

Original Research Article

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Scalable Production of Collagen Isolated from *Streptococcus pyogenes*

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ABSTRACT

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Streptococcus pyogenes was isolated from the throat swabs of infected individual and swabbed on the blood agar and sodium azide crystal violet blood agar plates. Isolated organism was grown on the semi-synthetic medium for enrichment and extraction of M protein. The extracted M protein was further purified. The purified M protein was estimated by techniques like Lowry's method of protein estimation. The M protein was subjected to confirmation techniques and SDS-PAGE analysis. The optimum conditions for the production of M protein like pH, temperature, glucose concentrations were standardized by analyzing the growth of M protein at different pH, temperature and glucose concentrations. Fermentation of collagen containing source with *Streptococcus pyogenes* and Streptococcal M protein in a culture medium was carried out at different conditions. The amount of collagen production before fermentation and after fermentation was estimated and confirmed. Amount of collagen production increased was estimated and confirmed by thin layer chromatography and SDS-PAGE analysis.

Introduction

Collagen is one of the principal component of the extra-cellular matrix in animals, It also act as a major structural protein in formation of connective tissues. In mammals 60% of total body protein is collagen. It is also a principal ingredient in fish skins. Bovine and Porcine skins are also used as a good source for collagen. Organic matter of skin, bones and

tendons is comprised of collagen. There are about 29 types of collagen have been reported, in which type 1 collagen is found in connective tissues, type 2 collagen is found in cartilage tissues. Many studies have been reported with the use of microorganisms in collagen production. Of which yeast is the most commonly used expression system for collagen production, then gram positive species of bacteria were used to enhance the

production of collagen.. In recent research, the gram positive bacteria *Streptococcus pyogenes* containing collagen –like protein were preferably used to enhance collagen production, production of collagen was expected to be high when compared to other expression systems as the organism itself has the (emm) gene containing collagen-like protein.

Streptococcus pyogenes is a species of gram positive streptococcus, popularly known as flesh eating bacteria, which are facultative, aerotolerant, non-sporing, non – motile bacterium. They are responsible for a number of invasive human infections, mainly respiratory infections like pharyngitis and impetigo. Streptococcus are classified into different types based upon their virulence and the causative agents.

Among which Group A *Streptococcus pyogenes* (GAS) is the causative agent for the wide variety of streptococcal infections. The severity of the infections may vary from mild to severe, they are responsible for both invasive and non-invasive infections.

M protein of Group A Streptococci is one of the cell surface anchored antigen, which confers the adherence associated virulence to the organism. These surface proteins are attached to the cell wall by three different mechanisms. Transmission electron microscopy studies proved that all human isolates of Group A Streptococci have M protein which appears like a fuzz on the extracellular surface of the cell wall.

It was found that the protection against the streptococcal infection was depend upon the presence of antibodies in the N-terminal region of the M protein. Rebecca Landfield was the first to detect the streptococcal M protein 60 years ago. The virulence of the streptococcal infection is based on the

presence of M serotype. M protein is strongly anti-phagocytic and binds to the serum factor H and prevents opsonisation by C3b. Antibodies generated against the M antigen helps in opsonisation and further destruction of the microorganism by macrophages and neutrophils.

Materials and Methods

Biochemical tests for confirmation of *Streptococcus pyogenes*

Bacterial Strain

Streptococcus pyogenes group A was isolated from throat swabs of an individual infected with strep throat and tonsillitis

Culture Techniques

Blood agar plate and sodium azide crystal violet blood agar was prepared by adding 3ml of defibrinated blood in nutrient agar and sodium azide crystal violet agar. Throat swabs of infected patients were swabbed on to the blood agar and sodium azide crystal violet blood agar plates. Growth and the morphology of the organism is observed in the blood agar plate after 24-48 hours of incubation at 37°C.

Microscopic Examination

Genus and the species of the organism isolated was confirmed by gram staining and various biochemical techniques..

Catalase Test

A clean glass slide was taken and small amount of culture is streaked on to it and heat smeared. Place a drop of hydrogen peroxide on to slide. Evolution of bubbles is seen if the organism is catalase positive, if the organism is catalase negative there will be no evolution of bubble.

Oxidase Test

Place the oxidase disc on the slide. Take a loop of bacteria and place on to the top of the disc. Observation of change in color of the slide from white to purple represents oxidase positive, absence of color change represents oxidase negative.

