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ORIGINAL RESEARCH ARTICLE

Production, Optimization And Spectroscopic Studies Of Hyaluronic Acid Extracted From *Streptococcus pyogenes*

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ABSTRACT

Hyaluronic acid (HA), a naturally occurring glycosaminoglycan, offers many unique advantages as a building block for biomaterials. Two different strains of *Streptococcus pyogenes* (442 & 1926) were obtained from MTCC and characterized. Optimization study for Hyaluronic acid production was carried out at different pH and different glucose concentration. Mutation studies were also carried as a mean for improving the Hyaluronic acid production. Hyaluronic acid compounds were qualitatively analyzed through HNMR spectroscopy. The Hyaluronic acid capsule of the two strains were quantified spectrophotometrically using stains all assay method. Based on the optimization study at different pH the maximum yield of Hyaluronic acid was obtained at pH 6.9. The yields of Hyaluronic acid from were *Streptococcus pyogenes* (442 & 1926) parallelly increases with increase in glucose concentration. The maximum Hyaluronic acid production was observed at 1% glucose concentration from *Streptococcus pyogenes* 442 marked under optimized pH 6.9. The production gradually increases due to improved energy yield. Mutation, with UV rays improves Hyaluronic acid production from *Streptococcus pyogenes* (marked 442) after 10 minutes of exposure. UV mutation produces positive effect on HAS gene (hyaluronats synthase gene). But the decline in the production of Hyaluronic acid was observed with Ethidium bromide treated calls. Since the survival rate of the bacteria is usually about 0.1% with chemical treatment. All three variable pH, glucose concentration and mutation have a major effect on the production of Hyaluronic acid by *Streptococcus pyogenes* (442 & 1926).

Key words: Hyaluronic acid, *Streptococcus pyogenes*, Mutation and HNMR spectroscopy.

INTRODUCTION

Hyaluronic acid is a naturally occurring biopolymer which serves important biological functions in bacteria and higher animals including humans. Naturally occurring Hyaluronic acid was found in the tissues of higher animals, in particular as intercellular space filler [1] (Balazs, 1995). In the body Hyaluronic acid is synthesized by many types of cells and extruded into the extracellular space where it interacts with the other constituents of the extra cellular matrix to create the supportive and protective structure around the cells. It is present as a constituent in all body fluids and tissues and is found in higher concentration in the vitreous humor of the eye and synovial fluids in the joints. In mammals, the highest reported concentration is found in umbilical cord [2]. The commercial source of Hyaluronic acid is rooster combs, which contain the polymer at a higher concentration with respect to other animal tissues. Another important source of Hyaluronic acid is from microorganism, such as Gram-positive *Streptococci*, where Hyaluronic acid appears as a mucoid capsule surrounding the bacterium.

Hyaluronic acid has been traditionally extracted from rooster combs and bovine vitreous humor. However, it is difficult to isolate high molecular weight Hyaluronic acid economically from these sources because it forms a complex with proteoglycans. Subsequent extraction and purification processes result in an inherent, molecular weight reduction [3]. The use of animal derived biochemicals for human therapeutics is being met with growing resistance, besides ethic arguments, because of the risk of viral infection.

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Industries have turned to bacterial fermentation processes with the hope of obtaining commercially viable polymer. In bacterial fermentation, extracellular polysaccharide is released in to the growth medium and control of polymer characteristics and product yields are feasible. The amount of biopolymer that can be produced by this route is theoretically unlimited.

Streptococci are nutritionally fastidious anaerobes, which produce lactic acid as a by-product of glucose metabolism. The Hyaluronic acid capsule is a biocompatibility factor, formed as a mucoid capsule around the cell, which enables the gram positive bacteria to evade host immune defenses and hence accounts for its characteristically high virulence level. In *Streptococcus*, Hyaluronic acid is produced as a secondary metabolite and the production is influenced by various factors that include genetic as well as nutritional. *Streptococcus* produces Hyaluronic acid both under aerobic and anaerobic condition [4]. Certain strains of S*treptococcus* produce Hyaluronic acid at a particular stage in their life cycle and the same organism secrete enzyme hyaluronidase at a later time, which degrades the Hyaluronic acid produced earlier, hence the strain selected for Hyaluronic acid production were negative for hyaluronidase activity and non pathogenic[5] .

