EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 9
Issue: 10
277-286
Year: 2022

EFFECT OF METAL IONS ON MICROBES FOR THE PRODUCTION OF HEPARINASE

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Article Received on 04/08/2022 **Article Revised on 25/08/2022** **Article Accepted on 14/09/2022**

ABSTRACT

Heparinase is an enzyme, known to be a coagulant that works by acting on heparin to degrade it and this property of the enzyme finds various uses and applications in medicine. In this study, the effect metal ions have on microbes for the production of heparinase was observed. For this, micro-organisms were isolated from soil samples using soil suspensions and M9 modified media containing heparin by pour plate method. The isolated colonies were screened on the basis of a coagulation test and a bioassay. The organism producing the highest amount of heparinase (2.02 units/ml enzyme) in the enzyme assay was chosen as the test organism and subjected to identification which involved Gram staining and a series of biochemical tests. Optimization of the physical parameters required for the production of heparinase was carried out for each parameter by bioassay method using broth cultures made from M9 modified media containing heparin. The optimum conditions were determined to be an incubation period of 48 hours (7.98 enzyme units/ml) and a pH of 9 (9.19 enzyme units/ml) at 35°C (10.32 enzyme units/ml). Another bioassay was then performed on broth cultures made from M9 modified media containing heparin, incubated at the optimum conditions along with the addition of $ZnCl_2$, $MgCl_2$, $MnSO_4$, $NiCl_2$ and $FeCl_3$ trace elements. The addition of $FeCl_3$ showed the most increase in production of the enzyme (11.97 units/ml enzyme) and upon testing various concentrations of the element, it was found that 500mg of the trace element increased the amount of heparinase even more (14.13 units/ml). The enzyme was then produced at optimum conditions with the addition of $FeCl_3$ in the broth and was purified by salt precipitation, dialysis, ion exchange chromatography and gel filtration. Characterization was done by SDS-PAGE.

KEYWORDS: Heparinase, purification, ion exchange chromatography, gel filtration, SDS PAGE.

INTRODUCTION

Heparinase is an enzyme that degrades heparin and functions as a coagulant. Due to this property of heparinase, it is used clinically to counterbalance the anticoagulant effect of heparin as heparinase is known to degrade heparin. Heparinase is also used industrially in the production of low molecular weight heparin (LMWH) which finds its uses clinically as it is anti-thrombotic and anticoagulant in nature and prevents blood clots and is also used to thin blood. Heparin lyase enzyme, hepase or heparinase is an enzyme that catalyses the cleavage of glycosidic linkage in a heparin molecule. Heparinase is monomeric basic protein (pI = 8.5) with a molecular weight of 45,000 Daltons (Joseph, 1988). Heparinase acts directly upon heparin, yielding 52% of a trisulfated disaccharide (O-(α-L-ido-4-enepyranosyluronic acid 2-sulfate)-(1–4)-2-sulfoamino-2-deoxy-D-glucose 6-sulfate) and 40% a tetrasaccharide, in addition to small amounts of hexa- and disaccharides (Chuan et al, 2019). Repeating disaccharide residues varying between one to three sulfate groups exist in heparin/HS, which results in domains of high and low sulfation. Based on this, heparinases are classified into three major types – Heparinase I, Heparinase II, and Heparinase III. Each of these enzymes recognize specific sequences of heparin/HS and cleave them. Heparinase I cleaves highly sulfated heparin/HS chains, Heparinase III cleaves less sulfated HS chains, while Heparinase II cleaves domains of both high and low sulfation on both Heparin and HS. Heparinase I, II and III used in combination can produce a near-complete depolymerization of Heparin/HS polysaccharide chains to disaccharides. All Hepases share similar catalytic mechanisms in which His-Tyr acts as a Brønsted base and acid. Notably, all identified Hepases belong to the family of endolytic lyases, which randomly cleave the HP/HS chains on the inside, while exo-type Hepases, which can be very useful tools for sequencing of HP/HS chains, have not been found until now (Qingdong et al, 2021).

