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Effects of Micronutrients on the Production of Heparinase from *Bacillus lentus*

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Abstract: ***Heparinases are lyases capable of recognizing and cleaving different sequences of heparin (a highly sulfated glycosaminoglycan). These enzymes have potential applications in several domains like treatment of Thromboelastography blood samples and removal of interference caused by heparin, monitoring of the alterations in coagulation function, protection against reperfusion injury following ischemia and reduction of restenosis following gangioplasty. Neutralization, detection and determination of plasma heparin and quality control in heparin manufacture also involve use of heparinases. In view of the extensive roles played by these enzymes, the current study aims to identify and isolate bacteria capable of producing this enzyme in the invitro study followed byenzyme extraction and purification. The work dealt withproduction of enzyme by adding various micronutrients so as to detect the best micronutrient for maximum yield. Once the production is done, the crude enzyme has been extracted and subjected to various purification protocols like ammonium sulfate precipitation, dialysis, ion exchange chromatography, gel filtration, etc. The enzyme assay, optimization and protein concentration estimations were also performed and Bacillus lentus resulted in the maximum yield in the presence of ferric chloride.***

Keywords: Heparinases, heparin, Thromboelastography, micronutrients, purification

1. Introduction

Heparinases (heparin lyases) are enzymes[1] that can eliminatively cleave polysaccharides, heparin, or heparan sulfate glycosaminoglycans (GAGs), into disaccharide and oligosaccharide products.Heparinase enzymes are classified into three types – heparinase I, heparinase II, heparinase III, which specifically recognizes and cleaves at different sequences of heparin. Heparinase I enzyme (Enzyme Commission number: 4.2.2.7)[2] cleaves heparin at the linkages between hexosamine and O-sulfated glucuronic acids, yielding oligosaccharides (mainly disaccharides). Heparinase II enzyme[3] cleaves at the 1-4 linkages between hexosamine and glucuronic acid residues of both heparin and heparan sulfate, yielding oligosaccharides (mainly disaccharides).Heparinas enzyme III (Enzyme Commission number 4.2.2.8)[4] cleaves heparan sulfate exclusively and does not cleave heparin.

These enzymes have potential applications in several domains like treatment of thromboelastography[5] blood samples and removal of interference caused by heparin, monitoring of the alterations in coagulation function[6], protection against reperfusion injury following ischemia and reduction of restenosis followingangioplasty. Neutralization, detection and determination of plasma heparin andquality control in heparin manufacture also involve use of heparinases.

In view of the extensive roles played by these enzyme heparinase, the current study aimed to identify and isolate bacteria capable of producing this enzyme in the *invitro* study followed byenzyme extraction and summary of enzyme purification.

2. Methodology

Isolation and identification of microbes for the production of heparinase

Five different soil samples from different locations such as milk parlours, tea shops, flour millsfrom Hosur, Berikai, Bagalur (TamilNadu) were collected. Production media containing 0.1% peptone, 0.1% NaCl, 0.1% yeast extract, 2% agar was prepared and sterilized. 0.5ml of heparin was added when the media had reached 55ºC. Samples from soil in sterilized water was poured and spread onto an agar plate and incubated at 37ºC for 24 hours.

Five slants using the media as described earlier were prepared for culture characterization[7]and selected colonies from each of the petri plate were streaked upon the slants. These test tubes were incubated at 37ºC for 24 hours. Gram staining technique was done for all the slant cultures to determine the morphological characteristics and the results were tabulated. The staining was followed by the use of various biochemical reagents and tests to get closer to the bacterial identification. Biochemical tests such as Gelatin test, Nitrate reduction test, Urease test, Hydrogen sulpide test, Fermentation of carbohydrates (Sucrose, Dextrose, Lactose), Indole production test, Methyl-Red and Voges-Proskauer test, Citrate utilisation test, Catalase test, Oxidase test, Starch hydrolysis test were performed to identify the microorganism.

Effect of micronutrients on the production of heparinase

Broth containing 0.1% peptone, 0.1% NaCl, 0.1% yeast extract was prepared and 0.5 ml of heparin was added to it. The broth was taken in 5 different conical flasks and the microbes from each of the slant culture were inoculated into it.The flasks were incubated in a shaker at 37ºC for 24 hours.

Enzymatic assay of heparinase
The broth was poured into five ependorf tubes and centrifuged at 6000 rpm for 15 minutes. The pellets were discarded and the supernatant was preserved, which served as the enzymatic solution. The enzymatic assay[8] was performed as per the protocol described for heparinase.

Estimation of protein
The broth with maximum enzyme activity was selected and protein estimationwas performed by Lowry's method[9].