Methyl Red and Voges Proskauer Test

MRVP broth was prepared in test tubes . Inoculated and incubated at 37° C for 24 hrs. Add some drops of methyl red reagent to the test tube, formation of red ring at the top represents the positive result for methyl red test, no formation of red ring represents the negative result for methyl red test.

Add 12 drops of Barrit's reagent to the test tube and add 1 drop of Barrit's reagent B observation of pink colored ring represents Voges proskauer test positive, absence of ring formation represents Voges proskauer test negative.

Indole Production Test

Tryptone broth was prepared and incubated at 37° C for 24 hrs. 10 drops of kovac's reagent was added and results were examined. Formation of red ring on the layer of reagent was represented as indole positive, absence of ring formation represents indole negative.

Beta – Hemolysis

Blood agar and Sodium azide crystal violet blood agar plates were prepared. The plates were swabbed with the throat swabs of the infected individual.

The plates were incubated at 37° C for 24-48hrs. Appearance of transparent surface around the colonies represents the beta-hemolytic property of the organism.

Urease Test

Urea agar was prepared and pH was adjusted . Inoculated and incubated at 37° C for 24 hrs. Color change from yellow to pink in the slant tubes represent urease positive and absence of pink color represents urease negative.

6.5% NACL Growth

Nutrient broth was prepared . Inoculated and incubated at 37° C for 24 hrs. Observation of turbidity in the sample represents organism positive for the test, no turbidity represents organism negative for the test.

DNase Test

Nutrient broth was prepared and DNA sample was added. Then the broth was inoculated and incubated at 37° C for 24 hrs. 0.8% agarose gel was prepared and the control and test samples was loaded. Run the gel electrophoresis at 150 V for 20 mins. Observe the results under UV Transilluminator. Appearance of DNA bands in control and faint bands or digested bands in the test proves that the organism has produced DNase so the band is faint or digested. No appearance of faints bands or appearance of proper band represents that the organism has not produced DNase.

Oxidative Fermentation

Hugh leifson's agar was prepared, inoculated and incubated at 37° C for 24 hrs. Conversion of green color of the medium to yellow color represents the oxidative and fermentative nature of the organism.

Bacitracin Sensitivity Test

Muller hinton agar plates were prepared. The test side of the plate was swabbed with the pure of isolated organism. Bacitracin discs were carefully placed on the both sides of the

plate. The plate was incubated at 37°C for 24-48 hrs. Absence of zone formation around the disc represents the sensitivity of the organism to bacitracin.

Fermentation with Various Types of Sugar

Peptone broth was prepared, autoclaved and distributed to all the tubes. 1g of each sugar (glucose, fructose, lactose, galactose, sucrose) was added to all the test tubes. All the test tubes were inoculated and incubated at 37°C for 24 hrs. Sugar fermentation can be observed through change in color of the broth to yellow color and gas production can be detected by presence of air bubbles in the Durham's tube.

Ribose Fermentation

Peptone broth was prepared, autoclaved and distributed to all the tubes. 1g of ribose was added to all the test tubes. All the test tubes were inoculated and incubated at 37°C for 24 hrs. No appearance of color change was detected, indicated the inability of the organism to ferment ribose.

Neuraminidase Enzyme Activity

E. Coli broth culture was prepared, inoculated and incubated at 37°C for 24 hrs. Sedimentation of cell debris at the bottom of the eppendorf represents the lysis of plasmid DNA due to the presence of neuraminidase activity to the organism.

16s rRNA Sequencing for Confirmation of the Isolated Organism

DNA was isolated from the culture containing isolated organism. Quality of the isolated organism was evaluated on 0% Agarose gel and a single band of high-molecular weight DNA has been observed.

Fragment of 16s rDNA gene was amplified by 27F and 1492R primers.

A single discrete PCR amplicon band of 1500bp was observed when resolved on agarose gel.

The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 cycle sequencing kit on ABI 3730xl genetic analyzer.

Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

The 16s rDNA gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program CLUSTAL W. Distant matrix was generated and the phylogenetic tree was constructed using MEGA 7.

M Protein Isolation And Purification

Culture Medium and Isolation

Semi-synthetic medium was prepared by adding all the components to their required composition by mixing in phosphate buffer solution (pH- 7.4). The medium was filter sterilized and a loop full of bacteria (*S. pyogenes*) was inoculated in the medium. The medium was incubated at 37°C for 5-6 days. The growth of M protein in the culture medium was estimated by Lowry's method.