Heparin and HS-degrading enzymes are produced by both mammals and microorganisms. The difference between them lies in their mechanism of action on glycosaminoglycans (Karthika et al, 2018). While heparin is known to come mainly from porcine intestinal

mucosa and the lungs and mucosal membranes of various animals for mass production, heparinase is produced mainly from microbes and no study using plants as the source has been done yet. Beef and rabbit liver have also been used for the extraction of heparinase in a study conducted by Cho and Jaques (1956). Microbial sources for the production of heparinase has been studied more widely due to the fact that heparinase produced from micro-organisms exhibits a wider range of applications clinically and pharmaceutically. Among the fungal strains, various species of *Aspergillus* such as *Aspergillus flavus* and *Aspergillus oryzae* are also reported to produce heparinases. To the best of information available till date no Actinomycetes is reported to produce heparinase, thus screening and investigation of novel microbial sources capable of producing different/modified heparinases is still a worth exploring area of research as heparinases have potential commercial, pharmaceutical and clinical applications (Vineeta et al, 2019).

Many experiments have been performed to produce heparinase enzyme from various bacterial sources. Genetic engineering of the various bacterial strains has also been performed in order to obtain higher yields of heparinase and to increase efficiency in the production of the enzyme. This study was carried out to understand the effect metal ions have on microbes for the production of heparinase.

MATERIALS AND METHODS

1. Collection of soil samples

Soil was selected as the source of micro-organisms since it exhibits a wide variety of micro-organisms and is rich in carbohydrates which is what makes up the enzyme heparinase. Six different samples of soil were collected, each from different sites, to obtain different types of micro-organisms which would be further screened for the production of the enzyme heparinase. The samples were collected from different spots in Bengaluru. The samples were taken from a hospital, a public park, a garage, a road, a market and a house garden.

2. Isolation of micro-organisms

The micro-organisms contained in the soil samples were isolated by preparing soil suspensions of each sample. The media which was used for the growth of these micro-organisms was M9 modified media containing heparin. The cultures were prepared by using the pour plate method for each soil suspension and incubating the petri plates for 24 hours to allow growth. The various colonies obtained after incubation were observed and a few were selected based on colony characteristics to be streaked on LB agar plates and again incubated for 24 hours to obtain pure cultures.

3. Screening of micro-organisms

Six colonies from the previously prepared LB agar plates were selected based on morphology for further screening and the screening of these micro-organisms was carried out by a coagulation test to determine the presence of heparinase in the culture broths of the micro-organisms, and a bioassay method to determine the amount of enzyme produced by each organism and single out the organism producing the highest amount of heparinase.

3.1. Primary screening

For the coagulation test, culture broths for all six micro-organisms were prepared by inoculating LB broth with the selected micro-organisms in separate conical flasks, and incubating them for 24 hours to allow growth of the micro-organisms after which the broth cultures were centrifuged and 200µl supernatant from each broth culture was tested by mixing it with 100µl of blood and observed for coagulation. The time taken for coagulation to occur in each tube was noted.

3.2. Secondary screening

Screening by bioassay method was done by inoculating the six micro-organisms in different conical flasks containing M9 modified media containing 50µl heparin each and incubating the flasks for 24 hours, after which the culture broths were centrifuged at 6000rpm for 10 minutes and the supernatants obtained were used in the enzyme assay as enzyme solutions. The reaction mix of this enzyme assay contained 450µl Tris HCl buffer at pH 7.5, 50µl heparin, pre-equilibrated for 5 minutes, 500µl of the enzyme solution, incubated for 10 minutes, followed by the addition of HCl to stop the reaction. The absorbance was then measured at 235nm and the highest obtained reading indicated which micro-organism produced the highest amount of heparinase and was selected as the test organism for this study.

4. Identification of the test organism

In order to identify the selected micro-organism, the test organism was subjected to Gram staining followed by a series of biochemical tests. After Gram staining, the first series of tests conducted were the IMViC tests, followed with an H_2S test, a gelatin test, casein and starch hydrolysis tests, cellulose degradation test, glucose, sucrose and mannitol fermentation tests, nitrate reduction test, urease test, TSI test and catalase test.

5. Optimization

5.1. Effect of incubation time for the production of heparinase

The effect of varying incubation periods on the production of heparinase was studied by preparing M9 modified media containing 50µl heparin, inoculated with the test organism and incubated. An enzyme assay was conducted every 24 hours of incubation, using the culture broth as enzyme solutions after centrifuging it, until a drop in the readings was observed. The incubation time after which the test organism showed the highest reading was concluded to be the optimum incubation period for the test organism to produce heparinase.

