Enhanced Hyaluronic Acid Production by a Mutant Strain, 3523-7 of *Streptococcus zooepidemicus*

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Abstract

Hyaluronic acid (HA) is a hydrated gel and comprises repeating units of glucuronic acid and N-acetylglucosamine. HA has been of considerable interest due to its clinical application. This study reports the optimization of fermentation conditions for HA production by *Streptococcus zooepidemicus* 3523-7, a mutant derived from MTCC 3523. In shake flask fermentation, CDM containing 2% glucose and 1.5% yeast extract at pH 7.2 and temperature 36°C with 1% inoculums favored maximum HA production by the strain 3523-7. Enhanced HA production (1.89 g/l) by 3534-7 was seen when the fermentation was carried out in a 10 L bioreactor under optimal conditions such as 400 rpm and aeration 0.6 vvm.

Key words: Hyaluronic acid production, fed-batch fermentation, *Streptococcus zooepidemicus* mutant strain 3523-7.

Introduction

Hyaluronic acid (HA) is a mucopolysaccharide, ubiquitously expressed in human and animal tissues as hydrated gel, which comprises repeating units of glucuronic acid and N-acetyl glucosamine (17, 20). It is widely used in biomedical, healthcare, food and cosmetics because of its unique physicochemical properties such as hydrophobicity, lubrication and biocompatibility (7). Many clinical applications of HA however, depends on its molecular size and there are several studies that focus on this aspect. HA has been conventionally extracted from animal tissues such as rooster combs and bovine vitreous humor (18). Since the isolation of HA from animal sources is economically difficult, technology has been developed to produce HA from microbial source through fermentation (13). HA is synthesized by many strains of group A and C *Streptococci* (1, 5, 14, 15, and 17) and number of separation procedures such as protease digestion, HA ion-pair precipitation (with e.g., acetyl-pyridinium chloride), membrane ultrafiltration, HA non-solvent precipitation and lyophilization (8, 9, 10, 12, and 16) have been employed to obtain a highly pure HA suitable for clinical application. Recently Patil et al (14) have shown that *S. equi* subsp. *zooepidemicus* MTCC 3523 is capable of producing exopolysaccharide, HA. The present study describes optimal conditions for enhanced production of HA by a mutant strain 3523-7 derived from *S. zooepidemicus* MTCC 3523 using a chemically defined medium.

Materials and Methods

**Bacteria and media:** A spontaneous mutant, 3523-7 derived from *Streptococcus equi* subsp.
zooepidemicus MTCC 3523 (obtained from Institute of Microbial Technology, Chandigarh, India) was defective in pathogenic factor, produced more than tenfold higher HA compared to its parent strain in shake flask fermentation (data not shown). Both the strains 3523 and its derivative 3523-7 used in this study were maintained as freeze dried cultures and stored at 4°C. Bacteria were sub cultured and grown as described by John et al. (4). One percent inoculum was used to propagate S. zooepidemicus in Todd Hewitt broth (THB), Brain heart infusion broth (BHI), Veal infusion broth (VIB) as recommended by the manufacturer (Difco) or a chemically defined medium (CDM, 19) containing carbon and nitrogen sources to determine the optimal media for cultivation.

HA production in shake flask fermentation: A multifactor experiment was carried out to optimize conditions for production of HA by the strain 3523-7 of S. zooepidemicus. Batch culture experiments were performed aerobically in 500 ml flask with a working volume of 100 ml for optimizing various conditions of pH (6.0-8.0), temperature (30-40°C), glucose (10-60 g/l), agitation (200-300 rpm) and harvesting time (16-24 h). The pH of the culture media was maintained by adding sterile 5 M sodium hydroxide for every 3-5 h (1). Different experiments were conducted to investigate the optimal concentrations of glucose, yeast extract, percentage of inoculum and favorable culture conditions to enhance hyaluronic acid yield.

HA production in fermenter: HA fermentation experiments were performed in 10 L fermenter (Scigenics, India) with a working volume of 4 L. Agitation was provided by three, four-bladed turbines, the pH of culture media was maintained as 7.2 ± 0.2 by automatic addition of 5 N sodium hydroxide and temperature at 36°C. As for the established conditions in shake flask experiments, and maintaining initial glucose concentration of 20 g/l by adding glucose from the stock (220 g/l) from the 8-10th h onwards. The impeller speed (300-800 rpm) and aeration (0.2 to 1.8 vvm) were optimized in the bioreactor for defined culture conditions.