Extraction and Purification of M Protein

M protein was extracted from the semi-synthetic medium by a standard method by using 1N NaOH and 1N HCl. The extracted

protein in the supernatant was collected and further purified. M protein extracted was further purified by 30% ammonium sulphate purification method.

Confirmation of M Protein

Serum Opacity Factor

Purified M protein sample was added to the serum obtained from the collected blood sample. Observation of opalescence produced in the serum represents the presence of M protein.

Radial Immuno Diffusion

1.2 % of agarose gel was prepared. A well was made in the centre of the gel with gel puncher. To the well serum was added which acts as an antibody. It was allowed to spread with gel for 10 minutes. Purified M protein sample added to the well acts as an antigen. Formation of precipitin ring around the well is represented as the antigen-antibody interaction and confirms the M protein.

SDS- Page Technique

SDS-PAGE technique was used to separate M protein based on its molecular weight by using standard protein marker of range 11-245 KDa.

Standardisation of M Protein

The temperature, pH, glucose concentrations of the M protein was altered and varied to identify the optimized condition at which the production of M protein is high. The optimum condition for the growth of M protein in semi-synthetic medium was found to be pH – 7, 37° C, non-shaking condition with glucose concentration 0.50 g in 50 ml of semi-synthetic medium.

Fermentation and Purification Of Collagen

Minimal medium was prepared and collagen containing source (collagen powder) was added to the medium. The medium was distributed in two conical flasks and one of the flasks was inoculated with Streptococcal M protein and other with *Streptococcus pyogenes* to increase collagen production. The medium was incubated in different conditions at 37° C. The amount of collagen production enhanced in 10 days was found by comparing with OD values of 0 hour collagen production.

Purification of Collagen

The collagen samples were purified using 5% NaCl. The samples were spun at 10000 rpm for 3 minutes. Supernatant was collected and it was used as the starting material for the further use.

Thin Layer Chromatography for Confirmation of Collagen

Solvent system was prepared using butanol, acetic acid and water in the ratio (4:1:5). Solvent system was allowed to get saturated overnight. TLC plate was taken thin line was drawn and the purified samples were loaded along with the standard sample in dots. TLC plate was kept inside the beaker containing saturated solution.

The solvent was allowed to run $\frac{3}{4}$ of the plate and the relative front was marked (till the level of the run of the sample). Then the plate was air dried. Spraying solution was prepared and sprayed on the TLC plate. Appearance of orange pinkish dots on the plate with the similar line of the standard collagen samples proves the collagen production by fermentation with *Streptococcus pyogenes* and M protein.

SDS- Page for Confirmation of Collagen

SDS-PAGE technique was used to separate collagen based on its molecular weight by using standard protein marker of range 11-245 KDa.

Applications of Collagen

Anti – Inflammatory Activity of Fermented Collagen

Packed RBC cells 10ml was mixed with equal volume of phosphate buffer solution. Then 10% v/v of RBC solution was made. Purified collagen was mixed in equal ratio with phosphate buffer solution.

To 1ml of prepared sample was mixed with 1ml of 10%v/v RBC solution and it was made into 2 duplicates. One set was incubated at 20° C for 20 mins and another set was incubated at 54°C boiling water bath for 20 mins. After incubation the tubes were spinned at 3000 rpm for 3 mins. Remove the supernatant containing hemoglobin content.

OD values were taken at 520 nm in colorimetry for the supernatant obtained. Range of OD values obtained determines the anti-inflammatory activity of the collagen

Application of Collagen in Wound Healing

Collagen pellets were collected by spinning the collagen sample at 10,000 rpm for 5mins. To the equal amount of the collected collagen pellet glycerol was added and dissolved.

The mixture was made as a paste and allowed to air dry at 37° C for 2-3 days. Biological skin like substance was formed which can be applied on the wounded surface and help in wound healing.

Results and Discussion

Isolation of *Streptococcus pyogenes*

Throat swabs collected from the infected individual were swabbed on to the blood agar and sodium azidecrystal violet blood agar plates. Appearance of beta-hemolytic colonies is due to the complete lysis of RBC cells in and around the colonies. This was confirmed by (Jawad Al-Khafaji et.al.,2016) on his study in observation of beta-hemolytic colonies in the blood agar plate.

Gram Staining of an Isolated Organism

Appearance of gram positive chain of cocci under the compound microscope after the gram staining procedure was the presumptive conformation for *Streptococcus pyogenes*. This was confirmed by (Holt et.al.,1994) on his microscopic observation of *Streptococcus pyogenes*.

