaccharides, lactic acid bacteria (LAB) are particularly interesting for food applications. Species associated with dairy fermentation, including *Streptococcus thermophilus*, have been investigated for their ability to produce HA and other extracellular polysaccharides (Abbas Mohammed and Niamah, 2022). Their association with fermented foods makes them potentially useful for developing HA-enriched dairy products without relying on the direct addition of purified HA.

Yogurt is a suitable food matrix for investigating microbial HA production because it provides a nutrient-rich environment for LAB growth and is already produced through controlled fermentation. In situ production of HA during yogurt fermentation may therefore provide an alternative strategy for incorporating HA into the final food product while potentially influencing its physicochemical characteristics.

HA production by microorganisms is influenced by environmental and nutritional conditions. Fermentation temperature can affect microbial growth, metabolic activity, and extracellular polysaccharide production. Previous studies on S. thermophilus and other HA-producing microorganisms have demonstrated that temperature is an important determinant of HA yield (Izawa et al., 2010; Abbas Mohammed and Niamah, 2022). Nutritional composition is also important because carbon and nitrogen availability can influence precursor formation, cellular energy metabolism, and biosynthetic activity.

Carbon sources can affect the availability of metabolic intermediates required for polysaccharide biosynthesis. Glucose, for example, can enter central carbohydrate metabolism directly and may therefore efficiently support extracellular polysaccharide production. Nitrogen sources can also affect microbial growth and metabolite production. Complex nitrogen sources such as yeast extract provide amino acids, peptides, vitamins, and other growth factors in addition to nitrogen, potentially supporting microbial growth and metabolic activity.

Although the effects of temperature and nutritional factors on microbial HA production have been investigated in defined culture media, limited information is available regarding the systematic evaluation of these factors in a real yogurt matrix using indigenous LAB. Therefore, the objective of the present study was to screen yogurt samples for high HA-producing LAB and evaluate the effects of fermentation temperature, carbon source, and nitrogen source on HA production in yogurt using a systematic One-Factor-at-a-Time (OFAT) approach. The study also evaluated syneresis and sensory characteristics of the optimized yogurt during refrigerated storage.

Materials and methods

Chemicals and culture media

De Man, Rogosa and Sharpe (MRS) agar and M17 culture media were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemical reagents, including trichloroacetic acid (TCA), ethanol, carbazole, sulfuric acid, $MgCl_2 \cdot 6H_2O$, glucose monohydrate, lactose monohydrate, yeast extract, and urea, were of analytical grade and purchased from Merck KGaA (Darmstadt, Germany). Amphotericin B was purchased from Sigma-Aldrich (St. Louis, MO, USA). All aqueous solutions were prepared using double-distilled water.

Yogurt sample collection and screening

A total of 20 yogurt samples, comprising 15 traditional samples (coded T1–T15) and five commercial samples (coded C1–C5), were aseptically collected from various locations across Zanjan Province, Iran, between May 15 and September 15, 2023. The samples were transported under refrigerated conditions (4 °C) and stored at 4 °C until microbiological and biochemical analyses.

Microbial isolation and preliminary phenotypic characterization

Lactic acid bacteria (LAB) were isolated from the collected yogurt samples. Ten-fold serial dilutions (10^{-1}–10^{-6}) were prepared in sterile 0.85% (w/v) NaCl solution. A 0.1 mL aliquot of the appropriate dilution was

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surface-spread onto MRS agar for the isolation of lactobacilli-like bacteria and M17 agar for the isolation of streptococci-like bacteria. The plates were incubated under 5% CO_2 at 37 °C for 48–72 h (Sterr, Weiss and Schmidt, 2009). Colonies with distinct morphological characteristics were selected and purified by repeated streaking for at least three cycles. The purified isolates were preserved at −80 °C in 20% (v/v) glycerol (Hajimohammadi Farimani et al., 2016).

The isolates were preliminarily characterized by Gram staining, cell morphology, and catalase and oxidase tests. Putative LAB isolates were Gram-positive and catalase-negative, with coccoid or rod-shaped morphology. Because MRS agar and M17 are selective or semiselective rather than species-specific media, the isolates were considered presumptive LAB based on their culture characteristics and phenotypic properties. Most isolates recovered on M17 agar showed coccoid morphology, whereas rod-shaped bacteria predominated among isolates recovered on MRS agar (Schillinger and Holzapfel, 2011). Fungal contaminants, including yeasts and molds, were inhibited by the addition of amphotericin B to the culture media, which facilitated the isolation and purification of LAB (Gemelas et al., 2013).

Screening of high-ha-producing yogurt samples

The LAB isolates obtained from the collected yogurt samples were screened for their ability to produce HA. Based on the HA production results, four yogurt samples showing the highest HA production were selected for subsequent experiments: C1, T2, T14, and T15. These codes refer to the original yogurt samples from which the selected LAB isolates were obtained.