Cell growth: The growth of the culture was determined by measuring optical density (OD) at A_{530} nm (1) with UV-Visible Spectrophotometer (Labomed Inc, USA) by using the medium without inoculation as reference blank. The culture samples were diluted with distilled water to give less than 1 OD at A_{530} nm.

Isolation and purification of hyaluronic acid: HA produced by S. zooepidemicus in fermentation broth was purified as described in literature (2) and some modifications in purification steps as indicated were done to improve the recovery. Purity of HA present in fermented broth during the growth and fermentation was estimated by the carbazole method (2). The fermentation broth containing HA was precipitated by addition of isopropyl alcohol (19). The precipitated HA was redissolved in 0.15 M sodium chloride solution. This solution was treated with activated charcoal (0.5-2%), and stirred for 1 h followed by centrifugation at 7000 rpm for 30 min at 4°C.

After centrifugation, HA solution was passed through 0.45 μ filters (Millipore, USA). The filtered HA solution was further purified by ultrafiltration in diafiltration mode after two dilutions with pyrogen-free water. Finally the retentate containing HA sample was concentrated to original volume. The concentrated HA solution was precipitated with isopropyl alcohol and vacuum (Biotron, Korea) dried.
**Statistical analysis**: Data were expressed as mean ± SD obtained from at least three independent experiments. Statistical significance of the obtained results was verified by Student's t-test and one way ANOVA using a commercial package (Sigma Plot 5.05). $p < 0.05$ in comparisons to controls was considered as significant.

**Results and Discussion**

Several studies have reported the optimum culture conditions for exopolysaccharide, HA production in batch mode (1, 3, 13, 14, and 17). Patil et al (14) have recently reported HA production by *S. zooepidemicus* MTCC 3523 and the yields were low. The present study reports the optimal conditions for efficient production of HA by a mutant strain, 3523-7 derived from *S. zooepidemicus* MTCC 3523 in a chemically defined medium (CDM). Effect of culture variables like pH, temperature, agitation rate and aeration, and CDM containing various media additives on HA production were also studied. HA production in shake flasks has been scaled up in 10 L bioreactor for enhancing HA production.

**Hyaluronic acid production**: The glucose concentration in the media for growing the organism might play a role in HA production. Initially, effects of glucose concentration in CDM on HA production in shake flask studies were compared. Maximum HA production (444 ± 11.3 mg/l) was obtained at 2% glucose (Fig. 1) and increasing concentration reduced HA production. Glucose molecules are converted into phosphohexoses such as glucose-6-phosphate and fructose-6-phosphate and further utilized for the synthesis of D-glucuronic acid and N-acetyl glucosamine moieties of HA (10). HA production of 506 ± 14.1 mg/l was obtained with 1.5% of yeast extract and further increase had no significant effect on HA production. Similarly adding 1% inoculum resulted in HA production of 605 ± 12.7 mg/l (Fig. 1) but no significant increase in HA was seen upon increasing inoculum size.

![Effect of initial glucose (Dextrose, Dex) concentration, yeast extract (YE) and inoculum (Inc) size on hyaluronic acid production by *S. zooepidemicus* 3523-7 in shake flasks.](image)

**Effect of culture conditions on hyaluronic acid production**: Since, the pH and temperature are the critical factors that play a major role in cell growth and favor the organism to produce the metabolites, the effect of pH and temperature on culture condition of *S. zooepidemicus* 3523-7 was compared for production of HA. The culture pH exerted a considerable influence on cell growth and HA yields (4). The maximum HA production (834 ± 10.6 mg/l) was obtained at pH 7.2 (Fig. 2A). This is inconsistent with that of Patil et al. (14) that they have reported the optimum pH as 7.5 for HA production. Further change in culture pH reduced HA production which is consistent with that of Johns et al., (4) as they have observed similar decline of HA yield at pH 6.0 and pH 7.9. Experiments were performed by varying the culture incubation temperature and this resulted in maximum cell growth and HA production (863 ± 3.5 mg/l) at 36 °C (Figure. 2B). HA production was less at lower temperatures and this suggest that this effect is result of a reduced growth rate of *S. zooepidemicus* 3523-7 at other temperatures (1).