Estimation of Amount of M Protein Produced due to the Effect of Growth in Semi- Synthetic Medium

Ontaking the values of BSA (Bovine serum albumin) as a standard, optical density of the M protein was read at 620 nm and the standard graph was plotted. The concentration of M protein produced on the effect of growth medium was found to be 200µg/ml. This technique was carried out based on (Lowry et.al,1951) for estimation of total protein as a reference.

SDS – Page Technique for Confirmation of M Protein

SDS-PAGE technique was used to separate proteins based on their molecular weight. Based on the SDS-PAGE it is proved that the M protein had a low molecular weight ranging from 20-40 kDa. This technique was

performed to identify the molecular weight of the M protein based on the (Eugene.N.Fox,1974) chronology of physical and chemical analyses of M protein as a reference.

Collagen Production

The amount of collagen production at 0 hour by *S.pyogenes* and M protein before fermentation was found to be 250 µg/ml. The amount of collagen by fermentation of 10 days with *S.pyogenes* and M protein was found to be 450µg/ml by using Lowry's method based on the (Komsa-Penkova R,et.al,1996) as a reference.

The amount of collagen production enhanced in 10 days was found by comparing with OD values of 0 hour collagen production. Collagen production enhanced was found to be 250 µg/ml.

Thin Layer Chromatography for Confirmation of Collagen

This technique was based on the principle of separation of chemical compounds according to their molecular weight. Appearance of pink colored spots on the TLC plate when the ninhydrin was sprayed proves the separated compound as collagen. This technique was performed by using (Sadaf Quereshi et.al,2010) as a reference.

SDS – Page Technique for Confirmation of Collagen

SDS-PAGE technique was used to separate proteins based on their molecular weight. Based on the SDS-PAGE it is proved that the collagen has a high molecular weight ranging from 175-245 KDa. This technique was performed to identify the molecular weight of the collagen based on the Sadaf Quereshi et.al,2010) as a reference.

Applications of collagen

Anti- inflammatory activity of collagen

Anti- inflammatory activity of collagen produced through fermentation with M protein and *S.pyogenes* was calculated by reading OD values of prepared RBC suspension at 520 nm in spectrophotometer. Calculation was done using the formula :

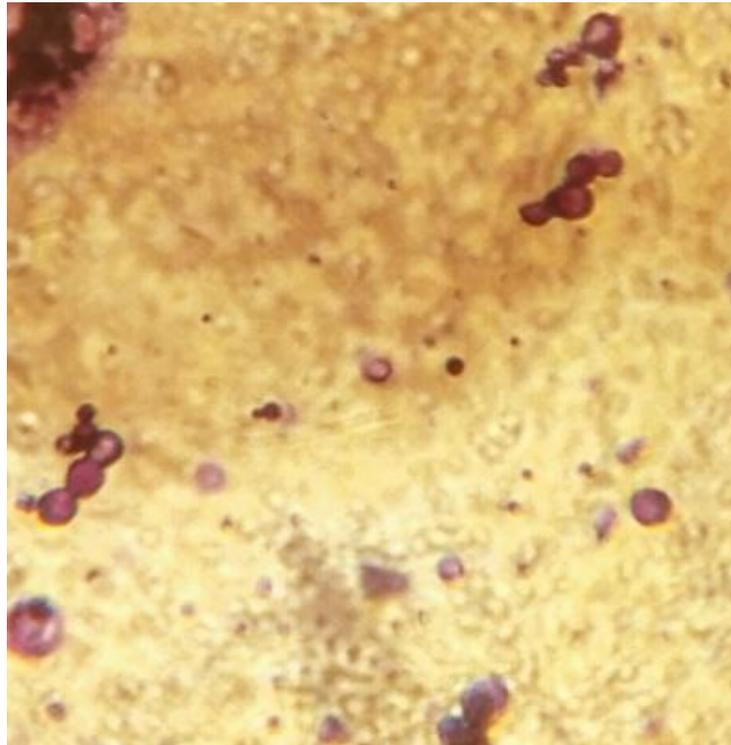
$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100.$$

This proved that the collagen produced through fermentation with M protein has a high anti-inflammatory activity when compared to the collagen fermented with *S.pyogenes*. Based on this property collagen produced through fermentation can be used for various biomedical applications. Anti-inflammatory activity of collagen is studied based on the study of (Suchita V Ghumre et.al,2017) assessment of invitro anti-inflammatory activity as a reference.