Extraction and quantification of HA

HA was extracted and quantified using a modified precipitation-based procedure based on previously described HA extraction and purification strategies (Rodriguez-Marquez et al. 2022; Callejas-Quijada et al., 2023) and extracellular polysaccharide recovery from fermented milk (Wa et al., 2022). Briefly, proteins were precipitated from yogurt samples on ice using 20% (w/v) trichloroacetic acid (TCA). The resulting supernatant was subjected to cold ethanol precipitation at 4 °C for 12 h. The recovered polysaccharide pellet was redissolved in distilled water and subjected to a second TCA treatment to reduce residual contaminants, followed by extensive washing.

HA concentration was determined spectrophotometrically using the carbazole-sulfuric acid method described by Bitter and Muir (1962). A standard calibration curve was prepared using HA concentrations ranging from 0.1 to 1.0 mg/mL, with a coefficient of determination (R^2) of 0.9951.

Yogurt production and fermentation conditions

Sterilized skimmed milk (Mihan Dairy Co., Tehran, Iran) was used as the fermentation medium. The milk was heat-treated at 92 °C for 5 min to promote whey protein denaturation and subsequently cooled to 42 °C before inoculation.

LAB cultures obtained from the selected high-HA-producing yogurt samples were used for the fermentation experiments. The cultures were inoculated into the sterilized skimmed milk at a concentration of 5% (v/v). Fermentation was continued until the pH reached approximately 4.4, after which the yogurt samples were immediately cooled to 4 °C.

Following the screening experiments, the selected yogurt sample showing the highest HA production was used for the optimization experiments. A systematic OFAT approach was applied to evaluate the effects of fermentation temperature and nutrient composition on HA production. The optimized conditions consisted of an incubation temperature of 37 °C, glucose concentration of 15 g/L, and yeast extract concentration of 20 g/L. These conditions were subsequently applied together to produce the optimized yogurt formulation.

A commercially available yogurt was used as the control sample. The control product was purchased from the local market and represented yogurt manufactured under routine industrial production conditions.

Experimental design and fermentation optimization

A systematic OFAT approach was used to evaluate the effects of fermentation temperature and nutrient composition on HA production by the selected LAB cultures. The factors investigated were incubation temperature (30, 37, and 42 °C), carbon source (glucose at 5, 10, and 15 g/L and lactose at 25, 30, and 35 g/L), and nitrogen source (yeast extract at 10, 15, and 20 g/L and urea at 0.4, 0.6, and 0.8 g/L).

Each factor was evaluated independently while the other experimental conditions were kept constant. The optimal level of each factor was selected based on the highest HA production. The selected optimal conditions (37 °C, 15 g/L glucose, and 20 g/L yeast extract) were subsequently applied together to produce the optimized yogurt. Each experimental condition was performed in triplicate (n = 3), and HA concentration was determined as described above.

Syneresis

Syneresis was determined for the selected yogurt samples (C1, T2, T14, and T15) and the optimized yogurt during refrigerated storage at 4 °C on days 1,4, 7, 14, and 21. Syneresis was measured according to the centrifugation method described by Amatayakul, Sherkat and Shah (2006), with minor modifications. Briefly, 5 g of yogurt

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sample was centrifuged at 1500 × g for 10 min at 4 °C. The separated whey was carefully collected and weighed, and syneresis was calculated as the percentage of whey released relative to the initial sample weight.

Syneresis (%) was calculated as follows:

Syneresis (%) = (Weight of separated whey / Initial weight of yogurt sample) × 100

All measurements were performed in triplicate, and the results were expressed as mean ± standard deviation.

Sensory evaluation

Sensory evaluation was performed using a panel of 12 trained assessors. Training focused on the sensory characteristics of yogurt, including aroma, taste, texture, mouthfeel, and overall acceptability. Each sensory attribute was initially scored using a 9-point hedonic scale, ranging from 1 (dislike extremely) to 9 (like extremely).

Sensory evaluation was performed on days 1, 7, 14, and 21 during refrigerated storage at 4 °C. The evaluated attributes included aroma, taste, texture, mouthfeel, and overall acceptability. Sensory testing was conducted under controlled lighting conditions to minimize the influence of external factors.

Statistical analysis

All experimental data are presented as mean ± standard deviation based on three independent replicates (n = 3). One-way analysis of variance (ANOVA) was performed separately for each experimental factor to determine whether significant differences existed among the tested levels of temperature, carbon source, and nitrogen source. When the ANOVA indicated a significant difference ($p \leq 0.05$), Tukey's HSD post hoc test was performed for pairwise comparisons. All statistical analyses were performed using IBM SPSS Statistics version 20 (IBM Corporation, Armonk, NY, USA).

Results and discussion

Initial screening of ha-producing strains in yogurt samples

The initial screening of 20 yogurt samples identified four samples with the highest HA production levels: T2, T14, T15, and the commercial sample C1. Their respective HA concentrations were 0.56, 0.42, 0.71, and 0.51 g/L (Figure 1). Statistical analysis revealed significant differences in HA production among the yogurt samples ($p < 0.05$), indicating variation in their HA-producing potential. Sample T15 exhibited the highest HA concentration (0.71 g/L), followed by T2 (0.56 g/L), C1 (0.51 g/L), and T14 (0.42 g/L).