5.2. Effect of temperature for the production of heparinase

Here, the effect varying temperature has on the production of heparinase was studied. M9 modified media containing 50µl heparin was inoculated with the test organism and incubated for the previously determined optimum incubation time at different temperatures (30°C, 35°C, 40°C, 45°C and room temperature). An enzyme assay was conducted using the culture broths as enzyme solutions after centrifugation, and the temperature at which the highest reading was obtained was concluded to be the optimum temperature for the test organism to produce heparinase.

5.3. Effect of pH for the production of heparinase

The effect of varying pH on the production of heparinase was then studied by preparing M9 modified media containing 50µl heparin, having pH ranging from 5 to 12. The media was inoculated with the test organism and incubated at the previously determined optimum incubation period and temperature. An enzyme assay was performed using the culture broths as enzyme solutions after centrifugation, after incubation and the pH which gave the highest value was concluded to the the optimum pH for the test organism to produce heparinase.

5.4. Effect of trace elements on the production of heparinase

After the effects of physical parameters had been studied, the effect various trace elements have on the production of heparinase by the test organism was studied. The media used was M9 modified media containing heparin (50µl) inoculated with the test organism and incubated at the previously determined optimum conditions, after the addition of trace elements. The different trace elements used for this experiment were $ZnCl_2$, $MgCl_2$, $MnSO_4$, $NiCl_2$ and $FeCl_3$. An enzyme assay was conducted using the culture broths as enzyme solutions after centrifugation. The highest reading obtained from the enzyme assay indicated which trace element increased the production of heparinase the most, after which the effect of varying concentrations of this trace element on the production of heparinase was studied. The culture broth that showed the highest reading indicated the concentration of the selected trace element that has the most effect on the test organism for the production of heparinase.

6. Purification and characterization of heparinase

For the purification and characterization of the enzyme, a production broth was prepared at the previously determined pH along with the optimum concentration of the determined trace element and was incubated at the determined temperature for the determined incubation time, after which the production broth was centrifuged and the supernatant was collected and labelled 'Crude'.

Purification and characterization of the enzyme was done in several steps that involved the methods salt precipitation, followed by dialysis, ion exchange and gel filtration chromatography. A series of assays were conducted to study the rate of reactions catalysed by the enzyme heparinase under different experimental variables like incubation time, pH, temperature and substrate concentration. Separate enzyme assays were performed for each variable to observe the effect of these variables.

RESULTS AND DISCUSSION

1. Collection of soil samples

The source of micro-organisms for this experiment was chosen as soil. This was due to the fact that soil contains a wide variety of micro-organisms and is also rich in carbohydrates. Since, the enzyme heparinase is also made up of carbohydrates, areas rich in carbohydrates are more likely to contain micro-organisms which are capable of producing heparinase. The samples were collected from a park, a garage, a market, a hospital, a house garden and a road. Thus, soil samples from six different places were collected for this experiment and used to prepare soil suspensions for culturing for further isolation of micro-organisms from the samples.

2. Isolation of micro-organisms

The isolation of the micro-organism to be used further in this study for the production of pharmaceutical grade heparinase was done by using soil samples and culturing the micro-organism found in those samples. Soil was picked as a source of micro-organisms owing to the fact that soil samples exhibit a large variety of micro-organisms as compared to air or water. Since the microbial count of a soil sample is high, the soil samples were used to create soil suspensions which were used with M9 modified agar containing heparin to culture the micro-organisms present in the soil suspension. The colonies obtained from these samples showed a wide variety of colonies ranging from colour of the colonies, to shape, size and elevation. The colonies obtained were found not only on the surface of the solidified agar but also within the agar, concluding that not only aerobic but also anaerobic colonies had been obtained as a result of isolation of micro-organisms using soil samples.

Musliu and Salawudeen (2012) created soil suspensions using soil samples which they used to inoculate a nutrient agar media. The incubation of this lead to the formation of colonies which they isolated. In their study, they sought to screen and isolate bacteria from a soil sample exhibiting antibiotic activity.

Alexis Gaete (2020) aimed to evaluate the beneficial features of soil bacteria for plants from two Chilean desert settings. He intended to extract and identify such bacteria present in soil which positively affected plant growth.

3. Screening of micro-organisms

Screening of the selected micro-organisms was done to single out the one micro-organism which showed the highest amount of heparinase production compared to the

rest. First, a coagulation test was performed on the selected organisms using 24 hour old culture broths prepared from LB broth to check for the production of heparinase and the time taken for the coagulation of each broth culture was noted down. After the first screening test, in order to determine which bacteria produced the highest amount of heparinase, an enzyme assay was performed to measure the enzyme levels of the culture broth of each micro-organism. The readings to determine the enzyme levels were taken after 24 hours of incubation to find out which organism showed the maximum production of the enzyme. The organism seen to produce the highest amount of heparinase (2.02 units/ml enzyme) was selected as the test organism.

