

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 assay⁸ was performed as per the protocol described for heparinase.

Estimation of protein

The broth with maximum enzyme activity was selected and protein estimation was performed by Lowry's method⁹.

Effect of micronutrients

0.1g of five different micronutrients such as MgCl₂, ZnCl₂, Na₂SO₄, CaCl₂, FeCl₂ 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 37°C 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 (20-60°C).

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 ₂ S	+
Indole	-
Nitrate	+
MR	+
VP	-
Citrate	+
Urease	+
Catalyse	+
Oxidase	+

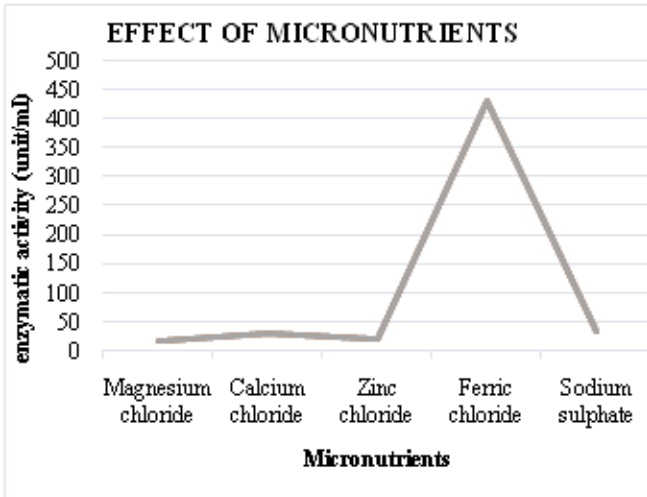
All strains showed positive results for starch hydrolysis, H₂S, citrate utilization, urease, oxidase, catalase and nitrate tests. The strains had a negative reaction to indole and VP tests. The results in the table reflect the ability of isolates to ferment sugars due to specific enzyme responsible for 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 ₂ SO ₄	35.78