Previous studies have also demonstrated that HA production varies depending on the microorganism and fermentation conditions. Abbas Mohammed and Niamah (2022) reported a maximum HA concentration of 0.598 g/L using *Streptococcus thermophilus* under optimized fermentation conditions. The concentration observed in sample T15 during the initial screening was higher than this reported value. However, a direct comparison should be made cautiously because differences in microbial composition, fermentation media, cultivation conditions, and analytical methods may influence the measured HA concentrations. In addition, Pan et al. (2014) reported that the nitrogen source affected HA production, with concentrations of 0.534, 0.192, and 0.063 g/L obtained using yeast extract, soybean protein, and whey protein, respectively. These findings highlight the importance of nutrient composition in HA production and support the investigation of nutrient sources during subsequent optimization. The variation observed among the yogurt samples in the present study may be associated with differences in their microbial populations and matrix composition; however, these explanations remain hypothetical because molecular identification of the bacterial isolates was not performed. Based on the screening results, T15 was selected as the most promising sample for further optimization of HA production.

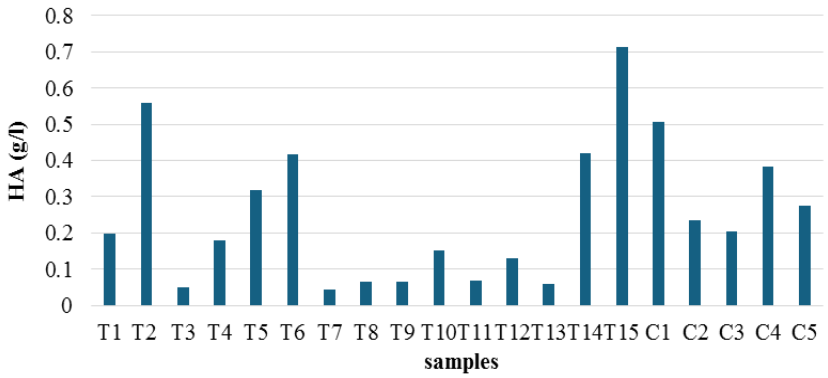


Figure 1: Initial HA content in yogurt samples

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Effect of temperature on HA biosynthesis

Temperature played a significant role in HA production. The highest HA yield was achieved with sample T15 at 37 °C, reaching 2.23 ± 0.01 g/L. Upon increasing the temperature to 42 °C, a marked decrease in HA production was observed across all samples ($p < 0.05$) (Figure 2). This indicates that the optimal temperature for HA production by the selected indigenous LAB strain is 37 °C. Identifying this as the optimal fermentation temperature aligns closely with previous reports regarding *Streptococcus thermophilus*. Izawa et al. (2010) identified the 33–40 °C range as suitable for HA production, while Girgin Öz and Öğüt (2025), comparing temperatures of 32 °C, 37 °C, and 40 °C, determined 37 °C to be the optimum for achieving maximum HA yield. Furthermore, these results support our hypothesis that this temperature range strikes an appropriate balance between microbial growth dynamics and the preservation of maximal hyaluronan synthase activity, avoiding thermal denaturation or the induction of hydrolytic enzymes (that occur at 42 °C and above). Indeed, the significantly lower HA production at 42 °C is likely attributable to accelerated proteolytic or glycosidic degradation; this phenomenon aligns with existing reports indicating that while increasing the temperature above 43 °C enhances biomass, it disrupts polysaccharide integrity (Liu et al., 2023).

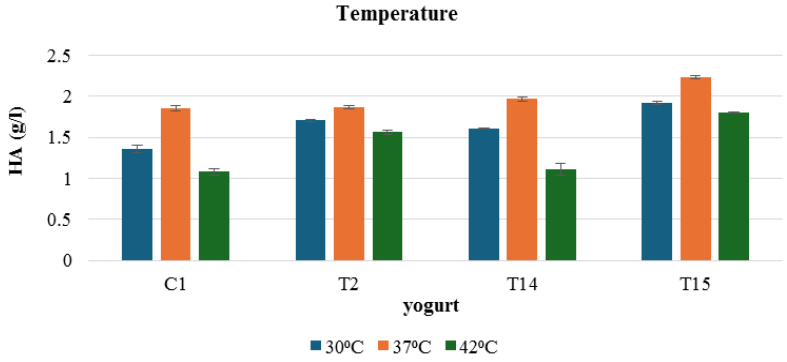


Figure 2: Effect of temperature on HA production by LAB

Effect of carbon source on HA yield

-Glucose

Compared to the control sample (without supplementation), glucose significantly increased HA production ($p < 0.05$). The highest yield was achieved at a concentration of 15 g/L, peaking at 2.38 g/L in sample T15—the highest production level observed under the tested conditions. In this series, the lowest production was recorded for sample C1 at a concentration of 5 g/L (1.61 g/L). At a concentration of 15 g/L, glucose resulted in HA production levels of 2.007, 2.11, and 1.74 g/L for samples C1, T14, and T2, respectively (Figure 3).