4. Identification of the micro-organism

The selected micro-organism was then subjected to tests like Gram staining and a series of biochemical tests for the identification of the micro-organism. Gram staining was done to identify the staining characteristics of these bacteria and it also helps in identifying the shape and type of the bacteria. It serves as an important step in the classification of the micro-organism, further leading to the identification of the micro-organism. From the Gram staining experiment performed, the test organism was found to be a Gram positive cocci.

The Gram staining is one of the most crucial staining techniques in microbiology. It gets its name from the Danish bacteriologist Hans Christian Gram who first introduced it in 1882, mainly to identify organisms causing pneumonia. Often the first test performed, gram staining involves the use of crystal violet or methylene blue as the primary color. The term for organisms that retain the primary color and appear purple-brown under a microscope is Gram-positive organisms. The organisms that do not take up primary stain appear red under a microscope and are Gram-negative organisms (Nishant and Amit, 2021).

The test organism was then subjected to a series of biochemical tests to determine the metabolic properties of the selected micro-organism. It was necessary to determine the properties of the bacteria in question for the purpose of further identification of the test organism. To meet this end, several biochemical tests were performed with the test organism and they were the IMViC tests, followed with an H_2 S test, a gelatin test, casein and starch hydrolysis tests, cellulose degradation test, glucose, sucrose and mannitol fermentation tests, nitrate reduction test, urease test, TSI test and catalase test. The indole, Voges Proskauer, citrate utilization, H_2 S, gelatin hydrolysis, sucrose and mannitol fermentation, urease and ctatalase tests gave negative results while the methyl red, casein and starch hydrolysis, cellulose degradation, glucose fermentation, nitrate reduction and triple iron sugar tests gave positive results.

Syahri (2019) sought to isolate and identify bacteria from post-mining lands in Pomalaa, having the ability to reduce nickel and chromium toxicity in soil. For this, bacteria were identified by means of Gram staining and biochemical tests and it was concluded that the isolated bacteria were Gram positive *Bacillus*, lacking the ability to produce catalase, utilise carbohydrates or produce indisposes. The other bacteria was a Gram positive *Clostridium* which could produce catalase and utilise carbohydrates. All of this could be concluded due to the biochemical tests the organisms were subjected to.

Zhang (2010) sought to disperse the allelopathic inhibition of plants by microbial breakdown and to detect and characterise microorganisms in soils with high phenolic content. By using p-coumaric acid as the only carbon source in a screening media, he identified four microorganisms from plant soils. Identification was done by biochemical analyses and the 16S or 18S rDNA sequences of the isolates.

5. Optimization

The test organism was tested for its ability to produce heparinase in different conditions like incubation time, pH and temperature. The organism was cultured at varying incubation periods, temperatures and pH to determine the amount of heparinase production at each condition. This was an important step in this project since the optimum conditions of the test organism needed to realized in order to obtain higher amounts of heparinase in the culture for further purification and characterization of the enzyme. The results of these experiments showed that the test organism produced higher amounts of heparinase after being incubated for 48 hours (7.98 enzyme units/ml) at a temperature of 35°C and a pH of 9, with the prodcution values being 9.19 and 10.32 enzyme units/ml respectively. The trace element recorded to have the most effect on the production of heparinase was seen to be ferric chloride (11.97 units/ml enzyme), for which another enzyme assay was conducted in order to determine the optimum concentration of the trace element required to produce the highest amount of the enzyme heparinase. The concentration of ferric chloride at which the test organism gave the highest enzyme value was recorded to be 500mg (14.13 units/ml).

Eiichi Yoshida (2002) optimised a heparinase with ability to cleave heparin as well as heparan-sulphate and concluded that the optimum conditions for the highest enzyme activity for that heparinase were a pH of 7.5 at 45°C. The presence of 5 mM CaCl2, BaCl2, or MgCl2 increased its activity.

