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Short Communication

HYALURONAN-A NOVEL POLYMER ISOLATED FROM MUTATED CLINICAL BACTERIAL ISOLATE

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ABSTRACT

Objective: This study was done to optimize the production parameters involved in the isolation of hyaluronan (HA) from UV mutated a clinical strain of *Klebsiella pneumonia* (M 3020).

Methods: Glucose and nitrogen enriched media (D-glucose, L-glutamic acid, and peptone) were utilized to cultivate the clinical isolate *K. pneumoniae*. The strain was Ultra Violet (UV) radiation mutated (254 nm, 25 min) and HA production was optimized by parameters such as pH and temperature. The isolated HA from the fermented broth was subjected to purification by isopropyl alcohol and silica gel and further dried by lyophilization. Produced HA was confirmed with UV and Fourier Transform Infra-Red (FT-IR) spectroscopy.

Results: UV treated strain at 254 nm for 25 min predominantly produce a high quantity of HA (3.5 g/l) in 37 °C, 300 rpm and pH 6.8 at 24 h run. UV and IR spectrum of produced HA showed strong similarity with the standard hyaluronan.

Conclusion: To conclude, high quantity and quality of HA can be isolated from mutated clinical strains of *K. pneumoniae*.

Keywords: HA, *Klebsiella pneumoniae*, Mutation, Enriched media

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HA is a carbohydrate–polymer of a disaccharide (D-Glucuronic acid and N-Acetyl glucosamine), linked by alternating β 1, 4 and β 1, 3 glycosidic linkages. Its molecular weight ranges between 1 and 14 KDa [1]. It can be considered as nature's moisturizer for its excellent hydrophilic property and has viscoelastic nature with bio-compatibility and non-immunogenic nature [2]. HA is used in the delivery of drugs to acute and chronic wounds [3] and hydrogels [4]. HA is one of the polymers for capsule development of encapsulated bacteria from the biosynthetic pathway which converts glucose into D-Glucoronic acid (N acetyl glucosamine) and finally, the HA synthase enzyme involved to synthesize HA [5]. Many natural sources have been described earlier for the HA isolation from plants, animals, microbes and even from avian [6]. But the microbial source seems to be effective in terms of sustaining biodiversity, easier process, quality and economy. An important gene involved in HA synthesis is arranged in *has* operan [7].

The economical and quality HA was isolated from microbial sources *Streptococcus zooepidemis* [8] and recombinant strains of *Bacillus subtilis* [9]. Native producer *S. zooepidemis* contains all the genes responsible for the production of HA while other strains need to be harboured with the lacking genes particularly *hasA.* Still, the molecular weight (MW) and quantity of HA biosynthesized, remains low [10]. There is a scope in identifying other encapsulated bacteria for isolation of quality (MW) and quantity HA. It is clear that the mucoid glistening colonies with viscid consistency formed by *K. pneumoniae* is of non-virulent capsular polysaccharide [11]. Hence the clinical isolate of *K. pneumoniae* was selected for the production of HA in this study.

The slant culture of *K. pneumoniae* M 3020 strain was obtained from Vijay clinical laboratory Madurai and chemicals were procured from Hi-media. The strain improvement was done by adding one-day old culture in nutrient agar media enriched with glucose and exposed to UV light for 25 min and incubated at 37 °C for 48 h. Sterile seed culture media was prepared using the following ingredient D-Glucose 50 g/l, Bactryo peptone 5 g/l, Yeast extract 10 g/l, Beef extract 10 g/l, Nacl 3.5 g/l and Na_2HPO_4 3.68 g/l. One loop full of *K. pneumoniae* M 3020 wild strain was inoculated in a 100 ml flask, followed by inoculation of UV exposed strain in another 100 ml flask using aseptic techniques. The inoculated media were incubated in a shaker at 37 °C in 180 rpm. Production media was prepared using the following chemicals D-Glucose 50 g/l, Bactryo peptone 5 g/l, Yeast extract 10 g/l, Beef extract 15 g/l, L-glutamic acid 0.8 g/l, sodium chloride 3.5 g/l, disodium hydrogen phosphate 3.68 g/l, potassium dihydrogen phosphate1.32 g/l, magnesium sulphate 0.4 g/l and distilled water up to 1L.