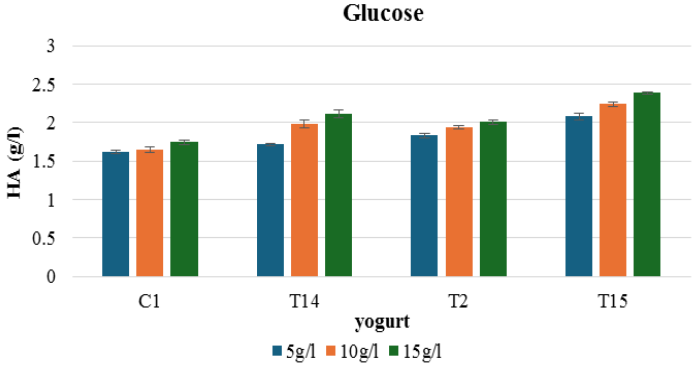


Figure 3: Effect of glucose on HA production

-Lactose

Lactose also supported HA biosynthesis, with the highest production observed at a concentration of 35 g/L; at this level, LAB isolates derived from sample T15 produced 2.28 g/L of HA. Under these conditions, HA production increased relative to the control group by 1.1-fold (for C1), 1.5-fold (for T14), 1.3-fold (for T2), and 1.6-

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fold (for T15). However, at equivalent concentrations, the amount of HA produced with lactose was lower than with glucose; this is likely due to the additional ATP cost and enzymatic steps associated with lactose hydrolysis and its entry into central carbon metabolism (Figure 4).

Under the conditions tested in this study, glucose proved to be a more efficient carbon source than lactose for HA production. At their respective optimal concentrations, LAB isolates derived from sample T15 produced 2.38 g/L of HA using 15 g/L of glucose and 2.28 g/L of HA using 35 g/L of lactose. The higher HA yield with glucose may be attributed to its direct entry into central carbohydrate metabolism—a process that supplies the carbon intermediates required for the biosynthesis of HA precursors, such as UDP-glucuronic acid (UDP-GlcUA) and UDP-N-acetylglucosamine (UDP-GlcNAc). In contrast, lactose must first be broken down (metabolized) into its constituent monosaccharides before entering central metabolic pathways. Previous studies have also demonstrated the importance of glucose concentration and carbon metabolism in HA production by the bacterium *Streptococcus zooepidemicus* (Pires et al., 2010). However, since glucose and lactose were evaluated across different concentration ranges in this study, the relative superiority of glucose should be interpreted based on the optimal conditions for each, rather than as a direct comparison at equal concentrations.

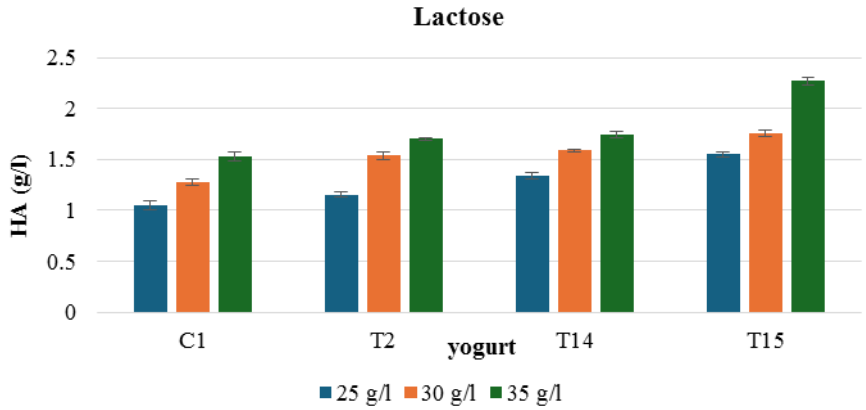


Figure 4: Effect of lactose on HA production

Effect of nitrogen source on HA production

-Urea

Urea served as an effective nitrogen source up to a concentration of 0.6 g/L, beyond which HA production declined. The highest HA concentrations obtained with 0.6 g/L urea for strains C1, T14, T2, and T15 were 1.64, 1.79, 1.73, and 1.66 g/L, respectively (Figure 5). Although urea increased HA production compared to the control, its effect was less pronounced than that observed with yeast extract.