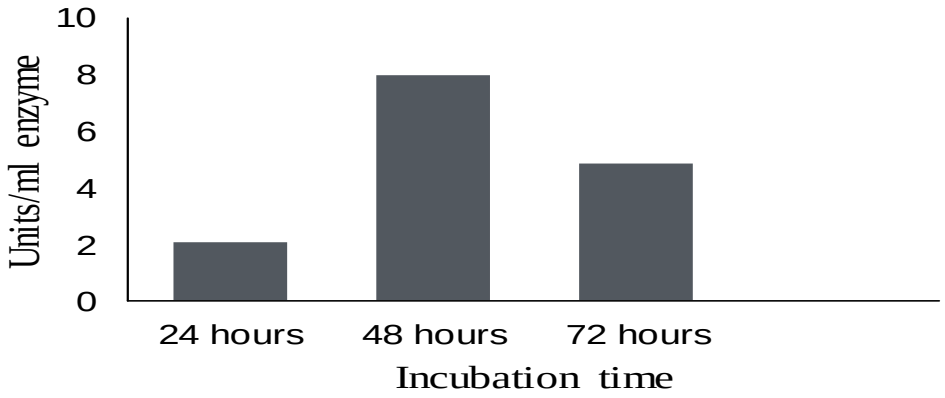

Fig 5.1. Effect of incubation time on the production of heparinase

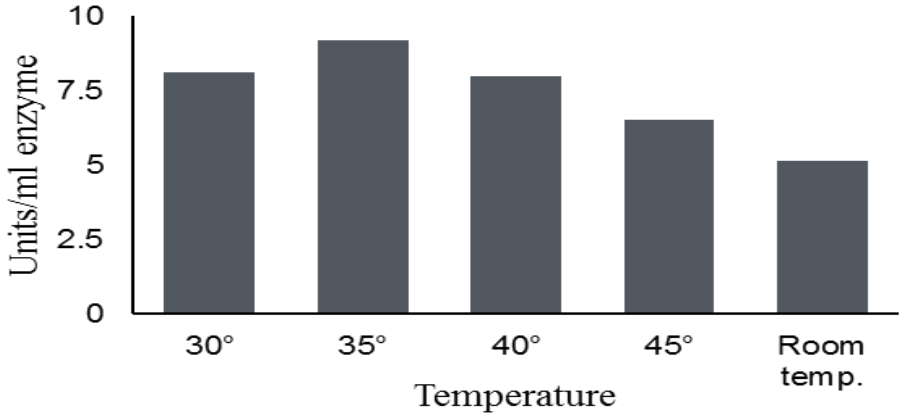

Fig 5.2. Effect of temperature on the production of heparinase

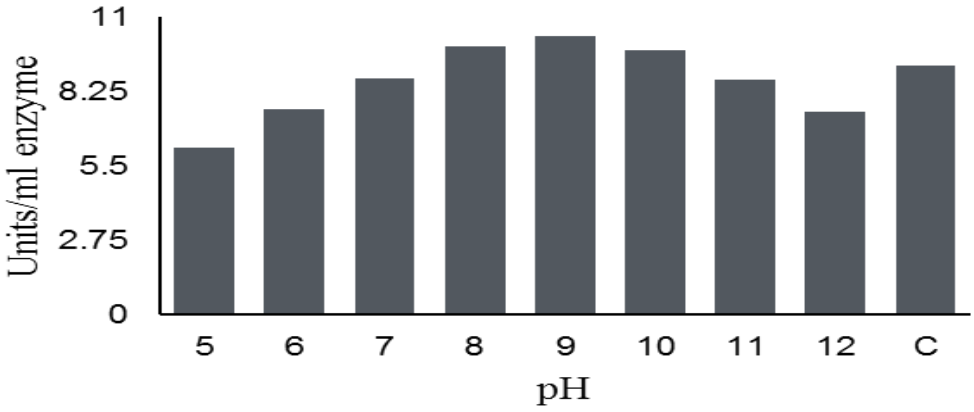

Fig 5.3. Effect of pH on the production of heparinase.