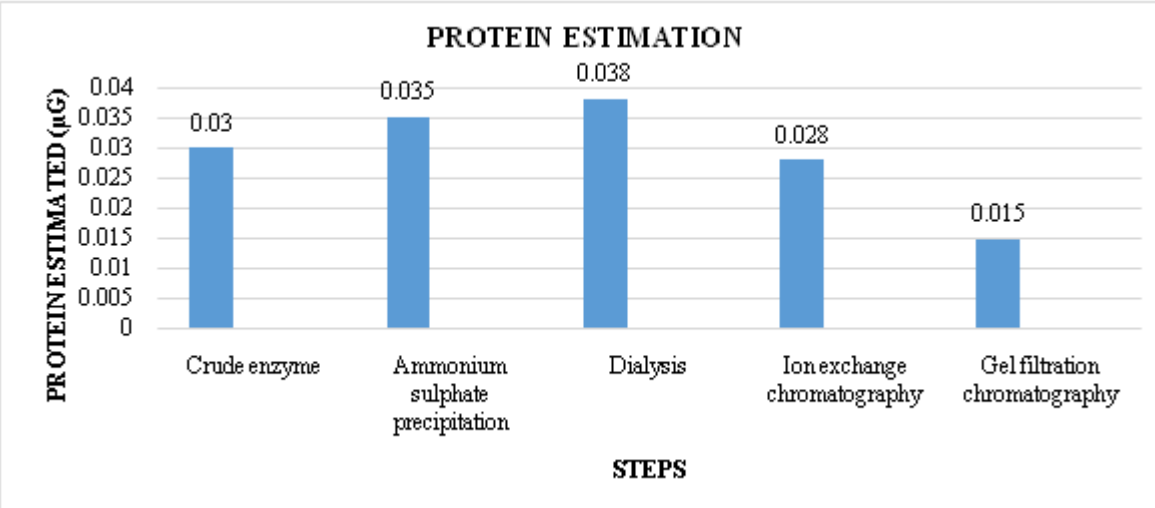
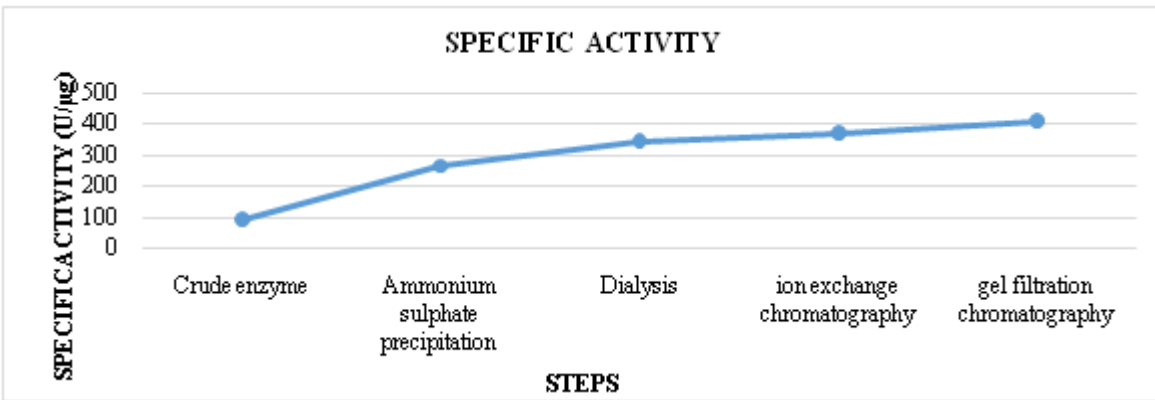
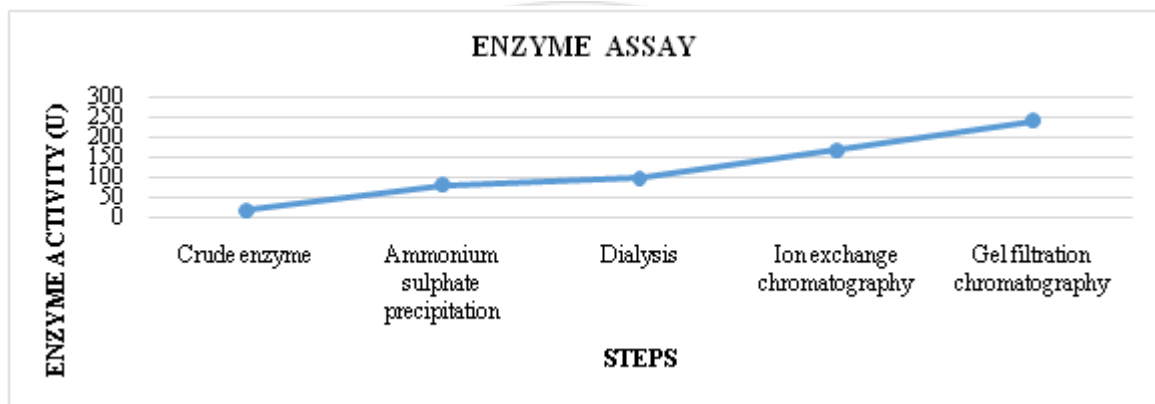


other micronutrients. Results of heparinase purification from *Bacillus lentus* are summarized in Table 3.

Table 3: Determination 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

From the assay, it was found that ferric chloride has the maximum effect on the production of heparinase than the