Spectrophotometric method was used to estimate the concentration of Hyaluronic acid present in the sample. Hyaluronic acid in the sample react with stains all reagents giving purple color. The color developed was read at 640nm [6]. Higher performances liquid chromatography (HPLC), size exclusion chromatography (SEC) coupled with multi angle laser light scattering photometry (Malls) were the other methods used to quantify Hyaluronic acid [7]. H^1-HNMR spectroscopy was used to qualitatively verify the Hyaluronic acid. The samples were dissolved and the spectra were record using a Varian Inova 500 spectrophotometer [8].

MATERIALS AND METHODS

Characterization of *Streptococcus pyogenes*

Two different strains of *Streptococcus pyogenes* with pathogenic characters were brought from MTCC, Chandigarh (MTCC No: 442 & 1926). The culture purity was checked as per the guidelines of Bergey's manual of bacteriology. The cultures were activated by introducing into sterile Nutrient broth and were incubated at 37°C with minimum shaking in Mechanical shaker. After incubation, a loopful of culture from each flask was transferred to separate Blood agar plates. The plates were incubated at 37°C under 5% CO_2 atmosphere for 24 hours and observed for the characteristic growth.

Isolation of Hyaluronic Acid producers

Hyaluronic acid producing organisms were selected based on their hemolytic character. Best hemolysis producing colonies were picked from each Blood agar plates (Marked 442 & 1926) and streaked on Todd- Hewitt agar plates. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 24hours.

Influence of pH on the production of Hyaluronic acid

The influence of pH on the production of Hyaluronic acid was measured by the following method. 10ml of production medium maintained at five different pH (6.3, 6.6, 6.9 7.2 and 7.5) were inoculated with loopful of S*treptococcus pyogenes* (442 & 1926), and incubated at 37°C for 16hours. Aliquots of the overnight cultures were inoculated into 10ml of fresh production medium and incubated at 37°C for 24hours under shaking condition. The resulting suspensions were centrifuged, washed with 10ml of 10mM Tris HCl (pH 7.5) and vortexed for 10 seconds.

Influence of Glucose concentration on the production of Hyaluronic acid

The influence of glucose concentration on the production of Hyaluronic acid was measured by following the method of Goh, 1994[9]. Five conical flasks containing 10ml of production medium each was supplemented with different glucose concentration (0.2, 0.4, 0.6, 0.8 and 1%) were inoculated with 15-30 colonies of *Streptococcus pyogenes* (MTCC 442 & 1926) and incubated at 37°C for 16hours. Aliquots of the overnight culture were sub-cultured in to 10ml of fresh production medium and incubated at 37°C for 24 hours under shaking condition. The resulting suspensions were centrifuged, washed with 10ml of 10mM Tris HCl (pH 7.5) and vortexed for 10 seconds.

Mutation studies

Two mutagenic agents, UV radiation and Ethidium bromide, were used to mutate *Streptococcus pyogenes* (442 & 1926) as a mean of improving the Hyaluronic Acid production.

Influence of UV Mutation on Hyaluronic Acid production

1ml of culture *Streptococcus pyogenes* (442 & 1926) grown on Todd-Hewitt broth (production media) was centrifuged and the pellet was resuspended in 1ml of nutrition free saline

medium. Cells suspended in saline were exposed to UV for different time intervals (5, 10, 15, 20, 25 min) UV exposed cells were streaked on Blood agar plates. As per the selection procedure, Hyaluronic acid producing colonies were selected and loopful colonies of *Streptococcus pyogenes* (442 & 1926) were inoculated in to the production medium, and incubated at 37^{o}C for 16 hours. Aliquots of the overnight culture were sub-cultured in to 10ml of fresh production medium and incubated at 37^{o}C for 24 hours under shaking condition. The resulting suspensions were centrifuged, washed with 10ml of 10mM Tris HCl (pH 7.5) and vortexed for 10 seconds.

Influence of Ethidium Bromide on Hyaluronic Acid production

Stock solution of Ethidium bromide (1mg/ml) was prepared. 1ml of *Streptococcus pyogenes* (442 & 1926) Todd-Hewitt broth (production media) were pelleted and resuspended in 1ml of nutrition free saline solution, to which 1ml of Ethidium bromide were added, from the stock, and incubated for 1hourr. Cells were streaked on Blood agar plates. After incubation for 24 hours, white mucoid colonies in the Blood agar plates were utilized for Hyaluronic acid production. 10ml of production medium was inoculated with a loopful of colonies, and incubated at 37^{o}C for 16 hours. Aliquots of the overnight culture were sub-cultured into 10 ml of fresh production medium and incubated at 37^{o}C for 24hours under shaking condition. The resulting suspensions were centrifuged, washed with 10ml of 10mM Tris HCl (pH 7.5) and vortexed for 10sec.