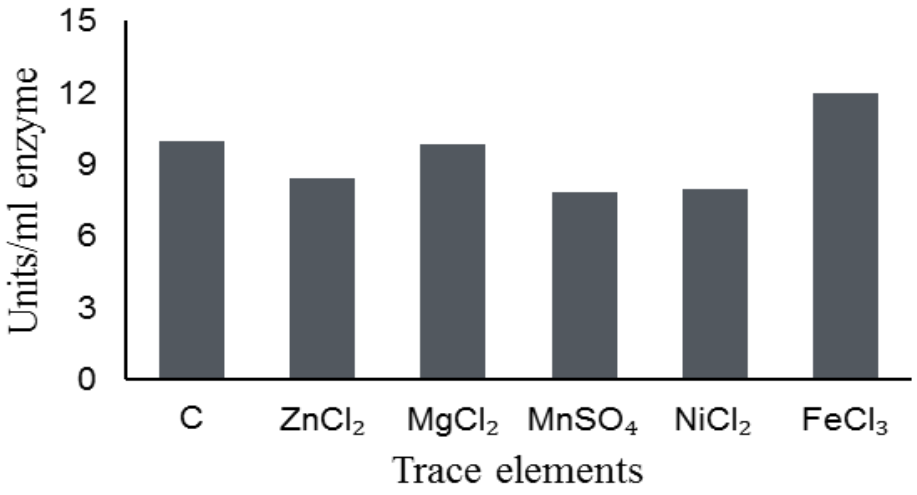

Fig 5.4. Effect of trace elements on the production of heparinase.

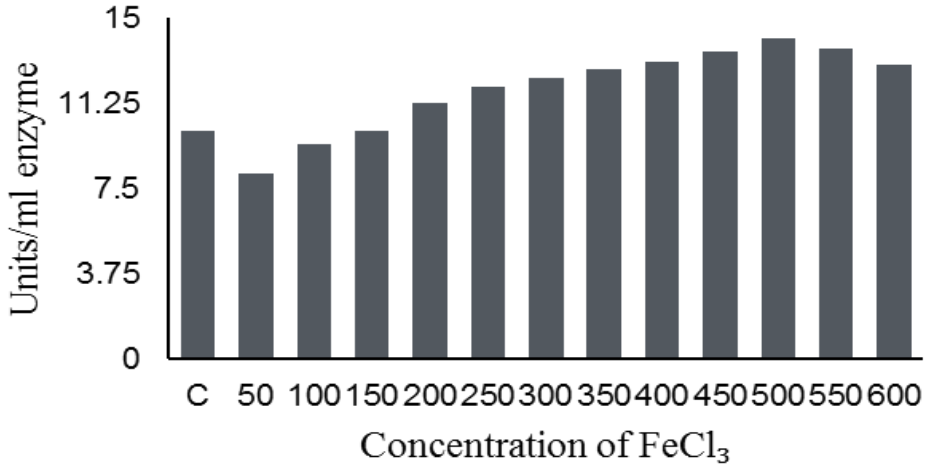

Fig 5.5. Effect of different concentrations of $FeCl_3$ on the production of heparinase

Bao- Cheng Yang (2016) purified heparinase I extracted from *Escherichia coli* and aimed to heighten soluble expression of the enzyme. In his study, the optimization concluded that the enzyme was most active at a pH of 7 at 30°C with the addition of 10 mmol/L Ca2+ in the reaction buffer.

6. Purification and characterization of heparinase

The enzyme produced in the broth culture has to be extracted from the broth culture and purified for use. This is done to ensure the purity and activity of the enzyme, and also gives insight into the structure of the enzyme. The methods used in this project to purify and characterise the enzyme heparinase were salt percipitation, dialysis, ion exchange chromatography, gel filtration chromatography, and SDS - PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis). Enzyme kinetic curves were made with showed the relation between enzyme production and the effect of incubation time, effect pf pH, effect of temperature and effect of substrate concentration.

