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ISOLATION, PURIFICATION AND CHARACTERIZATION OF HEPARINASE FROM BACTERIAL SOURCE

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ABSTRACT

Heparinases are a group of lyases that digest heparin and heparan sulfates by recognizing and cleaving the major glycosidic linkages. These enzymes belong to the family of carbon-oxygen lyases that act on polysaccharides. These enzymes have various clinical and therapeutic applications such as in the preparation of low-molecular-weight heparins and the deheparinization of heparin-treated blood, inhibition of neovascularisation, treatment of Thromboelastography blood samples, etc.In view of the various roles played by heparinases, the current study aims to identify and isolate bacteria capable of producing this enzyme in the in-vitro study followed by enzyme extraction, purification and charcterization. The work dealt with production of enzyme by optimizing the physical factors and the media in which various micronutrients were added so as to detect the best micronutrients for maximum yield. The strain was also mutated for better results. Once the production is done, the crude enzyme has been extracted and purified using ammonium sulphate precipitation followed by the dialysis of the salt precipitated protein. The enzyme assay, detection of optimum pH and temperature for enzyme activity were also performed. An experiment is also performed to study the role of heparinase in reversing the heparin-induced anticoagulation.

Keywords: Enzymes, Heparinases, Fermentation, Anticoagulation, Therapeutic

INTRODUCTION

Enzymes are the biocatalysts for the specific biochemical reactions which are the parts of several metabolic processes bring out in cells **[1].** They havehigh catalytic efficiency, greater affinity and specificity. Hence enzymes are broadly used for different enzyme therapies and therapeutic purposes. The enzymes medically important and produced by microorganisms have advantage of being economically convenient and consistent. They have high yield and are easy for product modification and optimization **[2].**

Heparin/heparan sulfate are highly sulfated glycosaminoglycans consisting of multiple repeating units of disaccharide forming*N*-acetyl-D-glucosamine and uronic acid residues (L-iduronic (IdoA) or D-glucuronic acid (GlcA)) **[3,4].** Heparin shows strong anticoagulation properties and present in mast cells.Heparin is generally used as blood anticoagulant and also possesses antithrombotic, antiheamostatic and antilipaemic activities **[5].**On the basis of various studies it was found that the unique sequence of pentasaccharide present in one third region of heparin polymer chains, which shows contribution in anticoagulant activity **[6,7,8,9].**

Derivatives of heparin shows several therapeutic applications such as antiproliferation, anti-inflammatory, antitumor, anti-viral, antiangiogenesis and antiatherosclerotic **[5]**.The heterogeneity structure of heparin is responsible for the physiological action and the bioactivity Unfractionated heparin (UFH).

Heparin and heparan sulfate can be digested by heparinases, a group of bacterial lyases that are capableof recognizing and cleaving different sequences of heparin. Heparinases employ two distinct mechanisms: β-elimination and hydrolysis. It belongs to lyases family, with C-O lyases activity on polysaccharides. The enzymes in known by heparin lyases systematically and commonly pronounced by heparin eliminase, and heparinise **[10].**

These enzymes have potential applications in several domains like neutralization, detection and determination of plasma heparin, treatment of Thromboelastography blood samples and removal of interference caused by heparin, to characterize the structure or endogenous activity of heparin and related compounds **[11]**.

There is also great interest in preparation of heparinase derived heparin fragments as alternative anticoagulantreagents with less side effects and as antitumor reagents **[12]**.Several heparinase producing micro-organisms have been isolated and characterized, but demand for novel heparinase is increasing, as the application spectra of heparinases are expanding in various industrial sectors **[13]**.

METHODOLOGY

Sample collection:

For the following study, soil sample was collected near slaughter house in order to find the desired species.

Isolation of bacteria from collected sample:

The soil sample was serially diluted in normal saline and then spread on sterilized nutrient agar plates. The grown cultures were selected on the basis of morphology, then streaked on separate nutrient agar plates to make pure cultures and incubated at 37°C for 24 hours. The pure cultures were re-streaked on minimal salt media supplemented with heparin**[14,15]**.

Biochemical tests:

Various biochemical tests were performed for the identification of culture.