Fermentation was done in 9 sets by which each set contains 15 flasks. Production media supplemented with carbon and nitrogen sources were adjusted to three different pH (6.8, 7.0, and 7.2) and temperature (30, 35, 37 °C). 1 ml UV exposed strain of *K. pneumoniae* was inoculated. The flasks were incubated in rotary shaker Incubator at 300 rpm. From all the resulting flasks after incubation, 20 ml of culture broth were transferred to centrifuge tubes aseptically and centrifuged at 15000 rpm for 20 min. After centrifugation, the pellets settled at the bottom were subjected to biomass estimation by dry cell weight method and other supernatants were subjected to HA estimation by carbazole assay method [12]. Determination of glucose utilized was done by phenol-sulphuric acid method [13]. HA was purified by treatment with IPA and silica gel [14]. *λ-max* for standard and biosynthesized HA were compared using UV double beam spectroscopy. IR spectroscopy was also performed to confirm the HA. Molecular weight was determined for the produced HA by agarose gel electrophoresis method [15].

The Nutrient agar medium with D-Glucose shows capsule development extensively. Optimization for production of HA results was given in table 1. Batch fermentations of *K. pneumoniae* were performed under standard conditions, and the effect of pH was examined over the range of pH 6.8, 7.0, 7.2. This range encompasses pH values at which the HA production rate and yield are optimal [10]. Biomass grew rapidly to a maximum level at 24th hour, which coincided with glucose depletion. HA concentration after glucose exhaustion was primarily due to the shedding of the polysaccharide

capsule from the cell [2] which was observed in this study also. The effect of pH on growth and HA production was similar to that observed by Johns *et al.* 1994 [16]. It was observed in this study, at pH 7.0, 7.2 the cell growth and HA productions were poor.

The maximum HA concentration and bacterial specific growth rate were also temperature dependent [7]. Results were tabulated in table 1 and the maximum growth was seen in UV exposed strain (4.52 g/l±0.26).

Table 1: Optimization of HA production

pH	6.8			7.0			7.2		
Temperature	**BM (g/l)**	**HA (g/l)**	**GU (g/l)**	**BM (g/l)**	**HA (g/l)**	**GU (g/l)**	**BM (g/l)**	**HA (g/l)**	**GU (g/l)**
30 °C	3.4±0.25	2.8±0.20	1.6±0.14	2.5±0.26	2.0±0.20	5.0±0.07	2.7±0.15	2.5±0.21	2.0±0.15
35 °C	3.8±0.02	3.0±0.26	0.5±0.20	2.8±0.18	2.2±0.12	5.0±0.16	3.1±0.10	2.7±0.21	0.9±0.01
37 °C	4.5±0.26	3.5±0.22	0.0±0.01	4.0±0.04	2.9±0.17	0.0±0.01	3.8±0.12	3.0±0.11	1.6±0.21

mean±SD., n=5. BM-Bio Mass, HA-Hyaluronan, GU-Glucose Utilisation

Experiments were limited to a maximum initial glucose concentration of 50 g/l. A very substantial increase in the maximum HA concentration was observed, although the yield of HA on glucose fell, indicating less efficient conversion of glucose to HA. A higher initial glucose concentration markedly increased the MW, which also increased with culture time. In marked contrast to results at an initial glucose concentration of 50 g/l demonstrated excellent reproducibility [15].