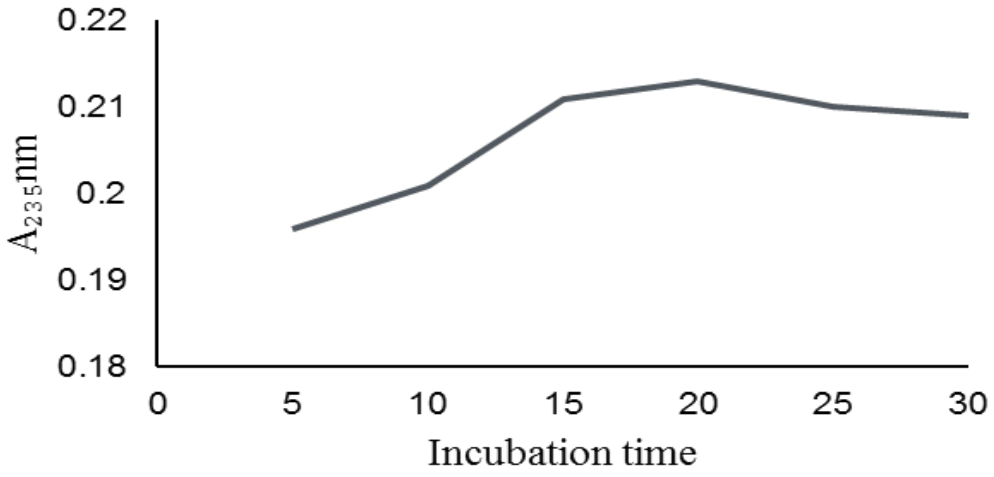

Fig 6.1. Effect of incubation time.

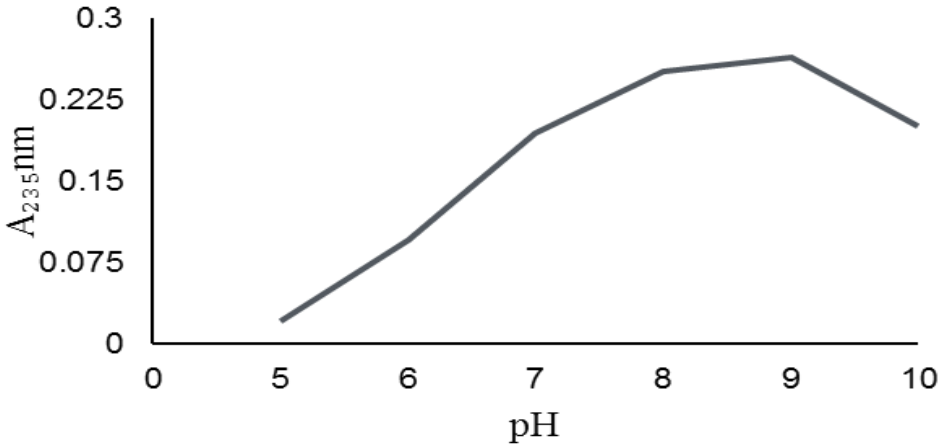

Fig 6.2. Effect of pH.

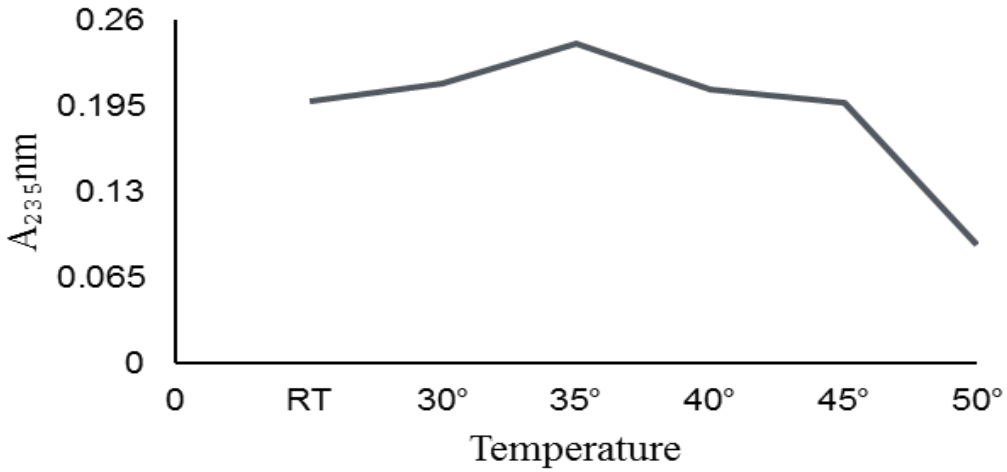

Fig 6.3. Effect of temperature.