Strain improvement:

Strain improvement of the culture was carried out by UV rays mutation **[16]**.

Enzyme assay:

The sample was added to the heparin and glycerol (1:1 ratio), incubated at 37°C for 15 min. Then DNS reagent was added to it and again incubated at 100°C for an hour. After that the absorbance was taken at 540 nm**[17]**.

Media selection and optimization:

The media selection was done on the basis of growth of culture in four different media termed as PM1 (g/l): tryptone-20, soya bean meal-3, dextrose-2.5, NaCl-5, K_2HPO_4 -2.5, PM2 (g/l): Tryptone-20, NaCl-5, 0.1M phosphate buffer, PM3 (g/l): Peptone-0.75, Yeast extract-0.5, K_2HPO_4 -0.1, NaCl-0.1, $MgSO_4.7H_2O$-0.02, PM4 (g/l): Peptone-0.1, NaCl-0.1, Yeast extract-0.1. Further optimization of the components was executed by replacing the components with other substitutes by using one factor at a time method**[18]**.

Fermentation and its downstream processing:

The production of heparinase was completed by inoculating culture in optimized media and incubating under optimum conditions by using shake flask fermentation. Further the purification of heparinase is ended by salt precipitation and dialysis. Even after that, the estimation of enzyme and the effects of pH and temperature on enzyme was carried out **[19,20]**.

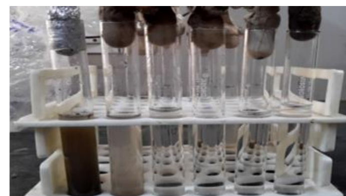
RESULTS

Collection of sample:

The soil sample is collected from slaughter house.

Figure 1:Soil sample from Slaughter House (Kanpur Road)

Isolation of heparinase producing bacteria: the soil sample was serially diluted in 0.85% NaCl solution. The whitish colours appearance colonies were observed after the completion of incubation period. On the basis of morphological parameters, the cultures were streaked in continuous quadrant pattern. Further the screening was carried out by restreaking the pure cultures on heparin agar plates. Where culture 3 obtained as positive culture.



a. Serial dilution

b. Spread plate

c. Streak plate of pure cultures

d. Culture growth seen on heparin agar plate

Figure 2: Representation of isolation of heparinase producing bacteria from soil sample.

Strain identification:

Table 1: Morphological characteristics

Characteristics	Result
Colony shape	Irregular
Colony margin	Undulate
Colony elevation	Flat
Colony texture	Hard
Colony Surface	Rough
Colony pigmentation	Off-white

Table 2: Biochemical characterization

Biochemical characterization	Result
Gram staining	Negative
Endospore staining	Positive
Catalase test	Positive
Indole test	Positive

Strain improvement by UV mutation: Colonies were picked from the plate exposed to UV treatment for different time interval and then the culture was streaked and allowed to grown on heparin agar plate, where 8 min treated culture was successfully grown on the heparin agar plate. Further the growth and enzyme production of wild as well as mutated strain was compared by DNS assay for estimation.

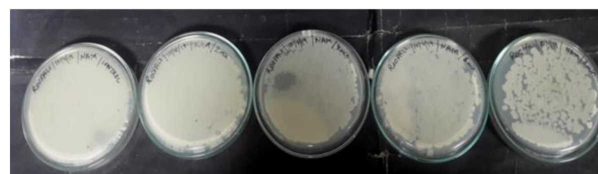


Figure 3: Growth of bacteria after UV treatment for different time intervals.

Table 3: Selection of culture after mutation

Culture	O.D. (620 nm)	O.D. (540 nm)
wild	0.25	0.85
UV mutated	**0.36**	**0.99**

Selection and optimization of production media:

The selection is done by taking the OD after 48 hours of incubation of bacterial growth and DNS assay.The best results were shown by PM-4. During optimization it was found that maltose (5g/l), Yeast + Peptone (0.2 g/l each),$CaCO_3$(0.1%) shows best results for the selection.