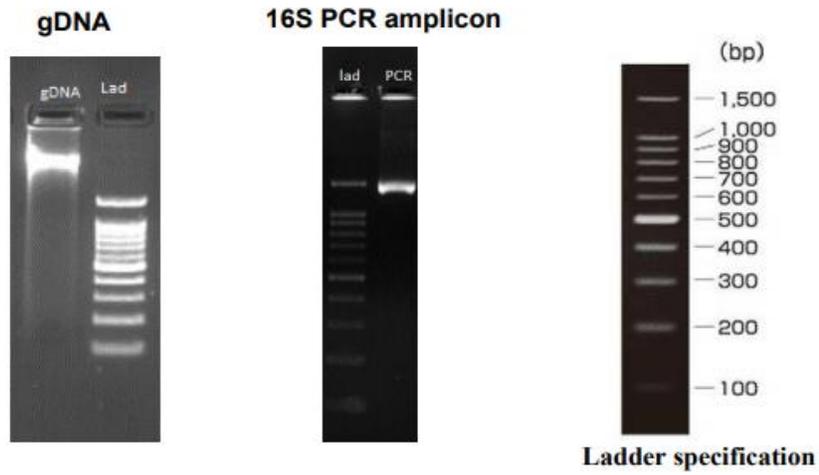
Application of Collagen for Wound Healing as a Bio-Skin

Collagen produced through fermentation of hydrolysed collagen powder with *S.pyogenes* and Streptococcal M protein was checked for its anti-inflammatory activity and used for the process of making bio-skin, which can be further used for the wound healing purpose during injury. Production of Bio-skin from collagen was done using (Lavanya Gunamalai,2017) use of collagen as a biomaterial for tissue engineering as a reference. The present study aimed to isolate a gram positive bacteria containing collagen – like protein. The organism isolated from the throat swabs of the infected individual was found to be *Streptococcus pyogenes*, a pathogenic cocci.

Fig.1 Microscopic Examination of Isolated *Streptococcus pyogenes* and observation of Gram positive chain of cocci



16S rRNA Sequencing for Confirmation Of An Isolated Organism



Phylogenetic Tree

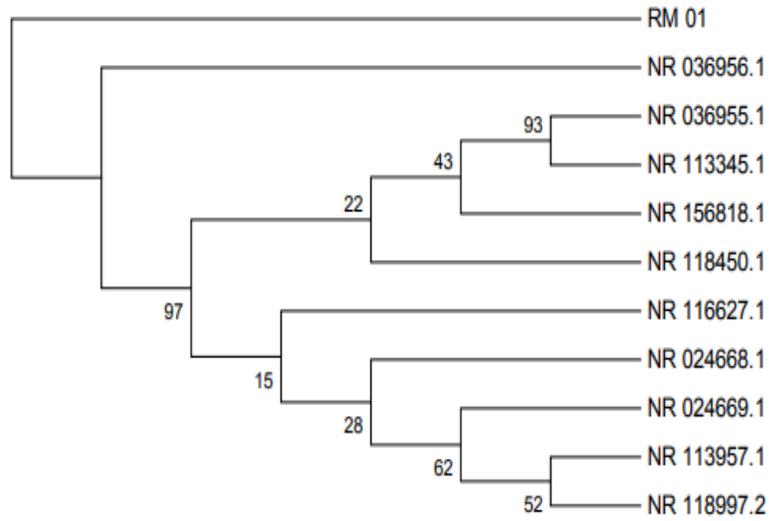


Fig.2 Estimation of production of m protein in a semi-synthetic medium by lowry's method