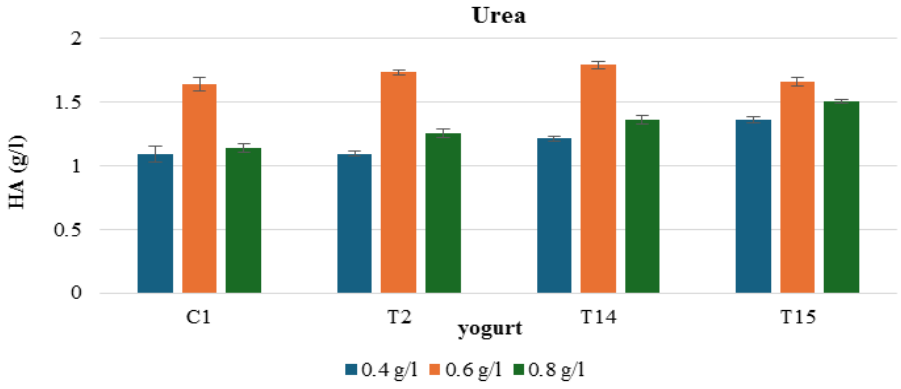


Figure 5: Effect of urea on HA production

-Yeast extract

Yeast extract was the most effective nitrogen source, and HA production increased steadily as the yeast extract concentration rose to 20 g/L. At this concentration, sample T15 achieved the highest HA concentration (2.48 ± 0.02 g/L), followed by samples T14 (1.64 ± 0.03 g/L), C1 (1.65 ± 0.03 g/L), and T2 (1.51 ± 0.01 g/L). The HA concentration obtained with 20 g/L of yeast extract was significantly higher than the values obtained at concentrations of 10 and 15 g/L ($p < 0.05$). The positive effect of yeast extract can be

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attributed to its complex nutritional composition (including amino acids, peptides, vitamins, and other growth-promoting factors) which supports microbial metabolism and biosynthetic activity. These nutrients may enhance cell growth and facilitate the synthesis of metabolic precursors required for HA production.

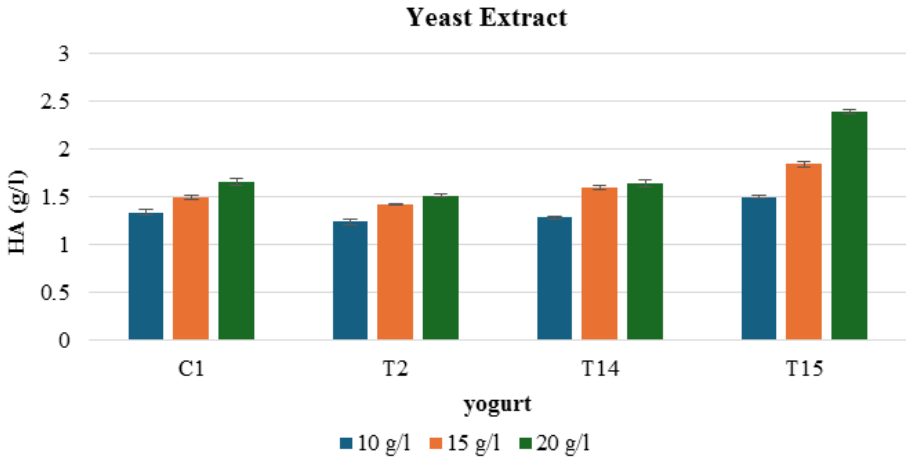


Figure 6: Effect of yeast extract on HA production

Table 1 presents the optimal performance for each factor. It is noteworthy that T15 achieved the highest HA yield under all examined conditions; the sole exception occurred in the presence of urea, where T14 performed slightly better.

Based on the OFAT experimental design approach employed in this study, the optimal conditions for HA production were determined to be a temperature of 37 °C, with concentrations of 15 g/L glucose, 20 g/L yeast extract, 35 g/L lactose, and 0.6 g/L urea. These findings indicate that optimizing temperature and nutrient composition can enhance HA production while simultaneously yielding a final yogurt product with desirable physicochemical and sensory characteristics.

Table 1: Optimal amount of HA production

Factor	HA (g/l)
Temperature (37°C)	2.23
Glucose (15g/L)	2.38
Yeast extract (20g/L)	2.48
Urea (0.6g/L)	1.79
Lactose (35g/L)	2.28

The potent stimulatory effect of yeast extract (resulting in an average HA yield approximately 40% higher than that achieved with urea (1.06 vs. 0.77 g/L)) is attributed to its rich composition of amino acids, nucleotides, and B-group vitamins; all of which play a role in the HA synthesis pathway, an energy-intensive process. Although urea serves as a nitrogen source, it lacks the complex micronutrients essential for coenzyme synthesis and nucleotide salvage pathways (required for UDP-sugar synthesis). This result aligns with the findings of Lee and Ha (2008), who observed a significant increase in HA production in *Streptococcus zooepidemicus* cultures upon the addition of yeast extract at a concentration of 2.5 g/L. Similarly, Pires and Santana (2010) demonstrated that complex nitrogen sources (such as hydrolyzed proteins) outperform their inorganic counterparts when combined with glucose, further underscoring the necessity of using nutrient-rich carbon sources to achieve maximum polymer yield.