Table 4: selection of production media

Production media	O.D. (620 nm)	O.D. (540nm)
PM-1	0.35	0.15
PM-2	0.47	0.12
PM-3	0.31	0.21
PM-4	**0.24**	**0.33**

Table 5: Selection of carbon source

Carbon source	O.D. (620 nm)	O.D. (540 nm)
Dextrose	0.08	2.0
Mannitol	0.10	0.12
Fructose	0.11	1.51
Maltose	**0.20**	**2.0**

Table 6: Selection of nitrogen source

Nitrogen source	O.D. (620 nm)	O.D. (540 nm)
Yeast + Peptone	**0.08**	**0.55**
NH_4Cl	0.02	0.42
Malt Extract	0.03	0.77

Table 7: Selection of metal ion

Metal ion	O.D. (620 nm)	O.D. (540 nm)
$MgSO_4$	0.02	0.59
$ZnSO_4$	0.00	0.47
$FeSO_4$	0.02	0.62
$CaCO_3$	**0.83**	**0.58**

Table 8: Selection of concentration of maltose

Conc. of Maltose (g/l)	O.D. (620 nm)	O.D. (540 nm)
2	0.01	2.00
5	**0.02**	**1.25**
8	0.02	0.84
10	0.01	0.22

Table 9: Selection of percentage of Yeast + Peptone

Yeast + Peptone(%)	O.D. (620 nm)	O.D. (540 nm)
0.1 + 0.1	0.11	0.79
0.2 + 0.1	0.10	0.86
0.1 + 0.2	0.09	0.76
0.2 + 0.2	**0.12**	**0.86**

Table 10: Selection of percentage of metal ion

$CaCO_3$ (%)	O.D. (620 nm)	O.D. (540 nm)
0.1	**0.68**	**0.56**
0.15	0.01	0.48
0.2	0.01	0.43
0.25	0.03	0.53

Effect of temperature on growth:

The bacteria showed no growth at 4°C and little growth at 20°C compared to that at 37°C and 50°C.

Figure 4: Effect of temperature on bacterial growth.

Growth curve:

It was found that stationary phase occurs after 48-72 hours

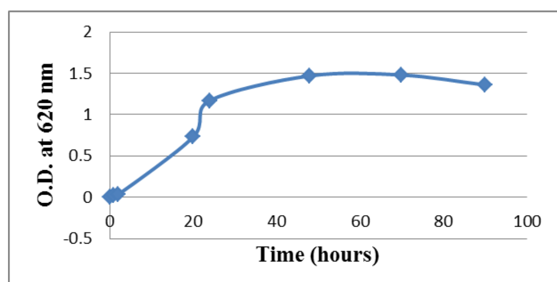


Figure 5: Growth curve study of culture.

Fermentation and Purification:

Fermentation was carried out by shake flask method. The crude enzyme obtained after fermentation was purified by ammonium sulphate precipitation followed by dialysis of the salt precipitated protein.

Figure 6: Dialysis bag containing protein placed in Tris buffer

Enzyme assay of crude and purified enzyme:

Sample	OD 540 nm	Concentration (μg/ml)	Enzyme activity (U/ml/min)
Crude enzyme	0.46	0.8	0.033
Pure Enzyme	0.62	1.05	0.0504

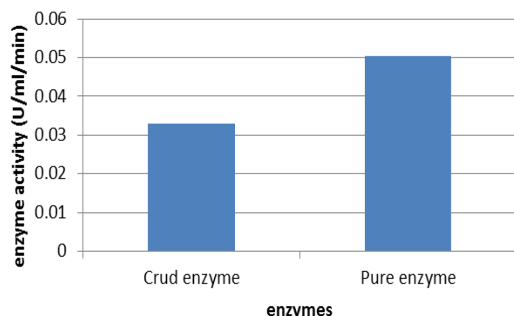


Figure 7: Graphical representation of DNS assay of enzyme

Table 12: given table represent the results of Lowry's assay of crud pure enzyme

Enzyme	O.D. AT 680nm	CONC. OF PROTEIN
Crud enzyme	0.96	0.22
Pure enzyme	0.49	0.48