Extraction of Hyaluronic Acid

Bacterial cells cultivated under different conditions were pelleted out and the cell pellets were resuspended in 1.5ml of water and vortexed for 10seconds. This washing sequence was repeated and the cell pellets were resuspended in water to a final volume of 1.5 ml. Hyaluronic acid capsule was extracted by adding 1.5ml of chloroform and vortexed for 60 seconds. Cells remained at room temperature for 1 hour and then were pelleted and 500µl of the aqueous phase was used for estimation.

Quantitative estimation of Hyaluronic acid by stains all assay

The quantitative estimation of Hyaluronic acid by strain all assay was measured by following the method of Gray Darmstadt *et al*., 1999[10]. The aqueous phase containing Hyaluronic acids were separated. 1ml of fresh stains all reagents were added to each sample. After vortexing for 25 seconds, the spectrophotometric absorbance was read immediately at 640nm. The amounts of Hyaluronic acid capsule were determined by interpolation from the standard graph for Hyaluronic acid. Standards were prepared containing 0.5, 1.0, 1.5, and 2.5, 5µg of Hyaluronic acid (sigma), which is plotted *via* stains all assay.

HNMR spectroscopy (Jennie Baier Leach *et al*., 2002)

In HNMR, a molecular sample, usually dissolved in a liquid solvent is placed in a magnetic field and the absorption of RF waves by certain nuclei (proton & others) is measured. This property is utilized to assign the structural and dynamic properties of the molecule. The samples were dissolved in heavy water (D_2O) tetramethylsilane (TMS) was added as internal standard to deduce the chemical shift of protons. The spectra were recorded using a Varian Inova – 500 spectrophotometer.

RESULTS AND DISCUSSION

Two different strains of *Streptococcus pyogenes* with pathogenic characters were brought from MTCC; Chandigarh (MTCC No: 442 & 1926) was characterized by preliminary tests, cultivating on selective media and standard biochemical tests. Based on the Conventional Biochemical tests, the organism was characterized as *Streptococcus pyogenes*. The results were shown in (**Table 1**). Large white, mucoid colonies were appeared on Todd – Hewitt agar plates. These colonies were utilized for Hyaluronic acid production.

In *Streptococcus*, Hyaluronic acid is produced as a secondary metabolite and the production is influenced by various factors that include genetic as well as nutritional. *Streptococcus* produces Hyaluronic acid both under aerobic and anaerobic condition [4]. Certain strains of S*treptococcus* produce Hyaluronic acid at a particular stage in their life cycle and the same organism secrete enzyme hyaluronidase at a later time, which degrades the Hyaluronic acid produced earlier, Hence the strain selected for Hyaluronic acid production were negative for hyaluronidase activity and non pathogenic [5].

HA content of the *Streptococcus pyogenes* (442 & 1926) incubated at five different pH 6.3, 6.6, 6.9 7.2 and 7.5) were analyzed according to the procedure Gray Darmstadt *et al*., 1999. Based on the results obtained (**Table 2**) the maximum HA yield was observed at pH 6.9 (0.69/10ml of culture 442). Decline in the production of HA

were observed at pH 6.2 (0.14/10ml of culture 1926) and pH 7.5 (0.03mg/10ml of culture 442).

Production media were maintained at optimized pH 6.9 and Hyaluronic acid production by *Streptococcus pyogenes* (442 & 1926) were estimated at five different Glucose concentrations (0.2, 0.4, 0.6, 0.8 and 1%) according to the procedures Gary Darmstadt *et al*., 1999. Based on the results (**Table 3**) the *Streptococcus pyogenes* (Marked 442) give maximum Hyaluronic acid yield at 1% Glucose concentration under optimized pH 6.9 yields gradually increases with increase in Glucose concentration.