Interaction effects between temperature and nutrient sources

Since the OFAT approach was employed in the experimental design, interactions between temperature, carbon source, and nitrogen source were not statistically evaluated; consequently, no specific conclusions regarding these interactions could be drawn in this study. Based on individual optimization experiments, a temperature of 37 °C and concentrations of 15 g/L glucose, 20 g/L yeast extract, 35 g/L lactose, and 0.6 g/L urea were determined as the optimal conditions for HA production. When these conditions were applied simultaneously, the highest HA concentration was observed in sample T15, reaching 2.8 ± 0.02 g/L. The optimized conditions also resulted in improved yogurt characteristics, including reduced syneresis and enhanced sensory acceptance. These findings indicate that optimizing temperature and nutrient composition can increase HA production while simultaneously achieving desirable physicochemical and sensory properties in the resulting yogurt.

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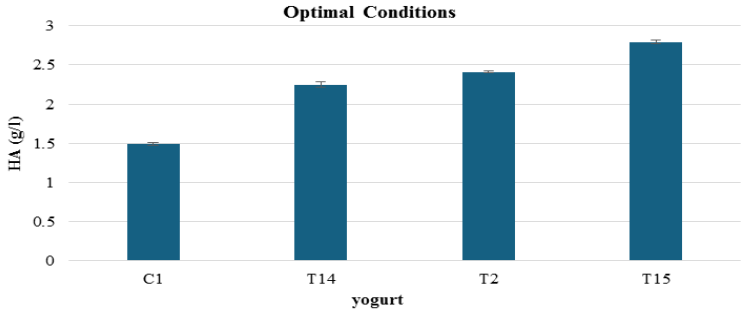


Figure 7: Interaction of temperature, nitrogen, and carbon sources on HA production

Syneresis

The phenomenon of syneresis (whey separation) was evaluated during refrigerated storage on days 1, 7, 14, and 21. On day 21, the optimized yogurt exhibited significantly lower syneresis compared to sample T14 (16.04% versus 26.02%). These findings indicate that the optimized formulation possessed a superior capacity for retaining whey during prolonged refrigerated storage compared to T14.

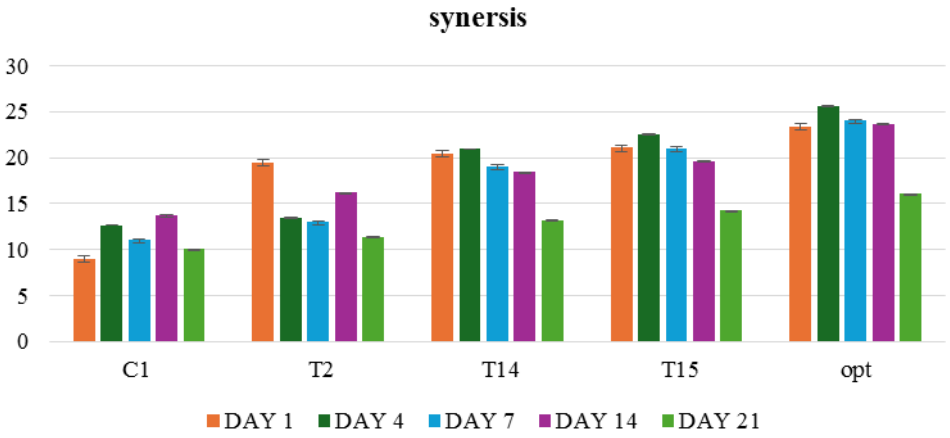


Figure 8: Syneresis (%) of yogurt samples during 21 days of storage

It is noteworthy that the functional value of endogenously produced HA extends beyond its mere quantitative yield. The significantly lower degree of syneresis observed in the optimized formulation (16.04% on day 21) compared to sample T14 (26.02% on day 21) suggests that HA likely contributed to improved water retention and the stabilization of the yogurt gel network. This effect can be attributed to interactions between the hydrophilic functional groups of HA (specifically carboxyl and hydroxyl groups) and the components of the casein protein network; such interactions can facilitate water stabilization and entrapment while enhancing structural integrity. In this regard, the functionality of HA is somewhat comparable to that of hydrocolloids like xanthan gum and beta-glucan, which are commonly employed to improve water-holding capacity and gel stability. However, unlike exogenously added hydrocolloids, the in situ production of HA by fermenting microorganisms offers added functional value without the need for separate hydrocolloid supplementation. Nevertheless, the proposed interactions between HA and casein should be viewed as a potential mechanism rather than a directly proven molecular interaction, as such interactions were not experimentally investigated or precisely characterized in the present study.

Sensory evaluation

Sensory evaluation of yogurt samples was conducted by 12 trained panelists on days 1, 4, 7, 14, and 21 of refrigerated storage at 4 °C. The panelists independently evaluated five sensory attributes: odor, texture, taste, mouthfeel, and overall acceptability. The mean score for each attribute was calculated across the 12 panelists. The sensory scores were analyzed to compare the sensory characteristics of the yogurt samples during refrigerated storage.

The scoring system used a 9-point hedonic scale, ranging from “liked very much” to “did not like at all.” Assessed sensory properties included aroma (superficial, salty, cooked, sweet, aromatic, and sour), texture (uniformity, texture, and stretch), flavor (intensity, sweetness, salty, sour, bitter, cooked, astringent, and

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pungent), mouthfeel (smooth, creamy, and custard-like), and overall acceptability.