Effect of micronutrients
0.1g of five different micronutrients such as $MgCl_2$, $ZnCl_2$, Na_2SO_4, $CaCl_2$, $FeCl_2$ was measured and added into five different conical flasks containing broth. The broth was sterilized and the microorganism was inoculated from the broth selected earlier and incubated at 37ºC for 24 hours in the shaker. Enzymatic assay for five different broths was performed to identify the micronutrient that had the maximum effect on the production of heparinase.

Purification of heparinase enzyme
The crude heparinase enzyme was subjected to purification by various steps such as Ammonium sulfate precipitation, dialysis, ion exchange chromatography (DEAE cellulose) and gel filtration chromatography(sephadex).SDS-PAGE used to determine the apparent molecular mass of the protein was performed.Gels were stained with coomassie brilliant blue to visualize the bands and estimate the molecular mass of the protein.

Optimization of heparinase production
The optimization study of the following parameters was done for better production of enzyme.

Effect of pH
The optimum pH was determined using various buffers of different pH that include glycine buffer(pH 4, 5), phosphate buffer (pH 6, 7), acetate buffer(pH 8, 9). The enzymatic assay using these buffers was performed.

Effect of temperature
Temperature dependence of the enzyme was investigated by measuring the enzymatic activity at different temperatures (20-60ºC).

Effect of incubation time
The incubation time required for enzymatic assay was varied from 10-40 minutes to determine the optimum time interval necessary for maximum production of heparinase.

Effect of substrate concentration
Various concentration of the substrate were incubated under optimal assay conditions and reactions were monitored and results were recorded at 235nm.

Effect of inhibitor
The effect of inhibitor on the enzyme activity was determined by using various concentrations of EDTA.

Effect of activator
The dependence of enzyme activity on the activator was measured by adding Magnesium Sulphate of different concentrations.

3. Results and Discussion

The results for morphological and biochemical tests of the strain selected are shown in

Table1.

Table 1: Biochemical characterization of isolated microorganisms

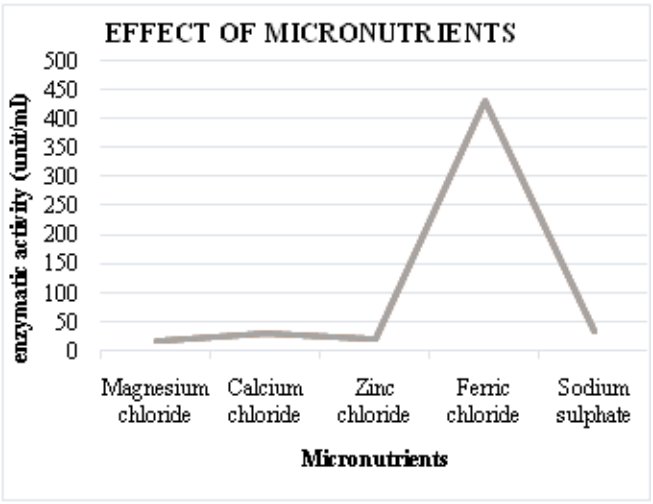
Tests	Results
Gram staining	+
Gelatin	+
Starch hydrolysis	+
Lactose	Acid and gas production
Sucrose	Acid and gas production
Dextrose	Acid and gas production
H_2S	+
Indole	-
Nitrate	+
MR	+
VP	-
Citrate	+
Urease	+
Catalyse	+
Oxidase	+

All strains showed positive results for starch hydrolysis, H_2S, citrate utilization, urease, oxidase, catalase and nitrate tests. The strains had a negative reaction to indole and VP tests. The results in the table reflect the ability of isolates to ferment sugars due to specific enzyme responsible for sugar fermentation and gas/ gas and acid production. From these results it has been identified that the isolates belong to Bacillus spp and the microorganism in the sample was identified as*Bacillus lentus.*

Effect of micronutrients on the production of heparinase
The results showing effect of micronutrients are given in Table2.