Table-1: Characterization of *Streptococcus pyogenes*

S.N	Biochemical test	MTCC	MTCC 1926
1	Gram staining	Positive	Positive
2	Motility	Motile	Motile
3	Catalase test	Negative	Negative
4	Growth with 6.5% NaCl	Negative	Negative
5	β – hemolysis in Blood agar	Positive	Positive
6	Hydrolysis of Hippurate	Negative	Negative
7	Bile esculin test	Positive	Positive
8	Acid from lactose	Positive	Positive
9	Bacitracin disk susceptibility	Positive	Positive

Table -2: Influence of pH on the production of Hyaluronic acid by *Streptococcus pyogenes.*

S.N	pH	Organisms	Concentration of culture(µg/10ml)
1.	6.3	*Streptococcus pyogenes* (442)	0.23
		Streptococcus pyogenes (1926)	0.14
2.	6.5	*Streptococcus pyogenes* (442)	0.43
		Streptococcus pyogenes (1926)	0.29
3.	6.9	*Streptococcus pyogenes* (442)	0.69
		Streptococcus pyogenes (1926)	0.52
4.	7.2	*Streptococcus pyogenes* (442)	0.3
		Streptococcus pyogenes (1926)	0.22
5.	7.5	*Streptococcus pyogenes* (442)	0.05
		Streptococcus pyogenes (1926)	0.03

Table-3: Influence of glucose concentration on the production of Hyaluronic acid by *Streptococcus pyogenes*

S.N	Glucose concentration (%)	Organisms	Concentration of culture(µg/10ml)
1.	0.2	*Streptococcus pyogenes* (442)	0.67
		Streptococcus pyogenes (1926)	0.51
2.	0.4	*Streptococcus pyogenes* (442)	0.75
		Streptococcus pyogenes (1926)	0.72
3.	0.6	*Streptococcus pyogenes* (442)	0.84
		Streptococcus pyogenes (1926)	0.76
4.	0.8	*Streptococcus pyogenes* (442)	1.02
		Streptococcus pyogenes (1926)	0.78
5.	1.0	*Streptococcus pyogenes* (442)	1.10
		Streptococcus pyogenes (1926)	0.81

Production media under optimized pH (6.9) and glucose concentration (1%) were inoculated with *Streptococcus pyogenes* (442 & 1926) mutated with UV – rays at different time interval (5, 10, 20 and 25 min). Based on the results obtained (**Fig 1**), the *Streptococcus pyogenes* (442 & 1926) which was UV exposed for 10 min gives maximum Hyaluronic acid yield under optimized condition. Increase in the time of exposure reduces Hyaluronic acid producing ability of *Streptococcus pyogenes*.

Production media under optimized condition were inoculated with *Streptococcus pyogenes* (442 & 1926) treated with Ethidium bromide for 1hour and analyses for Hyaluronic acid production. Based on the results (**Fig 2**) decline in the production of Hyaluronic acid were observed with Ethidium bromide mutated *Streptococcus pyogenes* (442 & 1926). The Hyaluronic acid extracted using chloroform were collected and used for estimation procedure. The Hyaluronic acid extracted from the *Streptococcus pyogenes* (442 & 1926) were estimated through stains all assay. The Hyaluronic acid content was measured at 640nm using spectrophotometer. The OD was plotted using Hyaluronic acid standard. The Hyaluronic acid content was expressed in micrograms (µg) per 10ml of the culture. The Hyaluronic acid capsule of the bacterial cultures *Streptococcus pyogenes* (442 & 1926) were estimated through stain all assay method. The results were tabulated and compared for different optimal conditions as mentioned above (III). The expected methyl peak appears at 1.9ppm. The other known aliphatic compound peaks were obtained between 2 and 5 ppm for Hyaluronic acid.

Fig. 1 Production of Hyaluronic acid by UV mutated *Streptococcus pyogenes* (442 & 1926)