To further evaluate the changes in sensory attributes during storage, detailed heat maps for each product are presented in Figure 10. The traditional yogurt samples (T2, T14, and T15) generally received higher scores for positive aroma attributes, such as aroma and sweet aroma, and lower scores for negative attributes, including salty and cooked aromas, compared with the commercial product (C1). Texture-related parameters, including uniformity, wateriness, and elasticity, remained relatively more consistent in the traditional samples, suggesting greater structural stability during storage. The traditional samples also showed higher scores for taste-related attributes, particularly sweetness, together with lower scores for sourness and bitterness. The most pronounced differences were observed in overall acceptability. The traditional samples maintained relatively high acceptability scores throughout the storage period, whereas the commercial sample showed a noticeable decline in overall acceptability by day 21.

These sensory findings are consistent with previous reports indicating that HA-enriched yogurts can exhibit favorable organoleptic properties without adverse effects on rheological characteristics (Hamad et al., 2017). More recently, Li et al. (2025) investigated the effects of different HA concentrations on the physicochemical, rheological, and organoleptic properties of yogurt and reported that 0.5% HA improved water-holding capacity, viscosity, hardness, and the continuity and uniformity of the yogurt structure. However, the effects of HA were concentration-dependent, as some lower concentrations did not produce the same beneficial effects. In the present study, the HA concentration in the optimized yogurt was 2.8 g/L, which was lower than the highest concentration evaluated by Li et al. (2025). Nevertheless, the improved sensory acceptability observed in the optimized yogurt is consistent with previous findings indicating that HA can be incorporated into fermented dairy systems without necessarily causing adverse sensory effects. These findings suggest that the HA concentration achieved under the conditions of the present study was compatible with the sensory characteristics of yogurt.

Overall, T14 showed higher sensory scores than T15 on days 1, 7, and 14, whereas T15 slightly exceeded T14 on day 21 (Figure 9). This finding indicates that the sensory superiority of T14 was evident during most of the storage period but was not maintained at the end of storage.