_Hyaluronic Acid from *Streptococcus zooepidemicus*_
Furthermore, the effect of aeration on HA production was compared and HA production was affected by different levels of agitation (200-300 rpm) in the shake flask cultures. Maximum HA production was achieved (939 ± 9.9 mg/l) when the agitation speed was at 250 rpm (Fig. 3A). Growth time also had a profound influence on HA production and maximum (990 ± 7 mg/l) was observed at 20th of fermentation (Fig. 3B).

**Effect of media on hyaluronic acid production:**

In order to determine the influence of the media on HA production under optimal conditions, *S. zooepidemicus* 3523-7 was grown in different media such as THB, BHI, VIB and CDM (Table 1) and HA production was compared. Maximum HA production was observed in CDM followed by THB, BHI and VIB. In CDM more HA production was seen compared to THB and BHI. In addition, HA production at various time intervals was also studied and maximum HA production was seen at 24 h of fermentation in CDM and HA concentration decreased beyond 24 hrs (Table 1) in all media possibly due to degradation.

**Fig. 2A.** Effect of pH on hyaluronic acid production by *S. zooepidemicus* 3523-7 in shake flasks. The change in hyaluronic acid yield (- ● -) along with cell growth indicated by OD$_{530}$ (- ■ -) are shown in line graph. **B.** Effect of temperature on hyaluronic acid production by *S. zooepidemicus* 3523-7 in shake flasks. The change in hyaluronic acid yield (- ● -) along with cell growth indicated by OD$_{530}$ (- ■ -) are shown in line graph.

**Fig. 3A.** Effect of agitation (rpm) on hyaluronic acid production by *S. zooepidemicus* 3523-7 in shake flasks. The change in hyaluronic acid yield (- ● -) along with cell growth indicated by OD$_{530}$ (- ■ -) are shown in line graph. **B.** Effect of growth on hyaluronic acid production by *S. zooepidemicus* 3523-7 in shake flasks. The change in hyaluronic acid yield (- ● -) along with cell growth indicated by OD$_{530}$ (- ■ -) are shown in line graph.
Enhanced hyaluronic production by *S. zooepidemicus* 3523-7 in fermenter: As we have obtained maximum HA production in CDM, fermentation process under optimal conditions was carried out in 10 L fermenter with CDM to enhance HA production by *S. zooepidemicus* 3523-7. Earlier studies have reported that aeration in bioreactor favors the growth of the organism as well as higher HA production compared to anaerobic fermentation (6). Although there are several studies reporting the use of aeration for increasing the yield in hyaluronic acid fermentations, the described aeration and agitation conditions are vague. The need for vigorous agitation is found to enhance oxygen transfer, yet the polymer chain is reportedly susceptible to mechanical stress (3). Very high agitation speeds may be deleterious to HA quality, since a high shear rate may also damage the polymer (4). In present study, maximum HA production of $1.82 \pm 0.098 \text{ g/l}$ (Fig. 4) was attained at an agitation rate of 400 rpm. Further, increase of the agitation speed from 400 to 800 rpm decreases cell growth and HA production that coincides with earlier reports which observed reduced HA production at high agitation speed (6). The cultures in 10 L bioreactor with aeration of 0.6 vvm resulted in HA production of $1.89 \pm 0.042 \text{ g/l}$ (Fig. 5). This higher HA concentration in aerated culture is probably due to the superior energy yield obtained by the use of molecular oxygen to oxidize nicotinamide metabolites and the diversion of pyruvate to acetate, rather than lactate (4, 11).

**Conclusion**

A spontaneous mutant, 3523-7 derived from *Streptococcus equi* subsp. *zooepidemicus* MTCC 3523 produced more than tenfold higher HA compared to its parent strain (data not shown).
shown). Therefore, strain 3523-7 was used throughout our studies to optimize fermentation condition for HA production. The present study optimized the media composition and growth conditions of 3523-7 for HA production in shake flask fermentation. CDM containing 2% glucose, 1.5% yeast extract at pH 7.2, temperature 36°C and 1% inoculums seem to favour HA production by the strain 3523-7. Further, we have optimized fermentation conditions (400 rpm and 0.6 vvm) in a 10 L fermenter for 3523-7 to obtain maximum HA production.

References


Hyaluronic Acid from Streptococcus zooepidemicus