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

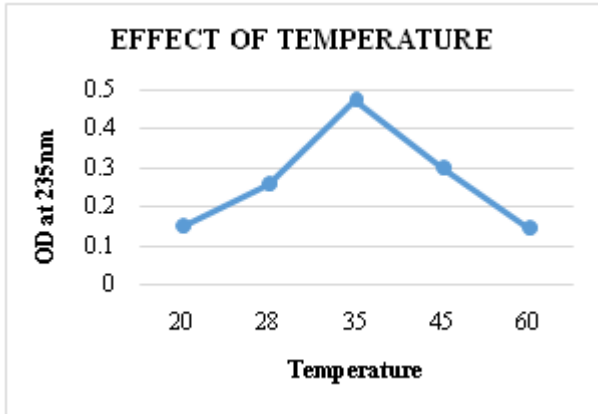


Table 5: Effect of pH

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

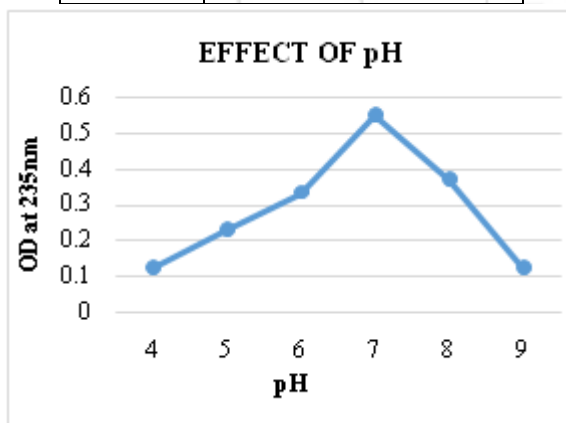


Table 6: Effect of substrate concentration

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

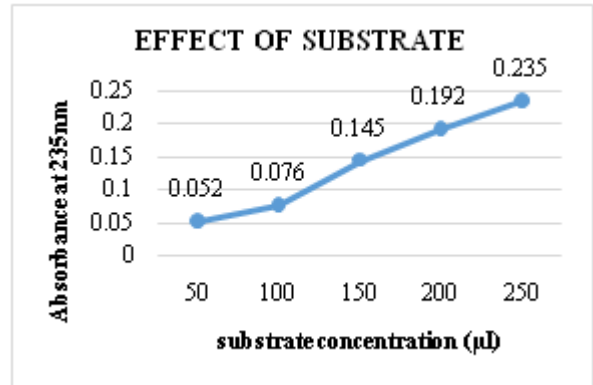


Table 7: Effect of activator

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

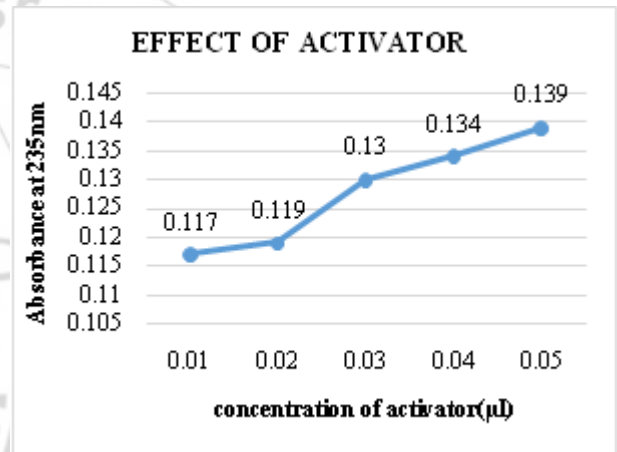


Table 8: Effect of inhibitor

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

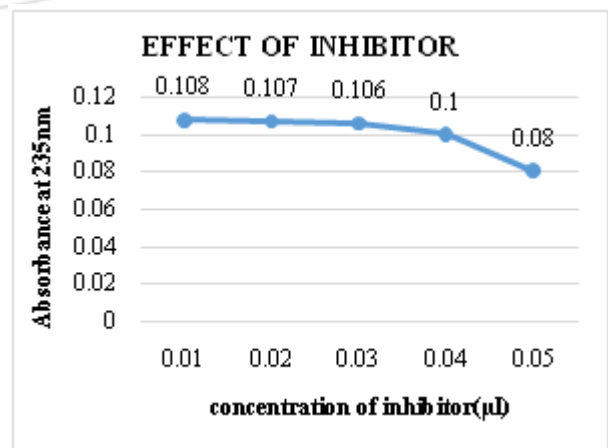
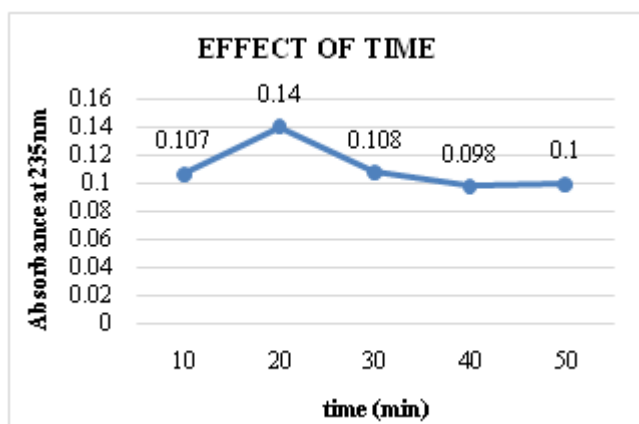


Table 9: Effect of incubation time

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 35°C, 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

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