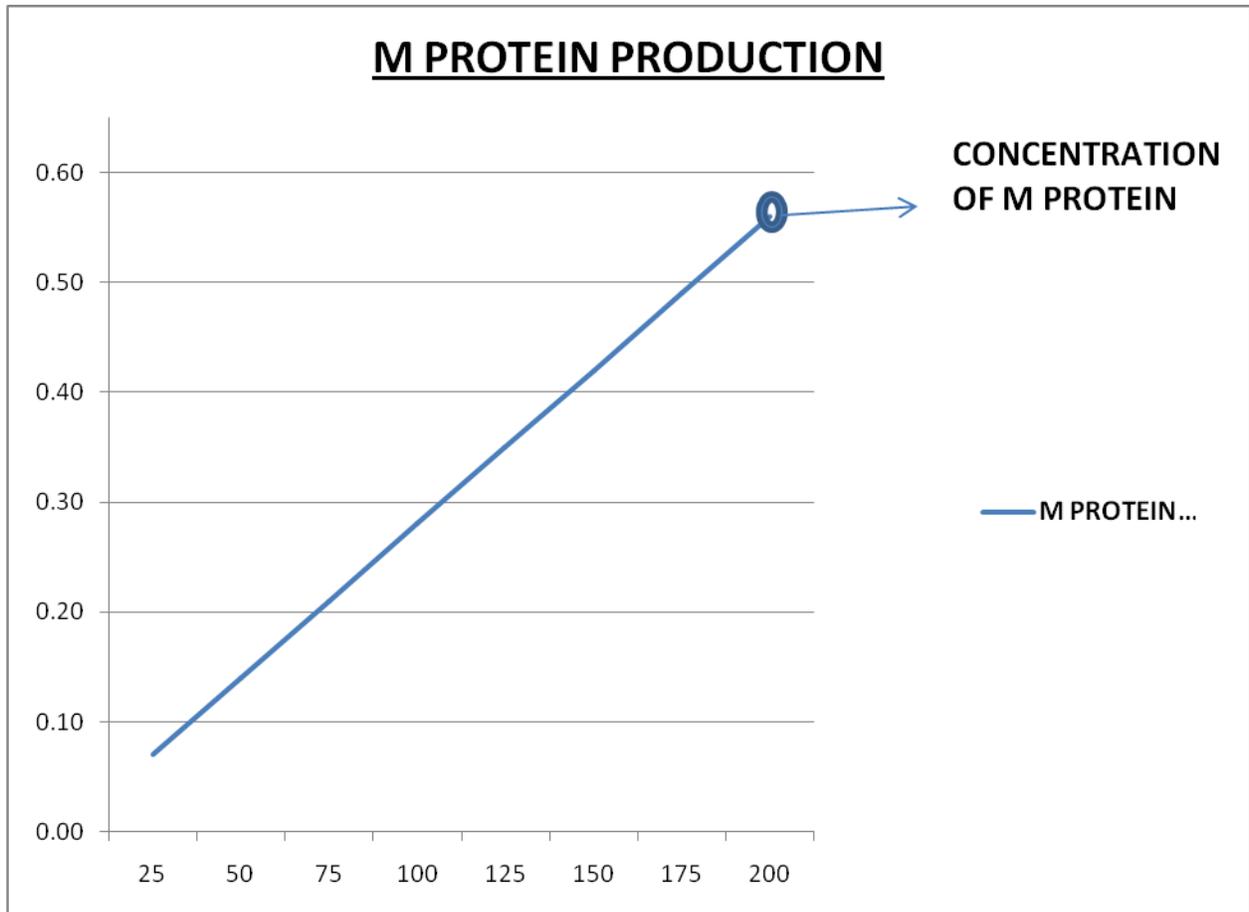


Fig.3 SDS PAGE showing M protein based on its molecular weight

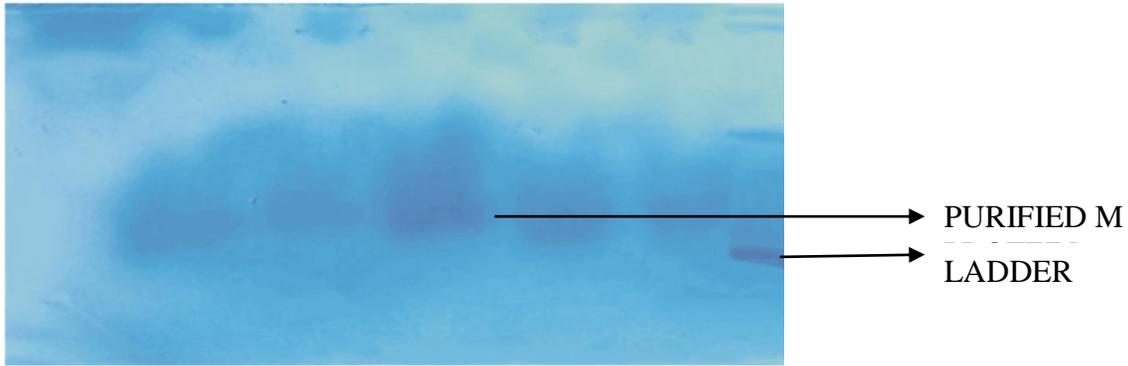


Fig.4 Estimation of collagen production at 0 hour

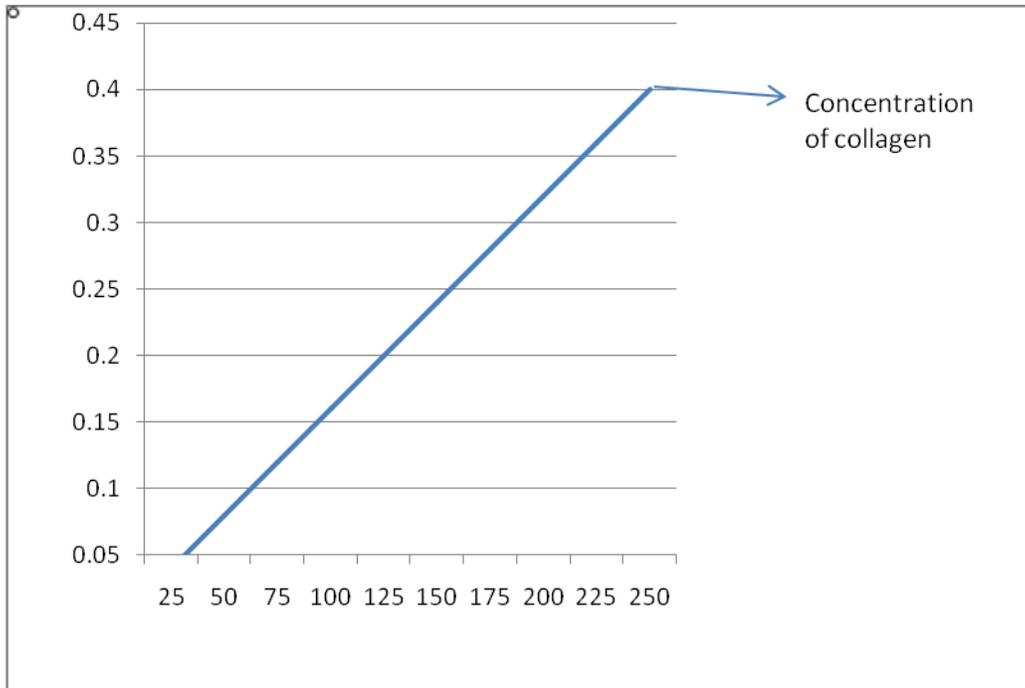


Fig.5 Collagen production after 10 days of fermentation

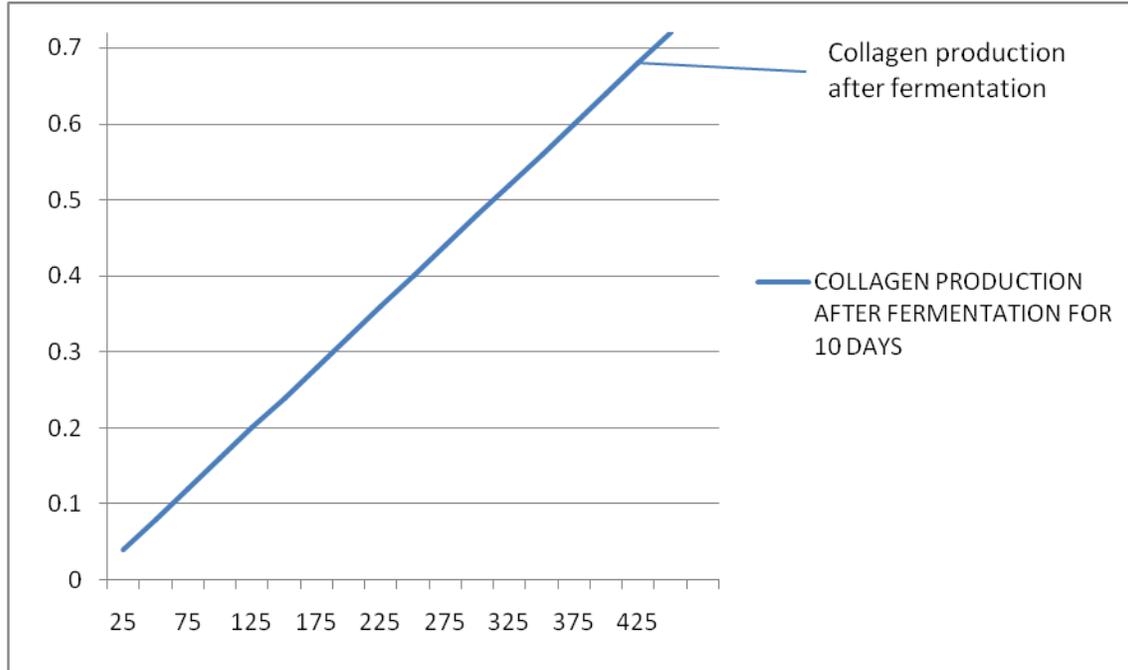


Fig.6 Thin layer chromatography for confirmation of collagen

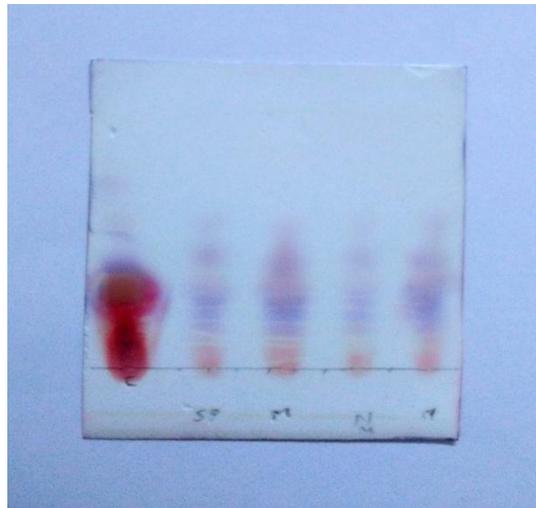
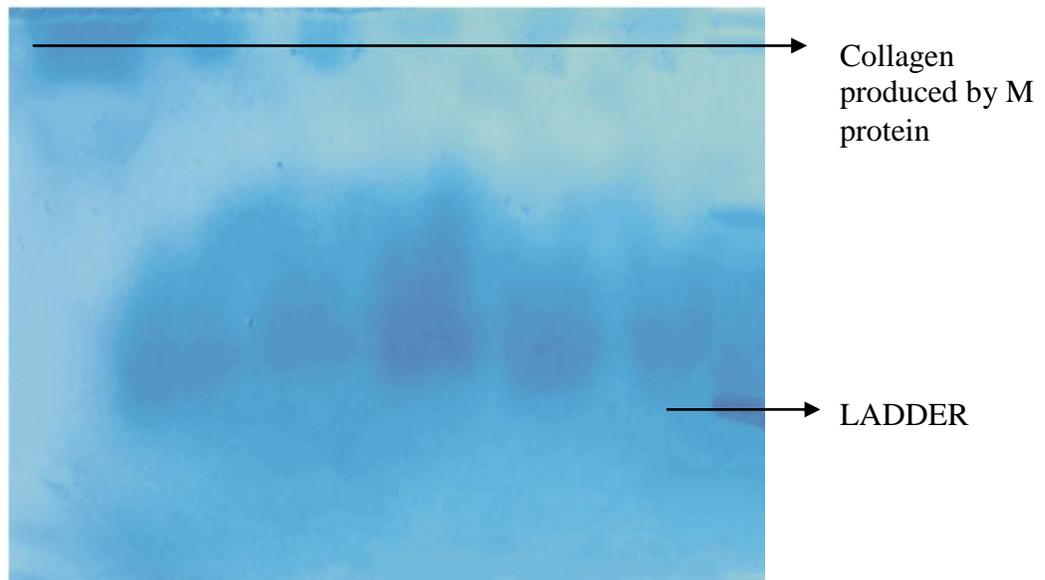


Fig.7 SDS – PAGE technique for confirmation of collagen



Further the M protein present in the cell wall of the *Streptococcus pyogenes* was cultured, extracted and purified by standard procedures. The extracted protein was confirmed by few techniques and used for further collagen production. As the main aim of the present study is to produce collagen from *Streptococcus pyogenes*, it was carried out by fermenting the collagen containing source with the M protein (containing collagen like sequences) and the *Streptococcus pyogenes*. Initial amount of collagen production was noted before fermentation and compared with the level of production after fermentation. On comparison the amount of collagen production increased was found to be 250 µg/ml. The collagen production was further confirmed by various techniques. The collagen produced was further used for biomedical applications such as wound healing.

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