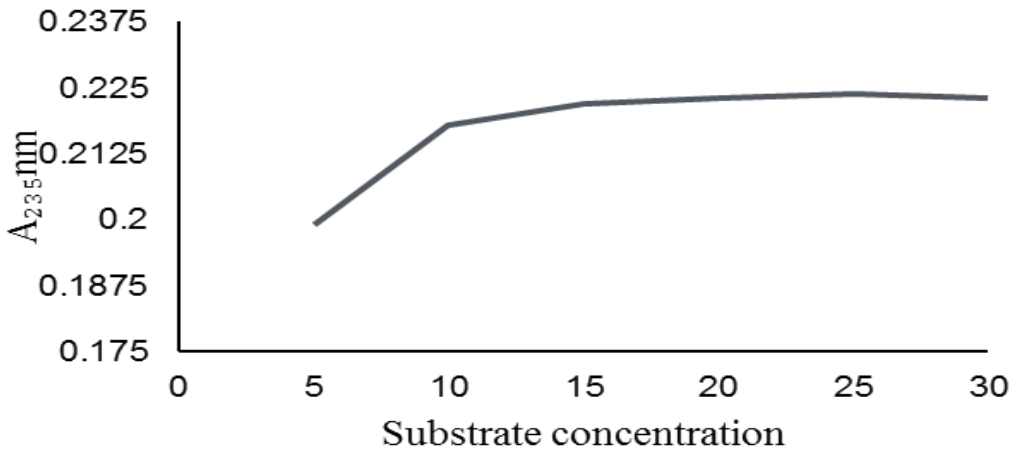

Fig 6.4. Effect of substrate concentration.

A heparinase that degrades both heparin and heparan sulfate (HS) was purified to homogeneity from the cell-free extract of *Bacillus circulans* HpT298. The purified enzyme had a single band on SDS-polyacrylamide gel electrophoresis with an estimated molecular mass of 111,000. The enzyme showed optimal activity at pH 7.5 and 45 degrees C, and its activity was stimulated in the presence of 5 mM CaCl2, BaCl2, or MgCl2. Analysis of substrate specificity and degraded disaccharides demonstrated that the enzyme acts on both heparin and HS, similar to heparinase II from Flavobacterium heparinum (Eiichi et al, 2002).

Heparinase (EC 4.2.2.7) isolated from Flavobacterium heparinum was purified to homogeneity by a combination of hydroxylapatite chromatography, repeated gel filtration chromatography, and chromatofocusing. The molecular weight of heparinase was estimated to be 42,900 +/- 1,000 daltons by gel filtration and 42,700 +/- 1,200 daltons by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (V C Yang et al, 1985).

A microbial strain of *Streptomyces* sp. showing antithrombotic activity was isolated from the soil sample collected from north India. The strain was characterized by using 16S rRNA homology technique and identified as *Streptomyces variabilis* MTCC 12266 capable of producing heparinase enzyme. This is the very first communication reporting Streptomyces genus as the producer of heparinase. It was observed that the production of intracellular heparinase was [63.8 U/mg protein (specific activity)] 1.58 folds higher compared to extracellular heparinase [40.28 U/mg protein]. DEAE-Sephadex A-50 column followed by Sepharose-6B column purification of the crude protein resulted 19.18 folds purified heparinase. SDS-PAGE analysis of heparinase resulted an estimated molecular-weight of 42 kDa (Vineeta et al, 2019).

The SDS polyacrylamide gel electrophoresis was done to separate the enzyme proteins according to their molecular weight and size.

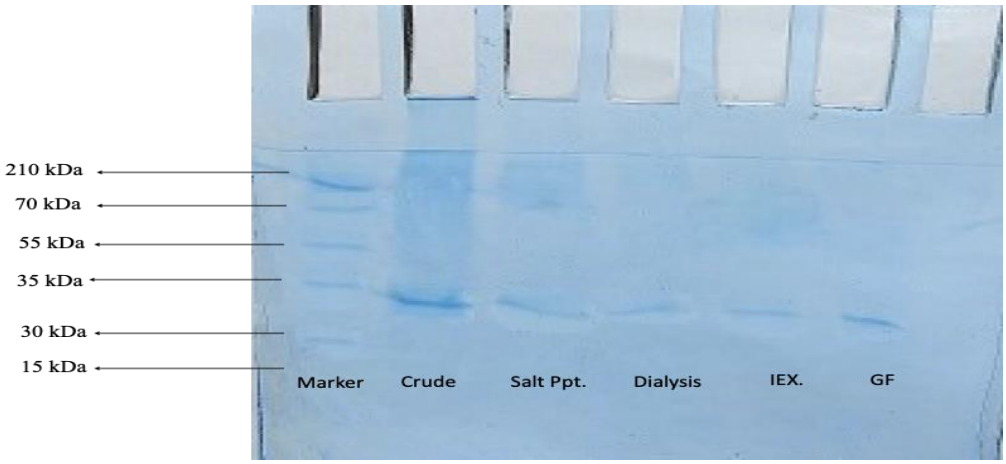

Fig 6.5. SDS-PAGE.

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