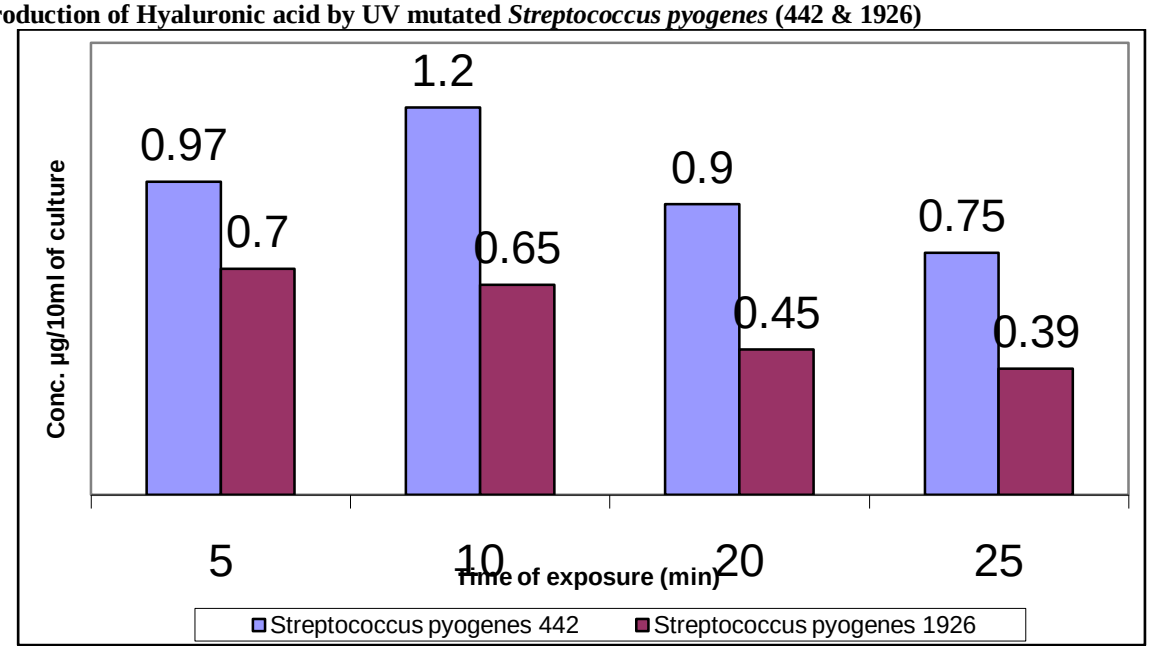


Fig 2: Production of Hyaluronic acid by Ethidium bromide treated *Streptococcus pyogenes* (442 & 1926)