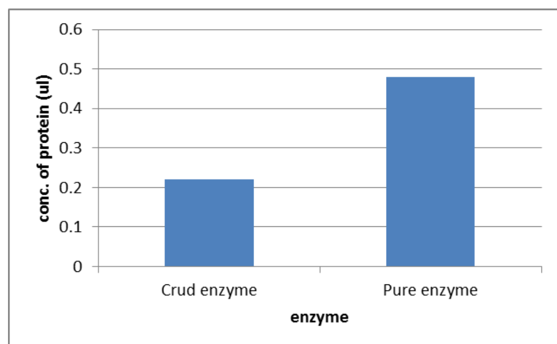


Figure 8: given graph represents the protein content of pure and crud enzyme;

Application of heparinase:

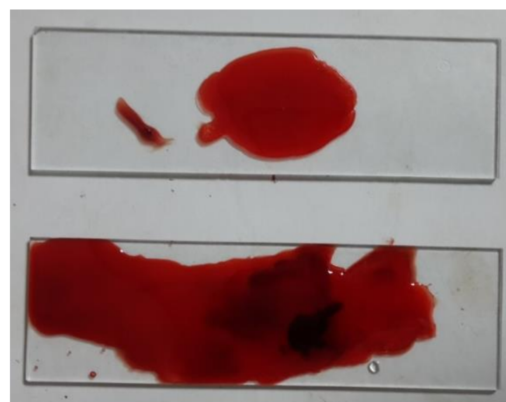


Figure 9: Application of enzyme. The top slide shows the blood sample with heparin added to it (no clotting) and the bottom slide shows the blood sample 2 minutes after addition of heparinase (blood starts clotting)

DISCUSSION

The bacterialspecies isolated from soil near slaughter house was found as an effective producer of heparinase evident fromthe findings. The enzyme has varied therapeutic applications. Bacterial isolates were obtained from soil sample. They were initially screened for heparinase production based on their growth oh heparin agar plates.

While characterizing the bacteria, the isolated strain was found to be gram negative, spore forming, and gave positive results for catalase and indole production test.

For the better production of enzyme, the strain was mutated by exposing to UV radiation for different time intervals. The colonies exposed to UV for 8 minutes showed better production as compared to others. Further, production of heparinase by culture in optimized media was carried out by shake flask fermentation.From various production media, the media that showed maximum growth and production of secondary metabolite was selected for the study.

The culture conditions and media components (carbon source, nitrogen source and metal ions) were optimized for maximum production of the enzyme. Maximum growth was found to be at temperatures of 37°C and 50°C. The most effective carbon source was found to be Maltose and it gave maximum results at a concentration of 5g/l. The nitrogen sources selected for the media were 0.2% Yeast and 0.2% Peptone as they together gave best results. Among the metal ions 0.1% $CaCO_3$showed the maximum effect on the growth of the microorganism and production of secondary metabolites.At the end of the fermentation period, the culture medium was centrifuged to obtain the crude extract, which served as enzyme source. The crude enzyme was purified using ammonium sulphate precipitation followed by the dialysis of the salt precipitated protein. Enzyme assay of crude and purified enzyme was performed by using DNS reagent and Lowry's assay for the concentration.

Heparin is an anticoagulant and heparinase is an enzyme that reverses heparin-induced anticoagulation i.e., inactivates heparin. So, to study the application of heparinase a blood sample was taken and heparin (16mg/ml) was added to it. The sample was observed for some time and it was found that the presence of heparin did not allow the blood to clot. Now, heparinase was added to this sample and observed. It was seen that the blood started clotting in 2 minutes which means that the enzyme had reversed the action of heparin. This whole process was carried out at room temperature (25°C).

CONCLUSION

The microorganism obtained from the soil sample was characterized morphologically and biochemically. This bacteria showed best results at temperature 37°C and 50°C.

Among the various components used maltose, yeast, peptone and $CaCO_3$ had maximum effect on production of heparinase. The enzyme was purified through various purification protocols. The stability of the enzyme at room temperature makes it useful for the generation of low molecular weight heparins, which are used as antithrombotics. Application of heparinase in reversing the anticoagulant activity of heparin has also been studied.

Further studies can be carried out to increase the activity of this enzyme and also finding out some new sources. Heparinases have already been implicated in inflammation, angiogenesis, and cancer progression. They are also found to have roles in viral pathogenesis. Due to their increasing therapeutical applications further studies should be carried out for elucidating some unique functions of heparinases.

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