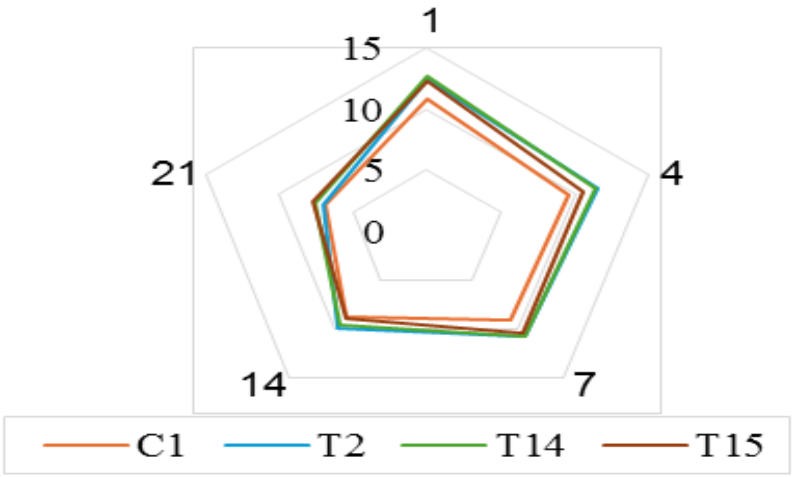


Figure 9: Sensory evaluation of optimal yogurts

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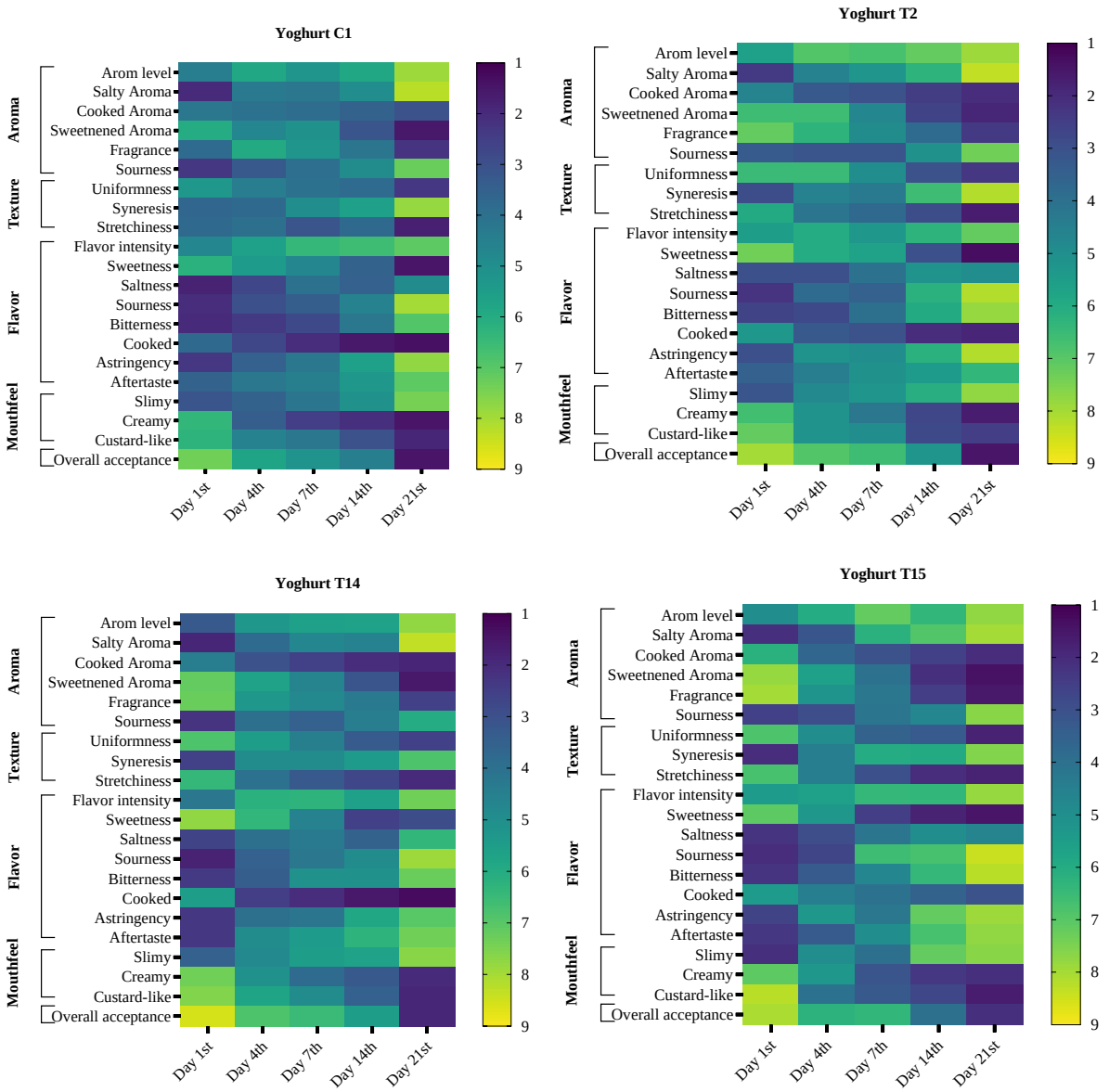


Figure 10: Sensory properties heat map

Conclusion

This study demonstrated the potential of indigenous LAB to enhance the in situ production of HA in yogurt by optimizing temperature and nutritional conditions. Using the OFAT method, the optimal conditions identified among those tested were a temperature of 37 °C and concentrations of 15 g/L glucose, 20 g/L yeast extract, 35 g/L lactose, and 0.6 g/L urea. Under these conditions, sample T15 achieved the highest HA concentration during the optimization experiments (2.8 ± 0.02 g/L), representing a 3.92-fold increase over the baseline value of 0.71 g/L.

The optimized yogurt also exhibited improved physicochemical and sensory characteristics. Notably, the syneresis level of the optimized yogurt on day 21 was 16.04%, compared to 26.02% for sample T14—a difference representing a reduction of approximately 38.3%. The optimized yogurt also demonstrated superior sensory acceptability during refrigerated storage. Since the OFAT design was employed in this study, interactions between the tested factors were not statistically evaluated; therefore, the observed improvements should be interpreted as the result of the selected optimal conditions rather than as proven synergistic effects. Overall, these findings confirm the potential of in situ HA production as an approach for manufacturing a functional yogurt with higher HA content and desirable physicochemical and sensory characteristics.

Limitations and Suggestions for Future Studies

This study was conducted with some limitations in mind. Identification of strains at the molecular level and

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determination of the molecular weight of HA produced were not performed. It is suggested that future studies use methods such as 16S rRNA sequencing to confirm the identity of strains and SEC-MALS chromatography to determine molecular weight and its distribution. The findings of this research have high commercialization potential. Adding indigenous HA-producing strains to yogurt starter can lead to the production of products with “natural moisturizing” and “hybrid probiotic” properties. Such dairy products can be considered as a functional option in health-oriented markets. From an industrial perspective, this technology does not require new equipment and can be implemented only by carefully adjusting fermentation parameters and providing appropriate nutritional resources. In the future, it is suggested to determine the molecular weight of the produced HA and investigate its effect on rheological properties, long-term stability, and skin anti-aging properties. Also, the development of semi-continuous models and the use of enzymatic pretreatments can increase production efficiency.

Author contributions

M.M. and A.S. designed and performed the work; A.S. supervised the research, reviewed the manuscript, and edited it; A.S. and M.M. accomplished the data analysis and wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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Ethical consideration

The sensory panel was provided with all the necessary information regarding the evaluation process, ensuring that they could make informed decisions in accordance with both ethical standards and legal guidelines.

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