Table 2: Effect of micronutrients

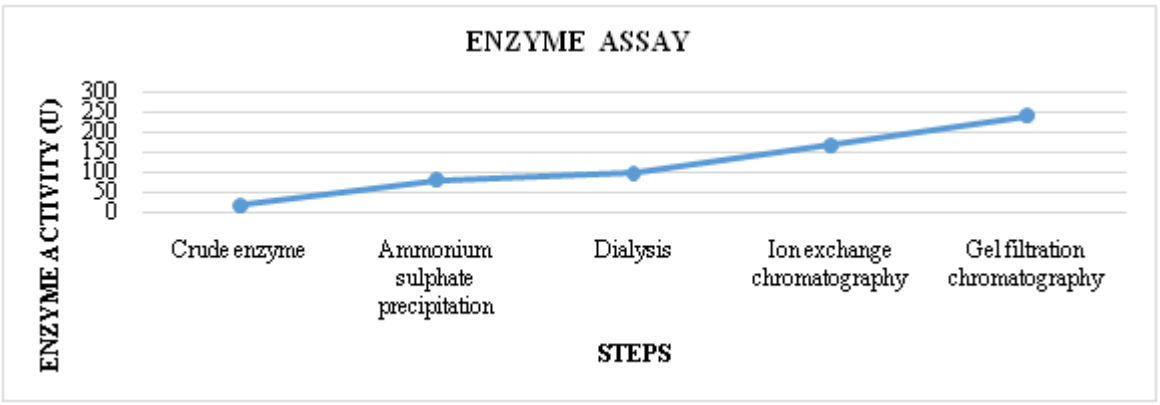
Micronutrients(µl)	Enzyme activity(units/µg)
$MgCl_2$	18.32
$CaCl_2$	30.10
$ZnCl_2$	20.07
$FeCl_3$	430.25
Na_2SO_4	35.78

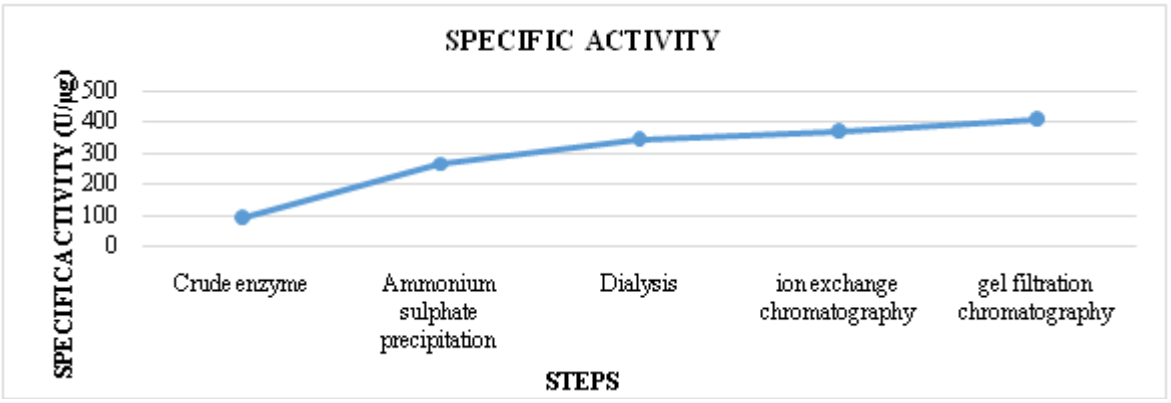


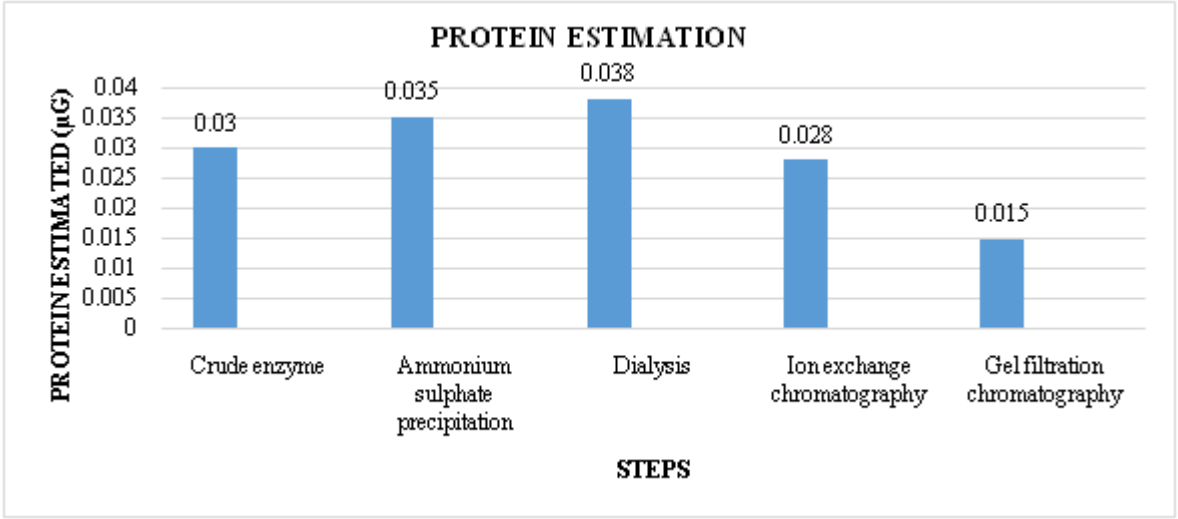
From the assay, it was found that ferric chloride has the maximum effect on the production of heparinase than the other micronutrients. Resultsof heparinase purification from *Bacillus lentus* are summarized in Table 3.

