Production Of Hyaluronic Acid By *Streptococcus Equi Subsp Equi* (MK156140) - Pilot Scale

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Abstract:
Hyaluronic acid (HA), is a mucopolysacharide produced from microbial fermentation has raised interest in medical and cosmetic industries due to its various promising biological functions. The present study reports the production of hyaluronic acid by S.equi subsp equi with of hyaluronic acid by S.equi subsp equi with optimized medium components and physical parameters such as beef extract, 12.15%, and yeast extract 7.64%, pH 7.0, agitation speed 180 rpm, pressure 0.5 kg /cm2, aeration 2.0 vvm and incubation time for about 24 – 26 hours at 35^0C temperature employed in the 5L fermentor. The yield in laboratory scale (500ml) is compared with that of pilot scale.

Key words: Hyaluronic acid, *Streptococcus equi subsp equi*, pilot scale, batch fermentation

Introduction:
The high molecular weight hyaluronic acid, one of the important mucopolysacharide has received immense interest in various pharmaceutical and cosmetic industries (Steven et,al., 2018). Generally, hyaluronic acid is being extracted from rooster comb and/ by microbial fermentation (Boeriu et al., 2013). In recent times, production of hyaluronic acid by bacterial fermentation has gained importance due to its low production cost, less environmental pollution, high molecular weight, easy purification and free from viral contamination, regardless with less yield when compared to that of traditional method of extraction from rooster comb (Lianhui et. al., 2014).
Among various human and animal bacterial pathogens, gram positive *Streptococcus* species belonging to both group A and C are reported to produce hyaluronic acid in their capsule, as a mode of defense mechanism against competent mediated phagocytosis (Brian burnish and Ivo van de rijn, 1998). This property is being exploited for the production of hyaluronic acid in industrial scale.
In the present study, the production, extraction and purification of hyaluronic acid by *Streptococcus equi subsp equi* (MK156140) isolated by us was carried out in 5L fermentor (Pilot scale) under optimized conditions.

Materials and Methods:
Several bacterial species were screened on blood agar medium, among which *Streptococcus equi subsp equi (MK156140)* isolated from horse nasal sample showed a promising result in producing hyaluronic acid under laboratory conditions and therefore taken for further study and therefore taken for further study.

Pilot Scale production of HA:
The selected isolate *Streptococcus equi subsp equi (MK156140)* was inoculated into 500 ml modified LB broth and incubated under shaken condition at 35^0C until the cell count reached to 108 CFU. The actively growing culture was transferred into 5 L modified LB broth in 10 L fermentor (Eppendorf) and incubated for 24 – 26 hours at 35^0C and pH of about 7.0. And various other parameters such as agitation speed 180 rpm, pressure 0.5 kg/cm^2 and aeration 2.0 vvm was maintained uniform throughout the fermentation process.(Chen et al., 2009) The hyaluronic acid produced by the isolate estimated at regular interval of time (2 hours) by CTAB method using UV- vis spectrophotometer (Labman, LMSP- UV1000B) (Blank et al., 2005).

Extraction and purification of HA:
Hyaluronic acid present in the capsule of *S. equi subsp equi (MK156140)* was extracted and precipitated with alcohol. The hyaluronic acid was extracted by blending the culture broth with 10% volume of 5% SDS for 10 min and centrifuged. The hyaluronic acid was precipitated by adding 3 volume of ethanol to one volume of supernatant and further centrifuged. The viscosity of the hyaluronic acid were reduced by adding one volume of NaCl and two volume of ethanol and centrifuged (Aroskar *et al.,* 2012).

Purification:
The crude hyaluronic acid was treated with isopropyl alcohol (IPA) (1:3v/v) to reduce the viscosity. The protein present in the crude extract was removed by adding 0.1% trichloroacetic acid (TCA) until the pH of the solution reaches 2.0. The extract was further purified by passing through the activated charcoal bed (2cm thickness). The collected filtrate was further centrifuged at 7000 rpm for 30 min at 4°C and the supernatant was filtered through 0.45µm filters. The hyaluronic acid present in the filtrate was precipitated with the addition of IPA and dried under vacuum (Jagadeeshwara et al., 2013).