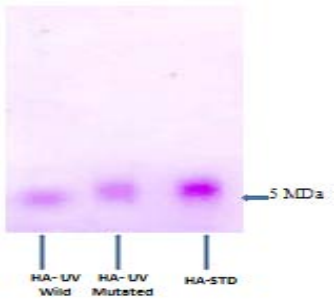
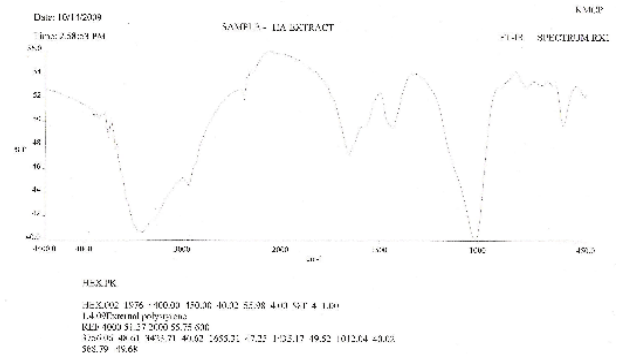


Fig. 1: HA Electrophoresis separation

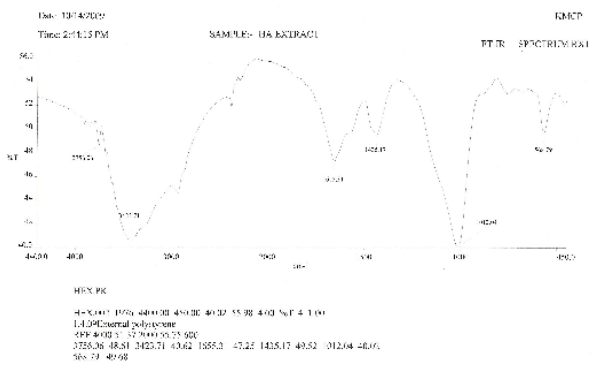
The initial observations with *K. pneumoniae* M 3020 appeared to indicate that total HA varied in molecular weight from 1 x 10^6 to 5 x 10^6. The 24th h culture of wild and UV exposed strains produced HA were comparatively studied with standard HA [17] by the absorbance (*λ-max*). The maximum absorbance for HA standard, extracted from wild strain and the mutated strain was found to be 252 nm, 244 nm and 253 nm respectively.

The FTIR spectrum (fig. 2) of wild and UV exposed strain of *K. pneumoniae* M 3020 produced HA were compared with IR reference spectrum of European Pharmacopoeia 5. The characteristics of spectrum bands of wild and mutant strain of *K. pneumoniae* M 3020 produced HA were similar to the reference spectrum of HA (table 2). The result indicates a strong similarity of reference standard, wild and UV exposed strain of *K. pneumoniae* M 3020 produced HA.

MW of reference sodium HA is 5 MDa. HA produced by UV exposed strain of *K. pneumoniae* possess commendable appreciation 5 MDa (fig. 1) when compared to reference standard, whereas the wild strain having low molecular weight. Previous investigators [5, 6] have demonstrated that HA isolated from Streptococci has an average molecular weight in excess of 1 x 106. The inherent viscosity of HA which increases with increase in MW presents a major obstacle during fermentation as well as in purification [16]. *Streptococcus* species are already demonstrated in HA production. Different capsular polysaccharides need to be understood and fermentation parameters other than pH and temperature may be optimized for better yield and quality. Results revealed that, the production of HA from *K. pneumoniae*, may be a good alternative source.



A) Wild strain



B) UV mutated strain

Fig. 2: HA FT-IR spectra

Table 2: FT-IR interpretation of HA

S. No.	HA standard (cm^{-1})	HA wild (cm^{-1})	HA mutated (cm^{-1})	Group assigned
1	1000	1012.04	1012	CO-Stretchning
2	1410	1435.17	1435	Carboxylate COO^-
3	1648	1655.31	1655	Mono Substituted amide
4	3412	3423.71	3424	OH group
5	3430	3756.06	3756	NH stretching

*-European Pharmacopeia, HA-Hyaluronan

AUTHORS CONTRIBUTIONS

Author's contributions are as follows, Natarajan K: Research idea, data analysis, and writing the manuscript. Vineeth Chandy: Compiled data and manuscript preparation. Sirajudeen MA: Data analysis and compilation of results.

CONFLICT OF INTERESTS

All authors state that they have no conflict of interest to declare

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