Table 3: Detemination of specific activity of heparinase

	Protein estimation (µg)	Enzyme activity (U)	Specific activity (U/µg)
Crude enzyme	0.03	18.76	93.8
Ammonium sulfate precipitation	0.035	81.6	265.4
Dialysis	0.038	97.7	345.9
Ion exchange chromatography	0.028	167.56	370.9
Gel filtration chromatography	0.015	239.27	410.6







SDS-PAGE electrophoresis of heparinase was performed and the molecular mass was found to be 60 KDa.The results of optimization parameters were tabulated.

Table 4: Effect of temperature

Temperature (ºC)	Absorbance at 235nm
20	0.15
28	0.26
35	0.475
45	0.3
60	0.145

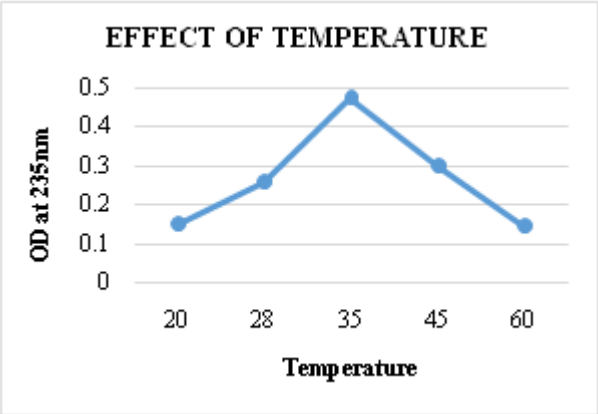


Table 5: Effect of pH

pH	Absorbance at 235nm
4	0.121
5	0.228
6	0.332
7	0.55
8	0.369
9	0.122

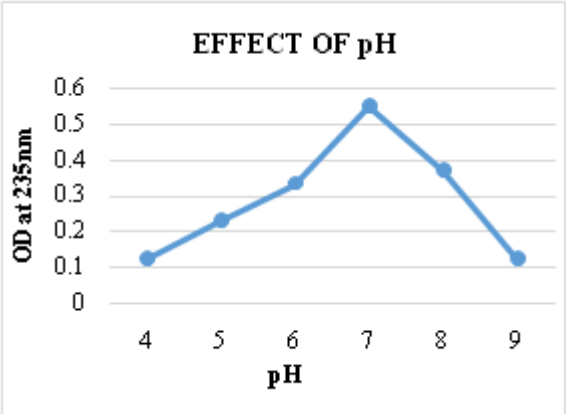


Table 6: Effect of substrate concentration

Substrate Concentration(µl)	Absorbance at 235nm
50	0.052
100	0.076
150	0.145
200	0.195
250	0.235

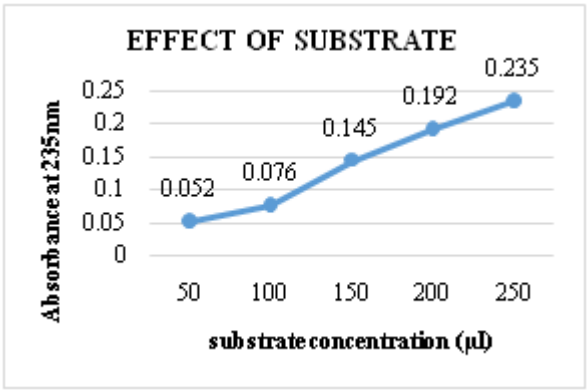


Table 7: Effect of activator

Activator concentration	Absorbance at 235nm
0.01	0.117
0.02	0.119
0.03	0.13
0.04	0.134
0.05	0.139