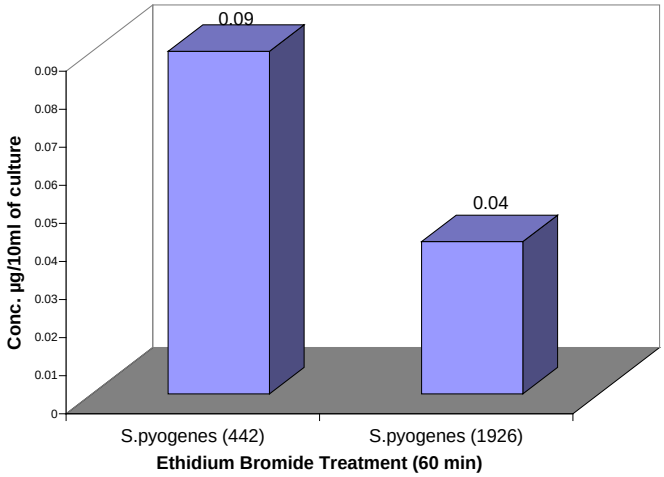


Fig 3: Quantitative analysis of Hyaluronic acid by UV Spectroscopy

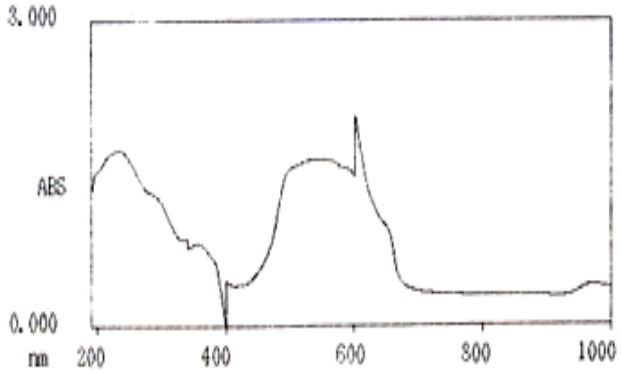
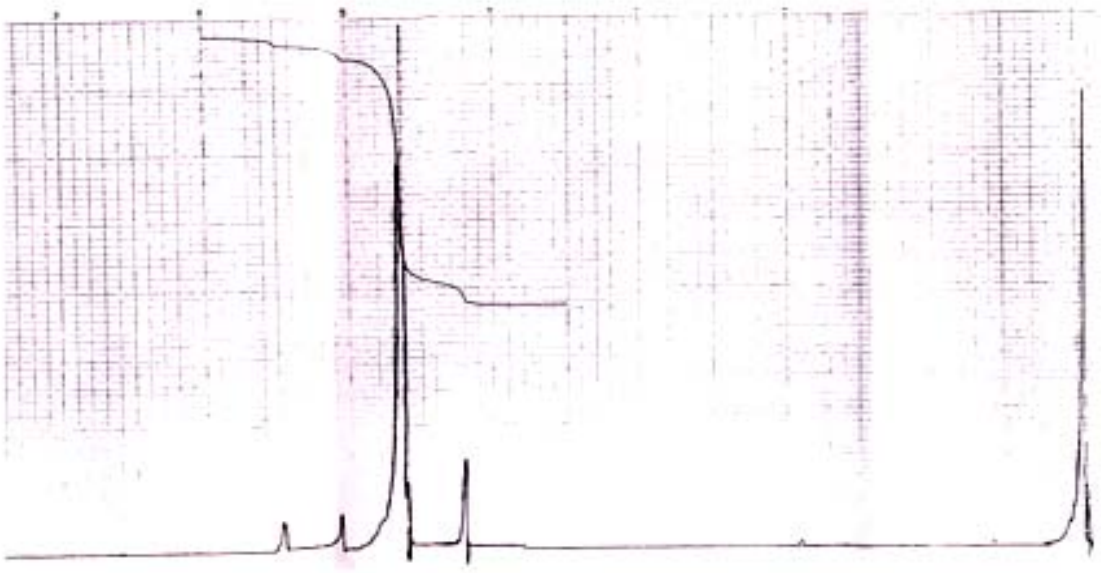


Fig 4. Qualitative analysis of Hyaluronic acid by HNMR Spectroscopy



* HA – METHYL PEAK AT 1.99PPM

CONCLUSION

In this study, it was concluded that, all the three variable pH, glucose concentration and mutation have a major effect on the production of Hyaluronic acid by *Streptococcus pyogenes* (442 & 1926). The optimal pH is 6.9 for Hyaluronic acid production; performance falls off significantly on either sides of this range. Mutation improved the yield from glucose under optimum pH probably due to improved energy yield.

REFERENCES

1. Balazs, EA. Hyaluronan biomaterials: Medical application in Hand book of Biomaterials and applications, ed. DL Wise et al., (Newyork: Marcel Dekkes) 1995: 2719 – 2741.
2. Balazs, E.A. 1979. Ultra pure hyaluronic acid and the use thereof, U.S. path.4, 141: 973.
3. Kitchen JR and Cysyk RL. Synthesis and release of Hyaluronic acid by Swiss BT 3 Fibroblasts. *Biochem.Journal* 1995, 309: 649 – 656.
4. Akasaka H, Susumu S, Yanagi M, Fukushima S and Mitsui T. *J Soc Cosmet Chem Japan* 1988, 22: 35 – 42.
5. Rosemans, Moses Fe, Ludowieg J and Dorfman A. Therapeutic effects of Hyaluronic acid on osteoarthritis of the knee. *J. Biol.Chem* 1950, 5: 203 -213
6. Laurel NE. "Hylan gel composition for precutaneous embolization". *J.Biomedical Materials Research* 1999, 25: 649 – 656.
7. Sany Okputsa, Kornelia Jumel and Stephen E. Harding. A comparison of molecular mass determination of Hyaluronic acid using SEC/MALLS and sedimentation equilibrium. *Eur. Biophys.J* 2003, 32: 450 – 456.
8. Jennie Baier Leach and Kathryn A Bivens. Photocross linked Hyaluronic acid Hydrogels: Natural biodegradable tissue engineering scaffolds. *Biotech. Bioeng* 2002, 82: 578 – 589.
9. Goh LJ. Effect of culture conditions on rates of intrinsic Hyaluronic acid production by *Streptococcus zooepidemicus*. Chemical engineering (Brisbane: University of Queensland) 1998
10. Gary L. Darmstadt and Laurel Mentele. Role of group A streptococcal virulence factors in adherence to keratinocytes. *Infect. Immuno* 1999, 59: 1811 – 1817