Results and discussion:
Hyaluronic acid being an important mucopolysaccharide its commercial production through microbial fermentation has received a great interest in recent times. In the present study the production of hyaluronic acid from *S. equi subsp equi (MK156140)* was studied by optimizing various physical and chemical parameters.
Media supplemented with sucrose as a carbon source and beef and yeast extract as nitrogen source, along with various physical parameters such as agitation speed 180 rpm, aeration rate 2.0 vvm, temperature 35ºC, pH 7.0 and pressure 0.5 kg/cm^2 were found to enhance the yield of hyaluronic acid 0.9 g/L to 7.8 g/L within 24 h of incubation period under pilot scale.
Similar such study was carried out by kim et al.,(2006) with *S. zooepidemicus*. Who reported the production of HA under optimized and large scale production as 5.4 g/L which is comparatively lesser than the production level (7.8 g/L) of S. equi *subsp equi (MK156140)*.
The quality of the product is one of the important criteria for any product to receive its commercial importance. The quality of the hyaluronic acid obtained after the microbial fermentation was enhanced with the help of various techniques, one such among them is by treating with activated charcoal which is found to be economical and effective method of purifying bacterial hyaluronic acid.
Many techniques in lab scale have been employed in purifying or enhancing the quality of the hyaluronic acid. Among them, activated charcoal was found to be economical and an alternative method for the purification of bacterial hyaluronic acid and it is been employed at pilot scale. In the present study 4.18 g/L, with (52.30%) of recover rate of HA was observed at pilot scale (5L).

There are very few reports available with respect to pilot scale fermentation for hyaluronic acid production from Streptococcus species. Among which Im *et al*., (2009), reported 6.94g/L crude hyaluronic acid from Streptococcus sp. ID9102 and kim *et al*., (1996) reported 6-7g/L hyaluronic acid from S.*equi subsp equi (MK156140) KFCC* 10830 which is lesser findings than our present study.
It is been stated that the production rate is closely associated to biomass of the organism. Recovery of the product ie., hyaluronic acid is been a demanding process in industrial scale much work yet to be carried out to increase the same. In our study we were able to get 4 fold increase in the yield and 52.30% recovery of the hyaluronic acid from *Streptococcus equi subsp equi (MK156140)* **(MK156140).**

Table 1: Comparision of hyaluronic yield between large scale and pilot scale for 2 consecutive generations of S.equisimilus

	Generation I yield g/L	**Generation II yield g/L**
Lab scale	7.16 g/L	7.18 g/L
Pilot scale	7.8 g/L	7.7 g/L

In the view of industrial production of any product it is necessary to know the viability of the organism in terms of production rate of the desired product, hence we performed the experiment for the production of hyaluronic acid in lab scale and pilot scale as well and did not find any major difference in the production rate. This may be the promising result to prove that the organism isolated by us is able to produce hyaluronic acid in industrial scale without any much decrease in the yield rate.

Conclusion: The present study focused on the production of hyaluronic acid in pilot scale fermentor (5L) from *Streptococcus equi subsp equi (MK156140)* . The parameters which have been studied under lab scale have been employed in pilot scale and obtained the crude hyaluronic acid extract 7.8 g/L. The resultant HA was purified by passing through the bed of activated charcoal in order to remove the impurities resulting in enhancing the hyaluronic acid content by 4.18 g/L (52.30% recovery). Based on the production rate of HA from S.equi *subsp equi (MK156140)* both in lab scale and pilot scale we conclude that this isolate can be used in industrial production as it has promising functions in both therapeutics and cosmetics field.

Declaration:
Conflict of interest with ethical standards:
Author declares that there is no conflict of interest. No animals or humans were used during the present study.

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References:

1. Chen, S. J., Chen, J. L., Huang, W. C., & Chen, H. L. (2009). Fermentation process development for hyaluronic acid production by Streptococcus zooepidemicus ATCC 39920. *Korean Journal of Chemical Engineering*, *26*(2), 428-432.
2. Boeriu, C. G., Springer, J., Kooy, F. K., van den Broek, L. A., &Eggink, G. (2013). Production methods for hyaluronan. *International Journal of Carbohydrate Chemistry*,
3. Steven B, Mohamed E.A, Mark W.H, Monte H, Sadanand F. Recent advances in hyaluronic acid based therapy for osteoarthritis Bowman et al. Clin Trans Med 2018, 7:6
4. Lianhui C, Shaopu L, Hongqun L, Xiaoli H. Spectrophotometric method for the determination of sodium hyaluronate with basic bisphenylnaphthylmethane dyes. J ChemPharma Res. 2014, 6: 1695-1698.
5. Kim, J. H., Yoo, S. J., Oh, D. K., Kweon, Y. G., Park, D. W., Lee, C. H., & Gil, G. H. (1996). Selection of a Streptococcus equi mutant and optimization of culture conditions for the production of high molecular weight hyaluronic acid. *Enzyme and Microbial Technology*, *19*(6), 440-445.
6. Im, J. H., Song, J. M., Kang, J. H., & Kang, D. J. (2009). Optimization of medium components for high-molecular-weight hyaluronic acid production by Streptococcus sp. ID9102 via a statistical approach. *Journal of Industrial Microbiology and Biotechnology*, *36*(11), 1337.