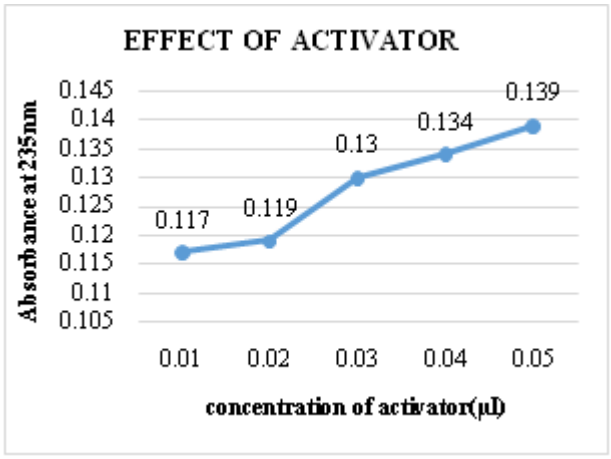


Table 8: Effect of inhibitor

Inhibitor concentration	Absorbance at 235nm
0.01	0.108
0.02	0.107
0.03	0.106
0.04	0.1
0.05	0.08

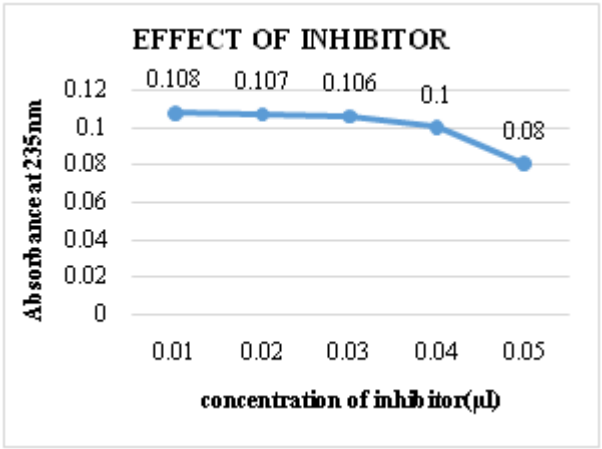
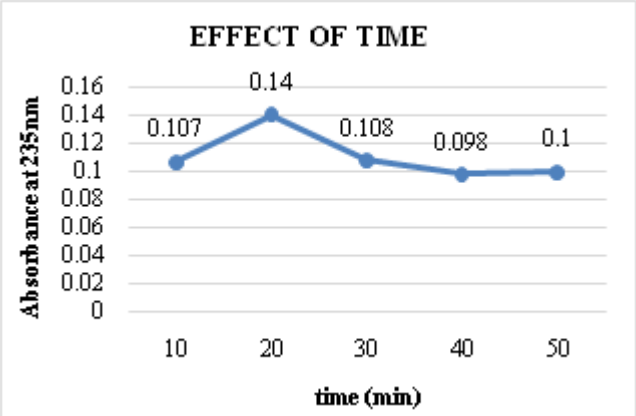


Table 9: Effect of incubation time

Time (min)	Absorbance at 235nm
10	0.107
20	0.14
30	0.108
40	0.098
50	0.1



Thus the optimum conditions required for the maximum activity of heparinase was found to be at temperature 35ºC, pH 7 and 20 minutes incubation. Also it has been showed that increase in concentration of substrate and decrease in inhibitor concentration tends to increase the activity of heparinase.

4. Conclusion

The microorganism obtained from the soil samples was identified as *Bacillus lentus* by morphological and biochemical tests confirmation. This microorganism showed best results at pH 7, temperature 35°C. Ferric chloride had maximum effect on production of heparinase among the various micronutrients used. The purity level was increased from 93.8 to 410 U/µg through various purification protocols. The molecular weight was determined as 60 kDa by SDS-PAGE.

References

[1] Jackson, R. L., Busch, S. J. and Cardin, A. D. (1991) Glysoaminoglycan: molecular properties, protein interactions, role in physiological processes. Physiol.rev.71, 481-539.
[2] Desai, U.R., Wang, H.M., and Linhardt, R.J. (1993) Substrate specificity of the heparin lyases from Flavobacterium heparinum. Arch. Biochem. Biophys. 306, 461-468.
[3] Ernst, S., Larger, R., Cooney, C.L., and Sasisekharan, R. (1995) Degradation of enzymaticglysoaminoglycan. Cit. Rev. Biochem.Mol. 30, 387-444.
[4] Bourin, M.C., and Lindahl, U. (1993) Glycosaminoglycans and the regulation of blood coagulation.Biochem.J. 289, 313-330.
[5] Folkman, J. and Shing, Y. (1992) Control of angiogenesis by heparin and other sulphated polysaccharides. Adv. Exp.Med.Biol. 30, 355-364.
[6] Linhard, R.J., Galliher, P.M., and Cooney, C.L. (1986) Polysacchride lyases, Appl, Biochem. Biotechnol. 12, 135-176.
[7] Smibert, R.M., Krieg and, N.R., (1994) Phenotypic characterization, p.615.
[8] Linker, A., and Hovingh, P. (1972) Methods in ezymology 28, 902-911.
[9] Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J.Biol. Chem 193: 265.

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